Her5 acts as a prepattern factor that blocks *neurogenin1* and *coe2* expression upstream of Notch to inhibit neurogenesis at the midbrain-hindbrain boundary

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Summary

Neurogenesis in both vertebrates and invertebrates is tightly controlled in time and space involving both positive and negative regulators. We report here that the bHLH factor Her5 acts as a prepattern gene to prevent neurogenesis in the anlage of the midbrain/hindbrain boundary in the zebrafish neural plate. This involves selective suppression of both *neurogenin1* (*ngn1*) and *coe2* mRNA expression in a process that is independent of Notch signalling, and where inhibition of either *ngn1* or *coe2* expression is sufficient to prevent neuronal differentiation across the midbrain-hindbrain boundary. A *ngn1* transgene faithfully responds to Her5 and deletion analysis of the transgene identifies an E-box in a *ngn1* upstream enhancer to be required for repression by Her5. Together our data demonstrate a role of Her5 as a prepattern factor in the spatial definition of proneural domains in the zebrafish neural plate, in a manner similar to its *Drosophila* homologue Hairy.

Key words: Her5, Hairy, Midbrain-hindbrain boundary, Zebrafish, Neurogenesis, Pre-patterning

Introduction

Neuronal differentiation in the vertebrate central nervous system (CNS) is under strict temporal and spatial control. First, populations of 'pioneer neurons' are defined at specific places during early stages and build a primary scaffold of neuronal tracts and connections (Easter et al., 1994). Later, differentiation spreads in the neural tube (Hollyday, 2001). The establishment of the neuronal differentiation pattern thus requires the precise coordination of patterning and neurogenesis.

Neurogenesis has been extensively studied in *Drosophila* (reviewed by Campos-Ortega, 1993). First, populations of cells competent to undergo neurogenesis are defined giving rise to so-called 'proneural fields' or 'proneural clusters', within which neuronal progenitors are selected. Progenitor selection relies on lateral inhibition mediated by the Notch receptor. Cells expressing high levels of the Notch ligand Delta will commit to neuronal differentiation and at the same time inhibit the neighbouring cells to enter the neuronal program (Simpson, 1997). After binding of Delta, the Notch receptor undergoes intra-membranous cleavage to generate a Notch Intra-Cellular Domain (NICD), which translocates to the nucleus, binds members of the Suppressor-of-Hairless (SU(H)) family and activates transcription of downstream effectors (Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998; Bray and Furriols, 2001; Mumm and Kopan, 2000). Major Notch targets are basic helix-loop-helix (bHLH) transcriptional repressors of the Enhancer-of-Split [E(Spl)] family, which prevent activity of proneural factors driving neurogenesis (Fisher and Caudy, 1998). Cells expressing high levels of Delta, by contrast, will maintain activity of proneural factors (such as the bHLH proteins Achaete, Scute and Atonal) and Delta transcription. Thus, initial differences in the levels of Delta expression among the cells of a proneural cluster are amplified, leading to the reinforcement of a neuronal fate.

Current evidence suggests that neurogenesis uses similar molecules in vertebrates as in invertebrates (Appel and Chitnis, 2002; Chitnis, 1999; Lewis, 1998). In these species, a number of Notch-, Delta-like and bHLH-encoding genes are involved in similar cascades within the neurogenic domains of the neural tube (Blader et al., 1997; Chitnis et al., 1995; Chitnis and Kintner, 1996; de la Pompa et al., 1997; Haddon, 1998; Ma et al., 1996; Takke et al., 1999). Vertebrate bHLH factors include the Neurogenin and Ath (Atonal-related), Ash (Achaete-Scute-related), and Hairy/E(spl) (Hes and Hairy in mouse and chicken, Her in zebrafish) subclasses, of which the first three have proneural activity, while most Hairy/E(spl) factors inhibit neurogenesis (Bertrand et al., 2002; Fisher and Caudy, 1998; Kageyama and Nakanishi, 1997; Lee, 1997).

Proneural bHLH factors are expressed with partially overlapping patterns. However, whether they play redundant or rather combinatorial roles remains in most cases unknown (Cau and Wilson, 2003; Mizuguchi et al., 2001; Parras et al., 2002).

