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Eph signalling functions downstream of Val to regulate cell sorting and boundary formation in the caudal hindbrain

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SUMMARY

Rhombomeres are segmental units of the developing vertebrate hindbrain that underlie the reiterated organisation of cranial neural crest migration and neuronal differentiation. *valentino* (*val*), a zebrafish homologue of the mouse bzip transcription factor-encoding gene, *kreisler*, is required for segment boundary formation caudal to rhombomere 4 (r4). *val* is normally expressed in r5/6 and is required for cells to contribute to this region. In *val*$^{-}$ mutants, rX, a region one rhombomere in length and of mixed identity, lies between r4 and r7.

While a number of genes involved in establishing rhombomeric identity are known, it is still largely unclear how segmental integrity is established and boundaries are formed. Members of the Eph family of receptor tyrosine kinases and their ligands, the ephrins, are candidates for functioning in rhombomere boundary formation. Indeed, expression of the receptor *ephB4a* coincides with *val* in r5/6, whilst *ephrin-B2a*, which encodes a ligand for EphB4a, is expressed in r4 and r7, complementary to the domain of *val* expression.

Here we show that in *val*$^{-}$ embryos, *ephB4a* expression is downregulated and *ephrin-B2a* expression is upregulated between r4 and r7, indicating that Val is normally required to establish the mutually exclusive expression domains of these two genes. We show that juxtaposition of *ephB4a*-expressing cells and *ephrin-B2a*-expressing cells in the hindbrain leads to boundary formation. Loss of the normal spatial regulation of *eph/ephrin* expression in *val* mutants correlates not only with absence of boundaries but also with the inability of mutant cells to contribute to wild-type r5/6. Using a genetic mosaic approach, we show that spatially inappropriate Eph signalling underlies the repulsion of *val*$^{-}$ cells from r5/6. We propose that Val controls *eph* expression and that interactions between EphB4a and Ephrin-B2a mediate cell sorting and boundary formation in the segmenting caudal hindbrain.

Key words: Zebrafish, Eph signalling, Ephrin, Valentino, Hindbrain segmentation, Rhombomere

INTRODUCTION

The developing vertebrate hindbrain is subdivided into a series of segments, visible transiently as morphological bulges, termed rhombomeres (Vaage, 1969). Rhombomeres form the foundation for segmental patterning of neurones and of cranial neural crest migration into the pharyngeal arches (Kimmel et al., 1985; Metcalfe et al., 1986; Lumsden and Keynes, 1989; Lumsden et al., 1991). Although all rhombomeres produce a similar array of cell types, the molecular identity of each individual rhombomere specifies distinct pattern elements.

Grafting experiments in chick show that boundaries form when rhombomeres from adjacent positions are juxtaposed, but not when rhombomeres from the same axial level are put together (Guthrie and Lumsden, 1991), suggesting that the apposition of cellular territories with distinct identities is required for formation of a boundary. Although the molecules responsible for rhombomere boundary formation are not fully understood, the Eph family of receptor tyrosine kinases and their obligate membrane-bound ligands, the ephrins, are candidates for a role. Using a dominant negative approach it has been shown that disruption of Eph signalling results in embryos showing a loss of the normal segmental restriction of gene expression within the developing hindbrain (Xu et al., 1995). More-recent experiments have shown that mosaic activation of Eph molecules leads to sorting of cells at rhombomere boundaries (Xu et al., 1999). Eph signalling has

also been implicated in segment boundary formation in the paraxial mesoderm (Durbin et al., 1998).

The Eph family of receptors and their ligands, the ephrins, are divided into two classes (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999). Class A ephrins are tethered to the plasma membrane by a glycosyl phosphatidylinositol (GPI) linkage and preferentially bind EphA receptors; class B ephrins (which preferentially bind EphB receptors) have integral transmembrane and intracellular domains and transduce signals back into the ligand-expressing cell via their intracellular domain following receptor binding (Bruckner et al., 1997; Eph Nomenclature Committee, 1997; Davis et al., 1994; Holland et al., 1996). Although binding is promiscuous within a class, different combinations of ligand and receptor interact with different affinities (reviewed in Flanagan and Vanderhaeghen, 1998). EphA4 is the only receptor shown to bind ephrins of both classes (Gale et al., 1996).

Functional studies show that in a variety of developmental contexts, cell repulsion is a major consequence of signalling between Eph receptors and ephrins. For example, localised expression of class A ephrins in the posterior optic tectum and of class B ephrins in the posterior half of each somite is required for repulsion of the axons from EphA receptor-expressing retinal ganglion cells and EphB receptor-expressing trunk motoneurones, respectively (Brennan et al., 1997; Bruckner and Klein, 1998; Monschau et al., 1997; Wang and Anderson, 1997).

Many members of the Eph family of signalling molecules are expressed in rhombomere-restricted patterns (reviewed in Flanagan and Vanderhaeghen, 1998) and are potential downstream targets of segmentally expressed transcription factors. For example, the transcription of *ephA4* in r3 and r5 is under the direct control of the segmentally expressed zinc-finger transcription factor, Krx20 (Theil et al., 1998).

The bzip transcription factor Val, a homologue of mouse Kreisler, is expressed in a stripe in the developing hindbrain corresponding to r5/6 (Moens et al., 1998). Zebrafish embryos homozygous for a null mutation for *val* have no visible rhombomere boundaries caudal to the r3/4 interface (Moens et al., 1996). The *val* mutant phenotype is first detected soon after the end of gastrulation, by reduced expression of *krx20* in r5 (r3 expression is unaffected). Analysis of the positions of reticulospinal neurones and mapping of marker gene expression indicates that the hindbrains of *val*$^{-}$ embryos are shorter than their wild-type and heterozygous siblings by the length of one rhombomere. The mutants have no region of r5 or r6 identity, instead they possess a region one rhombomere in length and of mixed identity, termed rX, that lies between (but does not form morphological boundaries with) r4 and r7 (Moens et al., 1996). Since Eph signalling is implicated in boundary formation, we have determined whether the rhombomeric expression of Eph molecules is controlled by Val, thereby addressing whether Eph receptors and ephrins are downstream of Val in a genetic hierarchy required for rhombomere boundary formation.

In addition to its role in boundary formation, Val function is required cell autonomously for cells to contribute to r5/6 of the developing hindbrain (Moens et al., 1996). When uncommitted *val*$^{-}$ cells are transplanted into the presumptive hindbrain of a wild-type host, the mutant cells are progressively expelled from r5/6. The converse experiment demonstrates that wild-type cells are not properly incorporated into rX of *val*$^{-}$ hosts and instead aggregate into clumps (Moens et al., 1996). These characteristic cell mixing behaviours seen in *val*$^{-}$↔wild-type genetic mosaic embryos reflect the normal cell movements at the rhombomere boundaries, although the movements take place over longer distances in the mosaics. Cell behaviour in the genetic mosaics is also reminiscent of the cell-sorting phenomena observed in chick rhombomere grafting experiments (Guthrie and Lumsden, 1991; Guthrie et al., 1993). Since interactions between Eph receptors and ephrins result in repulsive cell responses and cell sorting (Mellitzer et al., 1999), in this study we examined the possibility that Eph signalling underlies the cell sorting phenomena observed in *val*$^{-}$↔wild-type mosaics.

We show that zebrafish *ephB4a* and *ephrin-B2a* are expressed in complementary rhombomere-restricted domains in the developing hindbrain, and that the receptor and ligand they encode can bind together in situ. We show that Val function is required for activation of *ephB4a* expression in r5/6, and for repression of *ephrin-B2a* expression in this same region. The absence of alternating territories of receptor-expressing and ligand-expressing cells correlates with an absence of boundaries in *val* mutants. Furthermore, juxtaposition of *ephB4a*-expressing and *ephrin-B2a*-expressing cells (in wild-type→*val*$^{-}$ mosaics) results in ectopic boundary formation at the interface. Finally, by overexpressing *ephB4a* or by disrupting bi-directional EphB signalling in *val*$^{-}$→wild-type mosaics, we have rescued the inability of *val*$^{-}$ cells to contribute to r5/6 of wild-type embryos. These results indicate that Val controls expression of Eph molecules, and that repulsive interactions between EphB4a and Ephrin-B2a are important for cell sorting and boundary formation in the caudal hindbrain.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28.5°C on a 14-hour light, 10-hour dark cycle. Wild-type and *val*^{b337} embryos were collected by natural spawning and staged according to Kimmel et al. (Kimmel et al., 1995).

Whole-mount in situ hybridisations, alkaline phosphatase fusion protein detection, immunostaining and actin staining

Whole-mount in situ hybridisations using digoxigenin- or fluorescein-labelled antisense RNA probes were performed essentially as described (Xu et al., 1994; Hauptmann and Gerster, 1994). Hybridisation and detection of alkaline phosphatase fusion protein affinity probes was performed as described (Cheng and Flanagan, 1994). Immunostaining using a 1/500 dilution of a polyclonal anti-GFP antibody (Clontech) was performed as for other antibodies (Xu et al., 1994), except for the inclusion of an amplification step using the ABC kit (Vector Labs). Filamentous actin was stained by overnight incubation of fixed embryos with a 1/40 dilution of Alexa Red-phalloidin (Molecular Probes).

Cloning, synthesis and injection of RNA

The isolation and characterisation of a partial cDNA clone for *ephB4a* has already been described (*rtk5*; Cooke et al., 1997). This partial clone was used as a probe in a high-stringency screen of a 3- to 15-hour random-primed zebrafish cDNA library to isolate partial cDNAs

encompassing the 5′ coding region of *ephB4a*, using standard methods (Sambrook et al., 1989). The complete open reading frame of *ephB4a* was thus contained within two overlapping clones. To synthesise a construct reconstituting the entire open reading frame of *ephB4a*, a PCR-based technique was used (Horton et al., 1990). *ephB4a* was subcloned into the pCS2 vector for in vitro synthesis of RNA. A construct encoding soluble Ephrin-B2a was made by inserting a stop codon in-frame after residue 218. Capped RNA was synthesised and injected as previously described (Durbin et al., 1998). The GenBank Accession Numbers are AJ005026 for *ephB4a*, and AJ004863 for *ephrin-B2a*.

Mosaic analysis

Mosaic analysis was performed as described (Moens et al., 1996; Moens and Fritz, 1999). Donors and hosts were allowed to develop, and the genotype of mutants and siblings was ascertained either by visual inspection between 18 somites (18s) stage and prim-5 stage, by in situ hybridisation with *krx20*, or by PCR and *Pvu*II digestion (val^{b337}, the allele used throughout, is characterised by a *Pvu*II site polymorphism; Moens et al., 1998).

Biotin-labelled donor cells were detected in fixed host embryos using the ABC kit (Vector Labs) and a fluorescein-tyramide substrate (NEN; Moens and Fritz, 1999). Donor embryos injected with RNA encoding GFP (either alone or co-injected with RNA for Eph constructs) were screened for brightly fluorescent cells at epiboly stages. Owing to mosaic distribution of RNA in injected embryos, not all donor cells contain GFP protein. To ensure that essentially all the donor cells were GFP positive, the transplant procedure was monitored frequently with fluorescence microscopy. Localisation of donor cells in host embryos was detected in live embryos by GFP fluorescence or in fixed embryos by immunostaining for GFP or enzymatic staining for β-galactosidase. For statistical analysis (see Table 1), only host embryos in which donor cells were spread throughout the hindbrain and into the spinal cord were counted. The limits of r5 and r6 were ascertained by position with respect to the otic vesicle or by in situ hybridisation with probes for *ephrin-B2a* or *krx20*.

RESULTS

ephB4a and *ephrin-B2a* are expressed in complementary stripes in the presumptive hindbrain and are a receptor-ligand pair

Zebrafish *ephB4a* (previously *rtk5*; Cooke et al., 1997) and *ephrin-B2a* (previously *ephrin-B2*; Durbin et al., 1998) are expressed in complementary stripes throughout the presumptive hindbrain. *ephB4a* expression is strong in presumptive r2 from bud stage, with a sharp anterior boundary, but is graded caudally into presumptive r3 and there is low level expression in presumptive r5/6 at the late two-somite stage. From the three-somite stage (Fig. 1A), this caudal domain corresponds exactly to the r5/6 expression domain of *val* (seen at the seven-somite stage in Fig. 1B). Expression of *val* first appears at bud stage, slightly earlier than the first expression of *ephB4a* in presumptive r5/6 (Moens et al., 1998). Expression of *ephB4a* in the rostral hindbrain becomes restricted to r2, whereas caudally, strong expression of *ephB4a* is maintained in r5/6 between the eight and 18-somite stages (Fig. 1C) after which expression begins to decrease.

ephrin-B2a is expressed in a stripe in the presumptive hindbrain from late gastrulation stages (95% epiboly). Analysis of subsequent stages using probes for *ephrin-B2a* and for *krx20* (Oxtoby and Jowett, 1993) indicates that this stripe is in r4 (Fig. 1D,E). Additional expression in r1 is first detected at the one-somite stage, and by the two-somite stage, there are stripes of *ephrin-B2a* expression in presumptive r1, r4 and r7 (seen at the three-somite stage in Fig. 1D). Levels of expression in r1 and r7 increase during early somite stages (Fig. 1E, F) but are always weaker than the r4 domain. *ephrin-B2a* expression domains in the caudal hindbrain abut the r5/6 domain of *val* expression both rostrally and caudally (Fig. 1F).

Confocal time-lapse image analysis of living zebrafish embryos has demonstrated that the earliest morphological indications of rhombomere boundaries appear at the five-somite stage (for the r3/4 and r4/5 boundaries), shortly afterwards for the r6/7 boundary, and by ten somites for the r5/6 boundary (Moens et al., 1998). Therefore, the expression of *ephB4a* and *ephrin-B2a* in defined stripes in the developing hindbrain prefigures the formation of morphological boundaries.

Complementary expression of Eph receptors and ephrins on either side of a presumptive boundary is required for somite boundary formation (Durbin et al., 1998). Consistent with a role in rhombomere boundary formation, the expression domains of *ephB4a* and *ephrin-B2a* in the developing hindbrain are complementary to each other. Double in situ hybridisations with probes for *ephB4a* and for *ephrin-B2a* show that their domains of expression abut at the r1/2, r3/4, r4/5 and r6/7 boundaries during the stages at which the boundaries are becoming visible (Fig. 2A).