Although lateral inhibition is a major and evolutionarily conserved mechanism in restricting the extent of neurogenesis within proneural fields, the prepatterning mechanisms that specify these fields in the first place seem more variable and are less well understood. Both in invertebrates and vertebrates, a combination of positive and negative factors, the expression of which is controlled by the embryonic patterning machinery, establishes a grid of neurogenesis-competent domains along the anteroposterior (AP) and dorsoventral (DV) axes. Several cases of neuronal inhibition independent of lateral inhibition have been reported in vertebrates (Bellefroid et al., 1998; Bourguignon et al., 1998; Andreazzoli et al., 2003). Many local neurogenesis repressors belong to the Hairy family (Bally-Cuif and Hammerschmidt, 2003; Sasai, 1998). For example, Hairy restricts neuronal competence within the *Drosophila* peripheral nervous system (Fisher and Caudy, 1998). In a reminiscent manner, *Xenopus* ESR6e prevents neurogenesis in the embryonic superficial ectoderm (Chalmers et al., 2002), and mouse Hes1 negatively controls neurogenic domains within the olfactory epithelium (Cau et al., 2000). Hairy and the related E(Spl) proteins distinguish themselves from other bHLH factors by a proline residue in their DNA-binding domain and a C-terminal WRPW tetrapeptide. In contrast to E(spl), however, they can act independently of Notch signalling.

The midbrain-hindbrain (MH) is an interesting domain of the neural plate to study the mechanisms controlling the spatial extent of neurogenesis (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001), as the midbrain-hindbrain boundary (MHB) is characterised by delayed neuronal differentiation (Bally-Cuif et al., 1993; Palmgren, 1921; Vaage, 1969; Wullimann and Knipp, 2000). This 'intervening zone' (IZ) separates midbrain from hindbrain neuronal clusters and is believed to serve as a pool of precursor cells for the construction of MH structures during development. The functional importance of the IZ is highlighted in *Hes1*$^{-/-}$;*Hes3*$^{-/-}$ mouse mutants, where MH precursor cells differentiate prematurely, leading to the development of an abnormally small MH and to the lack of specific MH neuronal populations such as midbrain dopaminergic neurons, cranial neurons III and IV, or the locus coeruleus (Hirata et al., 2001). We recently demonstrated that, in the zebrafish, the Hairy/E(spl)-like bHLH transcription factor Her5 is crucially required for IZ formation at the onset of neurogenesis (Geling et al., 2003). *her5* (Müller et al., 1996) is expressed from 70% epiboly onwards in a domain of the neural plate that prefigures the early IZ and separates the first anterior neuronal cluster (ventrocaudal cluster, vcc) from presumptive motor- and lateral neurons in rhombomere 2 (r2M and r2L) (Fig. 1A-B′) (Geling et al., 2003). Impairment of Her5 activity leads to the ectopic generation of cells expressing *neurogenin1* (*ngn1*) and later of differentiated neurons across the medial (future ventral) aspect of the IZ (Fig. 1C,C′) (Geling et al., 2003). Thus, Her5 is crucial in inhibiting neurogenesis within the IZ and in maintaining the full MH precursor pool in zebrafish. However, to date, the molecular mode of action of Her5 has not been analysed.

We demonstrate that Her5 does not inhibit neurogenesis as a downstream effector of Notch. Rather, it blocks the establishment of a proneural field at the MHB. This is in striking contrast to most E(spl)-like factors, and identifies Her5 as a prepattern factor, similar to *Drosophila* Hairy. We further uncovered a cross-regulatory loop between the expression of Her5 and the non-basic HLH transcription factor Coe2, a likely orthologue of mammalian EBF2 (Dubois and Vincent, 2001). Epistasis experiments in backgrounds where Ngn1 or Coe2 activities are blocked demonstrate that *coe2* and *ngn1* are independent targets of Her5, but that blocking expression of either one of these genes is sufficient to prevent neuronal differentiation across the medial IZ. Finally, using reporter assays in transgenic embryos, we identify an E-box in a *ngn1* enhancer as the main element mediating repression of *ngn1* expression across the medial IZ in vivo.