To test if EphB4a and Ephrin-B2a can bind together in vivo, we ectopically expressed *ephB4a* by injecting DNA into two-cell stage embryos, then hybridised fixed embryos at gastrula stages to a fusion protein consisting of the Ephrin-B2a extracellular domain coupled to an alkaline phosphatase reporter (Ephrin-B2a-AP; see Cheng and Flanagan, 1994; Brennan et al., 1997). Alkaline phosphatase activity was detected in injected embryos (Fig. 2B), but not in uninjected embryos (Fig. 2C), indicating that the extracellular domain of Ephrin-B2a recognises and binds to the ectopically expressed receptor. Thus, EphB4a and Ephrin-B2a can recognise and bind each other in situ, and their territories of expression coincide with complementary rhombomeres in the developing hindbrain, suggesting that these molecules interact and signal to each other as a receptor-ligand pair in vivo at rhombomere boundaries.

Val function is required for activation of *ephB4a* expression and inhibition of *ephrin-B2a* expression in r5/6

The coincidence of *val* and *ephB4a* expression, and their complementarity to *ephrin-B2a* expression, suggests that Val may regulate the expression of these genes in the caudal hindbrain. To test this hypothesis, we analysed the expression of *ephB4a* and *ephrin-B2a* in val^{-} embryos. In situ hybridisation analysis of val^{-} embryos indicates that Val is indeed required to spatially regulate *ephB4a* and *ephrin-B2a* expression. The r5/6 stripe of *ephB4a* expression present in wild-type embryos from the late two-somite stage to the prim-5 stage (see Fig. 1A-C, Fig. 3A, and data not shown) is virtually absent in val^{-} embryos throughout the same range of stages (Fig. 3B,C). A low level of *ephB4a* expression is sometimes seen in the caudal hindbrain of val^{-} embryos at early somite stages (Fig. 3B), but this is lost by the 10-somite stage (Fig.

3C). Expression of *ephB4a* in the midbrain, in r2/3 and in the 3rd arch cranial neural crest appears unaffected (Fig. 3B,C).

In contrast to the downregulation of *ephB4a*, *ephrin-B2a* is upregulated in the caudal hindbrain of *val*$^{-}$ embryos. Instead of defined stripes of expression in r1, r4 and r7 (see Fig. 1D-F, Fig. 3D), in *val*$^{-}$ embryos, *ephrin-B2a* is expressed in r1, and in an enlarged caudal domain encompassing r4, rX and r7 (Fig. 3E,F). Initially (at the three- to four-somite stage), expression in r4 is stronger than in rX/r7 (Fig. 3E), however, by eight to ten somites, there is uniform high-level expression of *ephrin-B2a* throughout the caudal hindbrain of *val*$^{-}$ embryos (Fig. 3F). Therefore Val promotes expression of *ephB4a* and

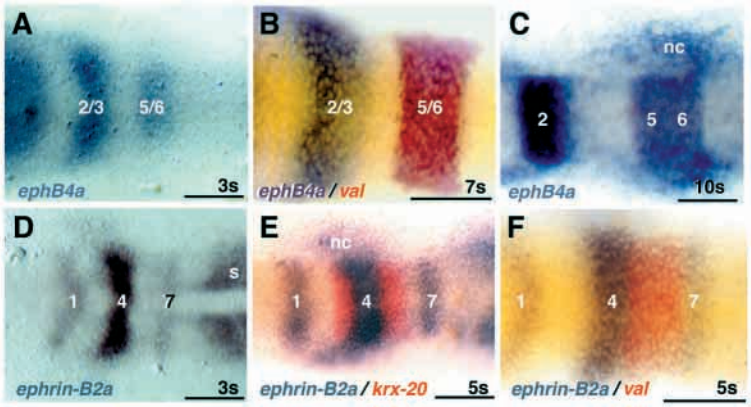

Fig. 1. *ephB4a* and *ephrin-B2a* are expressed in stripes in the presegmented hindbrain. Embryos processed for whole-mount in situ hybridisation (dorsal views, anterior towards the left). Embryonic stage is shown in the bottom right-hand corner (s, number of somites), in situ probe combination in the bottom left-hand corner of each panel. (A) *ephB4a* is expressed in rhombomeres r2/3 and r5/6 at the 3s stage. (B) *val* (red) and *ephB4a* (blue) are co-expressed in r5/6. (C) *ephB4a* expression (blue) is decreased in r3 by 10s, expression in r5/6 is stronger than at early somite stages. (D) *ephrin-B2a* is expressed in r1, r4 and r7 by the 3s stage. (E) *krx20* expression (red) in r3 and r5 abuts the r4 domain but not the r1 or r7 domains of *ephrin-B2a* expression (blue). (F) *ephrin-B2a* expression domains in r4 and r7 (blue) abut *val* expression in r5/6 (red). nc, neural crest; numbers refer to rhombomeres. Scale bars: 50 μm.

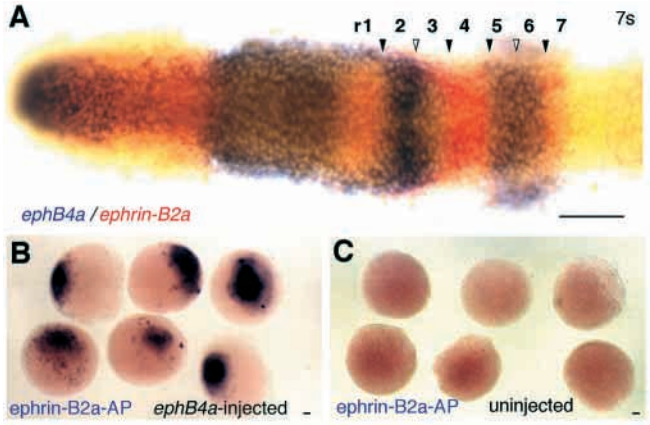

Fig. 2. EphB4a and Ephrin-B2a are a receptor-ligand pair. (A) Dorsal view of a seven-somite stage (7s) wild-type embryo with anterior towards the left, processed for two-colour double in situ hybridisation with probes for *ephB4a* (blue) and *ephrin-B2a* (red). Expression domains of *ephB4a* and *ephrin-B2a* are complementary in the hindbrain, and abut at the r1/2, r3/4, r4/5 and r6/7 boundaries (black arrowheads). The r2/3 and r5/6 boundaries lie within domains of *ephB4a* expression (white arrowheads). (B) 50-70% epiboly embryos overexpressing EphB4a, hybridised to Ephrin-B2a-AP (see text). Localisation of alkaline phosphatase (AP) activity (blue staining) indicates that the Ephrin-B2a-AP affinity probe recognises and binds to ectopic EphB4a in situ. (C) 50-70% epiboly uninjected embryos, hybridised to Ephrin-B2a-AP showing no AP activity. Scale bars: 50 μm.

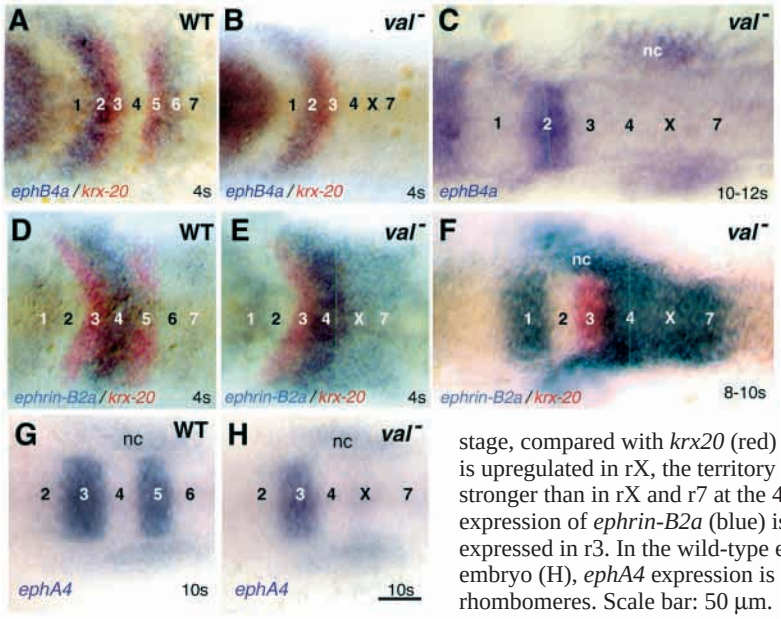

Fig. 3. *ephB4a* expression is induced and *ephrin-B2a* expression is repressed by Val in the caudal hindbrain. Dorsal views of wild-type (WT) and *val*$^{-}$ embryos processed for single colour or two-colour double in situ hybridisation, anterior is towards the left. Embryonic stage is shown in the bottom right-hand corner (s, number of somites), in situ probe combination in the bottom left-hand corner of each panel. (A) *ephB4a* (blue) is expressed in r2/3 and r5/6 in 4s stage wild-type embryos, partially overlapping with *krx20* expression (red) in r3/5. (B) r5 expression of *krx20* (red) and r5/6 expression of *ephB4a* (blue) are downregulated in the *val*$^{-}$ embryo at the 4s stage. (C) Expression of *ephB4a* persists in r2 and 3rd arch neural crest in the *val*$^{-}$ embryo, but is absent from the caudal hindbrain at the 10-12s. (D) *ephrin-B2a* (blue) is expressed in stripes in r1, r4 and r7 in the wild-type embryo at the 4s stage, compared with *krx20* (red) in r3 and r5. (E) In the *val*$^{-}$ embryo, expression of *ephrin-B2a* is upregulated in rX, the territory between r4 and r7 of *val*$^{-}$ embryos. Expression in r4 is stronger than in rX and r7 at the 4s stage. *krx20* (red) is expressed in r3. (F) By the 8-10s state, expression of *ephrin-B2a* (blue) is upregulated in rX and r7 of the *val*$^{-}$ embryo. *krx20* (red) is expressed in r3. In the wild-type embryo (G), *ephA4* is expressed in r3 and r5. In the *val*$^{-}$ embryo (H), *ephA4* expression is severely reduced in rX. nc, neural crest; numbers refer to rhombomeres. Scale bar: 50 μm.

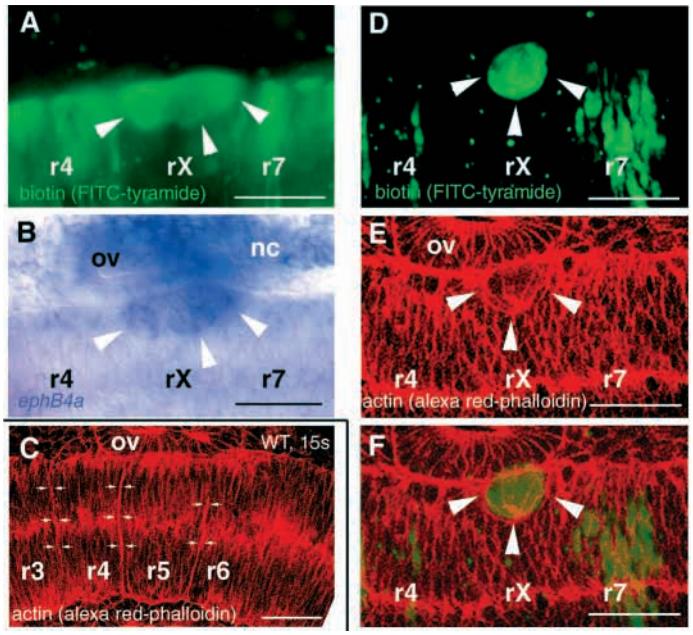

Fig. 4. Wild-type cells express *ephB4a* and form boundaries with *val*⁻ cells in rX of *val*⁻ host hindbrains. Dorsal views of right-hand side (A,B,D-F) or both sides of wild-type (C) hindbrain regions of fixed embryos processed for in situ hybridisation and biotin detection with a fluorescein-tyramide substrate (A,B); or actin detection with a fluorescent phalloidin conjugate (C), plus biotin detection (D-F); anterior is towards the left. (A,B) The wild-type cells that have formed a clump in rX of a *val*⁻ mutant host (green cells in A), express *ephB4a* (purple signal in B, same embryo as in A). Arrowheads in A,B indicate the same cells. (C) Actin accumulation (visualised in red with a fluorescent phalloidin conjugate) is a transient indicator of rhombomere boundary formation. Wild-type embryo at the 15-somite stage showing actin accumulation at r3/4, r4/5 and r5/6 boundaries (pairs of white arrows). (D-F) A boundary forms between the segregated wild-type (*ephB4a* positive) donor cells (green cells in D,F) and the rX cells of the *val* mutant host (*ephrin-B2a* positive). Actin detection with fluorescently labelled phalloidin (red staining in E,F) shows accumulation at the interface of the host cells with wild-type donor cells. Arrowheads in D-F indicate the same cells. nc, neural crest; ov, otic vesicle; r, rhombomere. Scale bars: 50 μm.

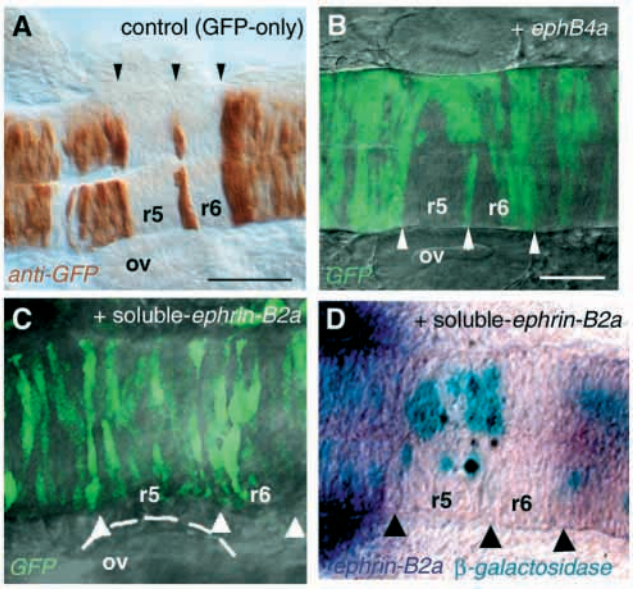

Fig. 5. Blocking of EphB signalling enables *val*⁻ donor cells to contribute to r5/6 of wild-type host embryos. Dorsal views of hindbrain regions of wild-type host embryos containing *val*⁻ donor cells. Anterior is towards the left. (A) Control GFP-overexpressing *val*⁻ donor cells (brown) are excluded from r5/6 of a wild-type host embryo. The *val*⁻ donor embryo was injected with RNA for GFP only. The wild-type host embryo was processed for anti-GFP immunohistochemistry to show localisation of *val*⁻ donor cells. *val*⁻ donor cells are present at the r5/6 boundary, as is often observed. (B) *ephB4a*-overexpressing *val*⁻ donor cells can contribute to r5/6 of wild-type host embryos. The *val*⁻ donor embryo was injected with RNA for full-length *ephB4a* and for GFP. Confocal image of living wild-type host embryo showing localisation of GFP-positive *val*⁻ donor cells (green) superimposed over brightfield image showing position of otic vesicle. (C,D) Soluble Ephrin-B2a-overexpressing *val*⁻ donor cells contribute to r5/6 of wild-type host embryos. The *val*⁻ donor embryos were injected with RNA for soluble Ephrin-B2a + GFP (C) or soluble Ephrin-B2a + GFP + β-galactosidase (D). (C) Confocal image of living wild-type host embryo showing localisation of GFP-positive *val*⁻ donor cells (green) superimposed over brightfield image showing position of otic vesicle (broken line). *val*⁻ donor cells are evenly distributed throughout r5 and r6. (D) Fixed wild-type host embryo showing localisation of β-galactosidase-positive *val*⁻ donor cells (blue) and in situ hybridisation for *ephrin-B2a* (purple), showing r4 and r7 stripes. The donor cells are located between r4 and r7. Arrowheads show positions of (from left to right) r4/5, r5/6 and r6/7 boundaries. ov, otic vesicle; r, rhombomere. Scale bars: 50 μm.

represses expression of *ephrin-B2a* in the territory between r4 and r7.

Val function is also required for r5 expression of *ephA4*, a second Eph receptor able to bind Ephrin-B2a (Durbin et al., 1998). *ephA4* is expressed in r3 and r5 in the wild-type embryo (Fig. 3G), but its r5 expression domain is severely reduced in *val*⁻ embryos (Fig. 3H). It has previously been shown that *ephA4* expression in r5 is under the direct transcriptional control of krx20 (Theil et al., 1998). Since *krx20* expression is also downregulated in *val*⁻ embryos (Moens et al., 1998), the effect of Val on *ephA4* expression is likely to be indirect.

The loss of alternating territories of receptor-expressing and ligand-expressing cells in the caudal hindbrain of *val*⁻ embryos correlates with the loss of some of the rhombomere boundaries associated with this mutant phenotype, and suggests that Eph signalling functions downstream of *val* in boundary formation in the caudal hindbrain.

Genetic mosaic studies reveal a link between Eph signalling, cell sorting and boundary formation in the hindbrain

Genetic mosaic experiments show that *val*⁻ cells cannot contribute to r5/6 of wild-type hindbrains and that wild-type cells do not contribute normally to rX of *val*⁻ embryos (Moens et al., 1996). We propose that spatial disruption of the normal signalling interfaces between *ephB4a*- and *ephrin-B2a*-expression domains is responsible for the loss of morphological boundaries in *val*⁻ embryos and for the characteristic cell-sorting behaviours observed in *val*⁻↔wild-type genetic mosaics.

Wild-type donor cells that form clumps in rX of *val* mutant

host embryos autonomously express *krx20* (when located at the rostral end of rX) and *val*, genes appropriate for cells in the equivalent region of a wild-type embryo (Moens et al., 1996; Moens et al., 1998). In situ hybridisations performed on wild-type→*val*⁻ genetic mosaic embryos show that *ephB4a* is also autonomously expressed by the clumps of wild-type donor cells present in rX (Fig. 4A,B). Thus, when wild-type cells are transplanted into rX of *val*⁻ hosts, a new Eph receptor/ephrin interface is established between the transplanted *ephB4a*-positive cells and the surrounding host cells which ectopically express *ephrin-B2a* (see Fig. 3E, F). This ectopic interaction between EphB4a and Ephrin-B2a may explain the repulsion of wild-type donor cells from the mutant host rX environment, resulting in their aggregation into clumps.

To assess whether a boundary forms at ectopic EphB4a/Ephrin-B2a interfaces established in wild-type→*val*⁻ mosaic embryos, we looked at actin localisation. Wild-type embryos stained with fluorescent phalloidin (which visualises filamentous actin) show an accumulation of actin at the rhombomere boundaries between approximately the 12-somite and 18-somite stages (Fig. 4C). This actin accumulation is transient and is not synchronous for all boundaries. Phalloidin staining of mosaic embryos demonstrates an accumulation of actin at the interface between the wild-type cell clumps and surrounding host cells (Fig. 4D-F). This observation indicates that a boundary is established at the interface between *ephB4a*-positive cells (clumped wild-type donor cells) and *ephrin-B2a*-positive cells (the surrounding host rX cells).

Our data indicate that in both wild-type and genetic mosaic embryos, cell repulsion and boundary formation occur when *ephB4a*-expressing (receptor-positive) cells are apposed to *ephrin-B2a*-expressing (ligand-positive) cells.

EphB4a can partially rescue the inability of *val*⁻ cells to contribute to r5/6

When *val*⁻ cells are transplanted into wild-type embryos, we predict that Eph signalling will be activated inappropriately, since *ephrin-B2a* will be expressed on the *val*⁻ donor cells in r5/6, and *ephB4a* will be expressed in r5/6 of the wild-type host hindbrain. This activation may underlie the repulsion of *val*⁻ mutant cells from wild-type r5/6. To test whether the loss of *ephB4a* expression in *val*⁻ donor cells contributes to their repulsion from r5/6, we reconstituted *ephB4a* expression in *val*⁻ donor embryos and analysed donor cell distribution in wild-type host hindbrains.

For these experiments, we injected mRNA encoding *ephB4a* into *val*⁻ donor embryos and green fluorescent protein (GFP), translated from co-injected RNA, was used as a tracer to identify cells ectopically expressing *ephB4a*. Co-localisation of protein products from these co-injected mRNAs indicates that the majority of GFP-expressing cells also express *ephB4a* (data not shown). To test the longevity of ectopic EphB4a protein, *ephB4a* mRNA-injected donor embryos were hybridised to the ephrin-B2a-AP affinity probe. Ectopic EphB4a protein could be detected up to and beyond those stages at which rhombomere boundaries are forming (data not shown). To control for nonspecific effects of mRNA overexpression on donor cell behaviour, we injected *val*⁻ donors with *GFP* mRNA alone. In the vast majority of cases, GFP-overexpressing *val*⁻ donor cells did not contribute to r5/6 of wild-type hosts (Fig. 5A, Table 1). The transplanted cells appeared healthy and behaved in an identical fashion to *val*⁻ donor cells injected with lineage tracers (Moens et al., 1996). *val*⁻ donor cells do frequently contribute to the r5/6 boundary region (Fig. 5A and Moens et al., 1996), indicating that cells in the boundary region are phenotypically distinct from cells in the body of the rhombomere.

When we overexpressed *ephB4a* and GFP in *val*⁻ donor embryos and transplanted cells from such individuals into wild-type hosts, we found that donor cells were often present in r5 and/or r6 (Fig. 5B, Table 1). These donor cells contributed to r6 about twice as frequently as to r5, and often exhibited abnormal morphology and unilateral distribution (Fig. 5B; see Discussion). In 50% of the wild-type hosts of mutant donors in which there was a good spread of donor cells from hindbrain to spinal cord, the *ephB4a*/GFP-overexpressing *val*⁻ donor cells were present in r5 and/or r6, the region from which GFP-overexpressing or lineage-labelled *val*⁻ donor cells are excluded. Therefore, overexpression of *ephB4a* can at least partially rescue the inability of *val*⁻ donor cells to contribute to r5/6 of a wild-type host, indicating that EphB4a is a downstream effector of *val*, whose function is required for proper cell sorting in the caudal hindbrain.

Blocking all EphB signalling efficiently rescues the inability of *val*⁻ cells to contribute to wild-type r5/6

Since overexpression of *ephB4a* by *val*⁻ donor cells resulted in an incomplete rescue of the *val*⁻→wild-type mosaic phenotype (see Discussion), we decided to use a complementary approach to test more rigorously the role of ectopic EphB signalling in the repulsion of *val*⁻ donor cells from wild-type r5/6. To do this, we used a soluble Ephrin-B2a construct (Durbin et al., 1998), which disrupts bi-directional signalling through all EphB molecules. Soluble B-class ephrins have been shown to block bi-directional signalling in other systems by preventing receptor clustering and competitively inhibiting endogenous ligand binding (Krull et al., 1997; Durbin et al., 1998). We injected mRNA encoding soluble Ephrin-B2a into *val*⁻ donor embryos, transplanted cells into wild-type hosts and analysed donor cell distribution in wild-type host hindbrains. In these experiments, GFP translated from co-injected RNA was used as a tracer to follow donor cells in living embryos. RNA for β-galactosidase was also injected in some experiments to enable localisation of donor cells in fixed embryos.

When soluble Ephrin-B2a and GFP were overexpressed in *val*⁻ donor embryos and cells from such individuals were transplanted into wild-type hosts, donor cells were often present in r5 and/or r6 and displayed a near-normal morphology (Fig. 5C,D; Table 1). In 62.5% of the wild-type hosts of mutant donors in which there was a good spread of

Table 1. Distribution of *val*⁻ donor cells in wild-type host hindbrains after injection of mRNA

	Wild-type hosts		
val⁻ donors injected with:	Gaps in r5/6	Cells in r5/6	*n*
ephB4a + GFP RNA	50%	50%	28
Soluble *ephrin-B2a* + tracer RNA	37.5%	62.5%	32
GFP RNA	91%	9%	11

n, total number of wild-type host embryos analysed (only hosts in which mutant donor cells were spread throughout hindbrain and into spinal cord were included). Data are pooled from several independent experiments.

donor cells from hindbrain to spinal cord, the soluble Ephrin-B2a-positive/GFP-positive *val*⁻ donor cells were present in r5 and/or r6, the region from which *val*⁻ donor cells are normally excluded. Therefore, blocking EphB signalling in *val*⁻→wild-type mosaics can rescue the inability of *val*⁻ donor cells to contribute to r5/6 of a wild-type host. This suggests that signalling between Ephrin-B2a and its cognate receptors (e.g. EphB4a, EphA4) plays a role in cell sorting in the *val*⁻→wild-type genetic mosaic embryos, and, more generally, may be required for establishing and maintaining segmental integrity during normal development.

DISCUSSION

Our observations of gene expression and cell behaviour in *val*⁻ embryos and in genetic mosaics indicate that the spatial control of *ephB4a* and *ephrin-B2a* expression by Val is crucial for cell sorting and boundary formation in the caudal hindbrain (see Fig. 6). We present several lines of evidence to support this. Firstly, EphB4a and Ephrin-B2a can bind together in vivo and are expressed in complementary domains with boundaries forming at the interface between these domains (Fig. 6A). Second, Val is required to induce complementary expression of *ephB4a* and *ephrin-B2a*, such that in *val*⁻ embryos, there is a loss of Eph/ephrin interfaces and a corresponding loss of boundaries (Fig. 6B). Third, an ectopic EphB/ephrin-B interface correlates with ectopic boundary formation (i.e. when wild-type cells populate rX of a *val*⁻ embryo; Fig. 6C). Fourth, the *val*⁻→wild-type mosaic phenotype (expulsion of donor cells from r5/6, Fig. 6D) is partially rescued by overexpressing *ephB4a* in *val*⁻ donors (Fig. 6E). Finally, blocking all bi-directional EphB signalling results in a more complete rescue, allowing *val*⁻ cells to contribute to wild-type r5/6 in most cases (Fig. 6F and Table 1). These results suggest that the repulsion of *val*⁻ cells from wild-type r5/6 is mediated by signalling between EphB molecules.

Control of EphB expression by Val is important for cell sorting and boundary formation

The complementary expression of *ephB4a* and *ephrin-B2a* arises after the r5/6 expression domain of *val* is established, but before the first appearance of morphological boundaries. Our results show that the receptor EphB4a requires Val for its expression in r5/6, whilst Val inhibits expression of the ligand Ephrin-B2a in these rhombomeres, either directly or indirectly. In some *val*⁻ embryos a very low level of *ephB4a* expression is detectable at early stages (e.g. four-somite stage), but is not detected later (e.g. ten-somite stage). This transient, low-level expression of *ephB4a* seen in rX of some *val*⁻ embryos may be a result of transcriptional activation by early-expressed *krx20* whose expression in the caudal hindbrain is initiated normally in *val*⁻ embryos before being lost (Moens et al., 1996). Even though some *ephB4a* expression may be retained in some *val*⁻ embryos, the high levels of *ephrin-B2a* in the same cells may suppress any activity of *ephB4a* co-expressed transiently at a low level (see below, and Bohme et al., 1996).

In the absence of Val, the consequent loss of Eph receptor/ephrin expression domain interfaces correlates with a failure of the r4/5 and r6/7 boundaries to form. The r5/6 boundary also fails to form in *val*⁻ embryos, probably because r5 and r6 identities are not specified. However, we have not addressed formation of the r5/6 boundary in this study, since it is not normally established by interactions between EphB4a and Ephrin-B2a. Further evidence supporting a role for EphB4a↔Ephrin-B2a signalling in boundary formation comes from the wild-type→*val*⁻ genetic mosaics, in which an ectopic boundary forms at the interface between the *ephB4a*-positive wild-type donor cells and the *ephrin-B2a*-positive host rX cells. This result suggests that introduction of an Eph/ephrin interface is sufficient to establish boundary features, even in the normally unsegmented caudal hindbrain of a *val*⁻ embryo.

Alterations in Eph signalling can also explain the behaviour of cells in *val*⁻→wild-type mosaics. When *val*⁻ donor cells are transplanted into a wild-type host hindbrain, those donor cells that find themselves in r5/6 will not be able to synthesise a functional Val transcription factor. The absence of Val in these cells means that they will also not activate *ephB4a* expression and will not repress *ephrin-B2a* expression. The *ephrin-B2a*-expressing donor cells will be surrounded by 'hostile' *ephB4a*-positive host cells of r5/6 and may therefore be repelled into the adjacent 'permissive' *ephrin-B2a*-positive host rhombomeres 4 and 7. Donor cells tend to pile-up at the edges of r5 and r6 (e.g. see Fig. 5A), suggesting that the cells move no further than to escape from rhombomeres 5 and 6. *val*⁻ donor cells are also often present at the wild-type r5/6 boundary, suggesting a difference in repulsive/adhesive properties between the body of the rhombomere and the boundary region. Indeed the unique extracellular matrix composition of rhombomere boundaries (as seen in the chick; Heyman et al., 1995) may lead to a restriction to the movement of repelled *val*⁻ donor cells in boundary regions.

In contrast to the *val*⁻→wild-type mosaics, wild-type donor cells in rX of a *val*⁻ host are not repelled to adjacent segments, indeed there are no adjacent 'permissive' (*ephB4a*-positive) segments, perhaps explaining why the wild-type donor cells aggregate into clumps in rX. Alternatively, the different cell behaviours of donor cells in the *val*⁻→wild-type mosaics (repulsion) and wild-type→*val*⁻ mosaics (clumping) may be a result of differences in the downstream signalling pathways activated by B-class ephrins and Eph receptors, respectively.

Reconstituting *ephB4a* expression in *val*⁻ donor cells increases the likelihood of incorporation into wild-type r5/6

When *val*⁻ donors were injected with mRNA for *ephB4a*, 50% of hosts analysed showed donor cells in r5 and/or r6, suggesting that cell-autonomous expression of *ephB4a* increases the probability that cells will contribute to r5/6. However, the *ephB4a*-positive *val*⁻ donor cells in wild-type r5/6 sometimes form clumps and are often unilateral (see Fig. 5B), in contrast to the appearance of such cells elsewhere in the host hindbrain, where they have a characteristic elongated morphology and form bilaterally symmetric clones. The unilateral appearance of the rescued cells suggests that some degree of repulsion may be taking place between the rescued cells and their wild-type neighbours. One possible explanation for this is that by the stages at which the host embryos were analysed (18 somites to prim-5), ectopic *ephB4a* expression is decreasing, so *val*⁻ donor cells may belatedly be repelled from host r5/6.

It should be noted that the *ephB4a*-overexpressing *val*⁻

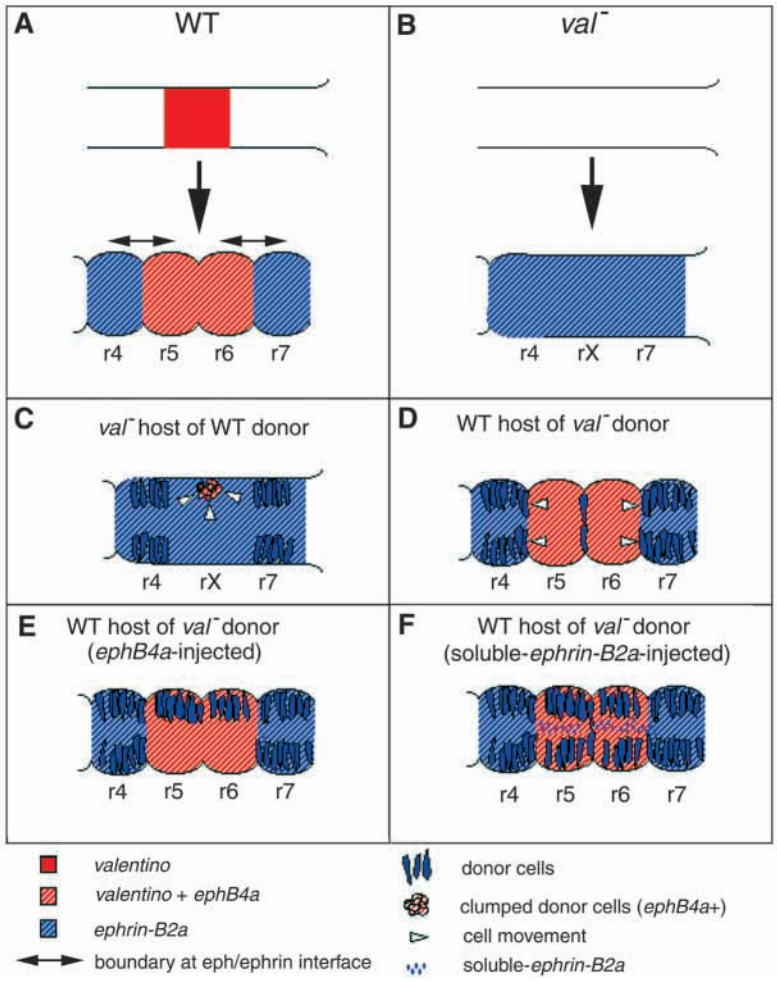

Fig. 6. Putative interactions between EphB4a and Ephrin-B2a in the caudal hindbrain of wild-type, *val*⁻ and genetic mosaic embryos. Dorsal views of developing caudal hindbrains, anterior is towards the left. (A) In the presegmented wild-type hindbrain (top), *val* (red) is expressed in the region that will give rise to r5/6; Val subsequently induces expression of *ephB4a* in presumptive r5/6 (red hatching, bottom), and represses *ephrin-B2a*, restricting its expression to r4 and r7 (blue hatching). Repulsive interactions between EphB4a and Ephrin-B2a at the r4/5 and r6/7 interfaces (double-headed black arrows) result in formation of rhombomere boundaries. (B) In the presegmented *val*⁻ hindbrain (top), no functional Val is present; in the absence of Val, *ephB4a* expression is not induced and *ephrin-B2a* expression is not repressed between r4 and r7 (bottom). *ephrin-B2a is* expressed throughout the caudal hindbrain of *val*⁻ embryos (blue hatching) and the consequent loss of alternation of Eph receptor-expressing and ephrin-expressing domains correlates with an absence of rhombomere boundaries. (C) Wild-type donor cells in rX of a *val*⁻ host autonomously express *ephB4a* (red hatching) and are repelled (white arrowheads) by surrounding *ephrin-B2a*-expressing host cells (blue hatching), leading to aggregation of the donor cells into clumps. A boundary forms at the interface between the receptor-expressing and ligand-expressing cell populations. (D) The expulsion (white arrowheads) of *val*⁻ donor cells (solid blue) from wild-type r5/6 (red hatching) is due to repulsive interactions between Ephrin-B2a, which we propose is expressed by the mutant donor cells, and EphB4a expressed by the host cells in r5/6. (E) Reconstituting *ephB4a* expression in *val*⁻ donor cells (blue) leads to a partial rescue of the *val*⁻→wild-type genetic mosaic phenotype, enabling donor cells to contribute to r5 and/or r6 (red hatching). (F) Blocking of EphB signalling using soluble Ephrin-B2a (blue dots) rescues the inability of *val*⁻ donor cells (blue) to contribute to r5/6 of a wild-type host hindbrain (red hatching), indicating that ectopic EphB signalling is indeed responsible for the expulsion of *val*⁻ donor cells from r5/6 of wild-type host hindbrains (D).

donor cells do not necessarily reflect the wild-type situation, since we have not ruled out the possibility that these cells also express *ephrin-B2a*. Evidence from in vitro co-transfection assays suggests that activity of B-class Eph receptors is reduced when ephrin B ligands are co-expressed in the same cell (Bohme et al., 1996). This raises the possibility that signalling by overexpressed EphB4a may be compromised in the *val*⁻ donor cells by co-expression of Ephrin-B2a. This co-expression may explain why there is only partial, and abnormal, contribution of these cells to r5 and r6. It may also help explain another curious observation that the *ephB4a*-expressing *val*⁻ cells appear to contribute normally to r4 and r7, both of which express *ephrin-B2a*. Again, we suggest that co-expression of ligand and receptor may alter the Eph signalling-mediated events within these cells.

It is interesting to note that *ephB4a*-overexpressing *val*⁻ donor cells incorporate into wild-type host r6 approximately twice as often as into r5. The *ephA4* receptor, which also binds *ephrin-B2a* (Durbin et al., 1998) is expressed with a similar timecourse to *ephB4a* in r5, but is not expressed in r6. Similar to *ephB4a*, expression of *ephA4* is downregulated in rX of *val*⁻ embryos. Perhaps EphB4a and EphA4 interact within the cells of r5 such that signalling through both receptors is required for incorporation of cells into r5. Overexpression of both receptors together may increase the probability that *val*⁻ donor cells incorporate into r5. The possibility of a role for other Eph family members is supported by the observation that blocking all bi-directional ephrin-B-mediated signalling produces a more efficient rescue of the *val*⁻→wild-type mosaic phenotype than simply upregulating expression of *ephB4a*.

A pan-EphB signalling blocker efficiently rescues the *val*⁻→wild-type mosaic phenotype

Blocking all bi-directional EphB signalling results in a more complete rescue of the *val*⁻→wild-type mosaic phenotype than reconstituting *ephB4a* expression, confirming the importance of the EphB signalling pathway in mediating expulsion of *val*⁻ cells from wild-type r5/6. Using soluble Ephrin-B2a to prevent interactions between Ephrin-B2a and receptors such as EphB4a and EphA4 (Krull et al., 1997), the repulsion of *val*⁻ donor cells from wild-type r5/6 can be overcome, allowing the cells to contribute to this region. The improved efficiency of this rescue, compared with that achieved with *ephB4a* overexpression, suggests a possible involvement for additional family members such as *ephA4*. It will be interesting to see which cell types these rescued donor cells are able to give rise to, as this may provide a clue as to the importance of segmentation in hindbrain neuronal patterning.

Our results implicating Eph signalling in the expulsion of *val*⁻ donor cells from r5/6 of wild-type host hindbrains suggest that similar repulsive interactions in the wild-type hindbrain function to separate cells from adjacent rhombomeres. The cell

movements seen in *val*$^{-}$→wild-type mosaics take place over relatively larger distances than the movements that occur during normal boundary formation, nevertheless, they appear to be governed by the same molecular interactions. Supporting a role for Eph-mediated repulsion in rhombomere-dependent cell sorting, recent work using a co-culture system of zebrafish cells expressing different Eph receptor and ephrin constructs has shown that bi-directional Eph signalling results in restriction of cell intermingling and loss of intercellular communication (Mellitzer et al., 1999). Interactions between activated Eph molecules and components of the cytoskeleton are likely to mediate the repulsive response (reviewed in Bruckner and Klein, 1998).

Val may mediate proliferation and fate-determination independently of Eph signalling

Eph molecules are important downstream targets in the Val pathway, providing a mechanistic link between position along the anteroposterior axis and the sorting of cells into segments. However, Val function is required not only for proper boundary formation in the caudal hindbrain, but also for expansion of a precursor region of one segment in length into definitive r5/6. Since no direct link between Eph signalling and cell division has been demonstrated, we suspect that Eph molecules do not play a role in this process. Segmental identity is likely to be imparted by Hox genes (e.g. Bell et al., 1999), and so targets of Val (other than Eph molecules; such as Hox genes), may be responsible for growth of the protosegment rX into r5/6 and for acquisition of appropriate regional identity. Indeed, Kreisler/Val has been shown to regulate expression of *hoxb3* in both the mouse (Manzanares et al., 1997) and the zebrafish (Prince et al., 1998).

Eph signalling does not simply mediate odd/even alternations of rhombomeric cell-surface properties

Grafting experiments in chick suggest that an odd/even segmental alternation of cell-surface properties normally restricts the mixing of cells from adjacent rhombomeres and show that juxtaposition of odd and even rhombomeres is required for boundary formation (Guthrie and Lumsden, 1991; Guthrie et al., 1993). Could Eph molecules provide the basis for this odd/even alternation of cell-surface properties shown experimentally in the chick? In mouse and *Xenopus*, there is alternating expression of an interacting Eph receptor-ligand pair, with *ephrin-B2* expression in r2/4/6 (Bergemann et al., 1995; Smith et al., 1997) and *ephA4* expression in r3/5 (Gilardi-Hebenstreit et al., 1992; Hirano et al., 1998; Nieto et al., 1992; Winning and Sargent, 1994; Xu et al., 1995). Thus, Ephrin-B2 and EphA4 are good candidates for mediating cell sorting between odd and even rhombomeres, at least in mouse and *Xenopus*.

We have shown that zebrafish Ephrin-B2a is expressed in r1/4/7, and an interacting receptor, EphB4a, is expressed in double-rhombomere domains r2/3 and r5/6. This interacting ligand-receptor pair do not, therefore, observe a simple single-rhombomere-alternation of expression domains. Zebrafish EphA4 (which also interacts with Ephrin-B2a) is, like its counterpart in mouse and *Xenopus*, expressed in odd-numbered rhombomeres r3 and r5 (Xu et al., 1995), partially overlapping with *ephB4a* expression domains. There are also additional, yet to be characterised, members of the zebrafish Eph family expressed in rhombomere-restricted patterns. For instance, a second zebrafish *ephrin-B2* orthologue, *ephrin-B2b*, is expressed in r1 and r4 (L. D., unpublished observations). This complexity may in part be the consequence of a postulated genome duplication in the lineage leading to teleosts (Amores et al., 1998; Meyer and Malaga-Trillo, 1999). One might predict that an ephrin able to interact with EphA4 but not with EphB4a might be expressed in r2 and r6, and be required for formation of the r2/3 and r5/6 boundaries. Thus, the presence of additional Eph family members in the zebrafish may result in increased levels of complexity or redundancy in the genetic regulation of hindbrain patterning, compared with other vertebrates.

In this study, we have addressed the role of the Val bzip transcription factor in establishing segment boundaries in the hindbrain. We have shown that Val regulates rhombomere-restricted complementary expression domains of an EphB receptor-ligand pair in the developing hindbrain. This receptor-ligand pair interact repulsively at the interfaces of their expression domains, leading to cell-sorting and subsequent formation of rhombomere boundaries.

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