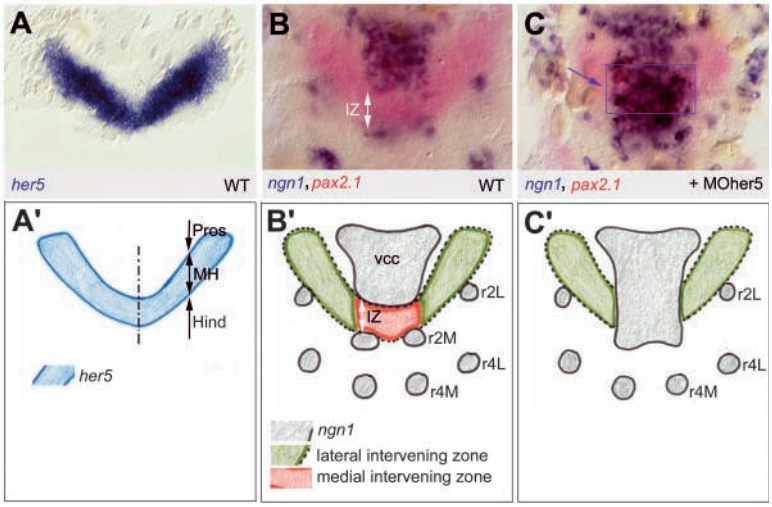

Fig. 1. Her5 activity at the midbrain-hindbrain boundary and nomenclature. All top views (A-C) are flat-mounted embryos at the three-somite stage, dorsal views with anterior upwards, revealed by in situ hybridisation for expression of the genes indicated (colour-coded, left corner) (see also Geling et al., 2003). Bottom panels (A′-C′) are interpretative drawings of the embryos in A-C to introduce the specific nomenclature used in this work. At the three-somite stage, *her5* expression (A) encompasses most of the presumptive MH (Tallafuss and Bally-Cuif, 2003) and separates the first *ngn1*-positive clusters (B) within the anterior neural plate. These are the ventrocaudal cluster (vcc), located in the basal diencephalon and anterior midbrain, the presumptive motoneurons (r2M) and lateral neuronal precursors (r2L) in rhombomere 2. The non-neurogenic domain identified by *her5* positivity and *ngn1* negativity around the MHB is called intervening zone (IZ) (white arrow in B,B′). (C) Upon blocking Her5 activity by injection of a *her5* morpholino (MO^{her5}) into wild-type embryos, the medial (future basal) part of the IZ domain is bridged by ectopic *ngn1*-positive cells (blue arrow and blue box in C, compare with B). Thus, the IZ is composed of a medial domain (red in B′, absent in C′, blue box in C) that crucially requires Her5, and of a lateral domain (green in B′ and C′) that exhibits additional blocks towards neurogenesis. Interpreted from (Geling et al., 2003). Hind, presumptive hindbrain; MH, mid-hindbrain domain; Pros, presumptive prosencephalon; r4M, motorneurons of rhombomere 4; r4L, lateral neuronal precursors in r4.

Materials and methods

Fish strains

Wild-type embryos were obtained from natural spawning of AB adults, raised according to Kimmel et al. (Kimmel et al., 1995). *deadly-seven* (*des*^{p37a}) and *ngn1*$^{-/-}$ (*neurod3*^{hi1059}) mutant embryos carry non-functional *notch1a* and *ngn1* alleles, respectively (Golling et al., 2002; Holley et al., 2002). They were obtained by pair-wise mating of heterozygous adult carriers.

Transgenic lines

ngn1 transgenic reporter lines (*–8.4ngn1:gfp*, *–3.4ngn1:gfp*, *–3.1ngn1:gfp*) (Fig. 6A, left panel, 6B-H′) have been described previously (Blader et al., 2003). Ectopic activation of *her5* expression was achieved by applying to *pzhsp70:her5* (homo or heterozygote) transgenic embryos a heat-shock pulse between 80% epiboly and tail-bud stage, as described (Geling et al., 2003). *pzhsp70:her5* transgenic embryos were identified by PCR following in situ hybridisation (Geling et al., 2003).

Generation of the *–3.3ngn1:gfp* mutated construct (*–3.3ΔEboxngn1:gfp*) and transient reporter assays

The *–3.3ngn1:gfp* fragment was obtained by restriction digestion of a 100 bp 5′ fragment of *–3.4ngn1:gfp*. In *–3.3ΔEboxngn1:gfp*, the E-box located in the ANPE element (CATGTG) was selectively replaced by an unrelated sequence (TCTAGA), using standard procedures. Details of these constructs are available upon request. Both constructs were then flanked by I *Sce*I restriction sites, which allow efficient integration in the Medaka genome in co-injection with the I *Sce*I meganuclease enzyme (Thermes et al., 2002). For transient reporter assays, 50 ng/μl of *–3.3ngn1:gfp* or *–3.3ΔE-Boxngn1:gfp* circular plasmid DNAs were injected together with 1 U/μl I-*Sce*I meganuclease (Roche, 10 U/μl) into wild-type embryos at the one-cell stage. Embryos were left to develop at 28°C upon injection and fixed at 1-3 somites for in situ hybridisation analysis.

Antisense experiments

The morpholino antisense oligonucleotide MO^{her5} (Gene-Tools Inc., Oregon, USA) was described previously and demonstrated to fully and specifically inhibit the translation of endogenous *her5* mRNA (Geling et al., 2003). It was dissolved to a stock concentration of 2 mM in H_2O and injected into one-cell stage wild-type or transgenic embryos at 2 mM.

RNA injections

To prepare *coe2* capped RNA, the full-length coding region of *coe2* (Bally-Cuif et al., 1998) was PCR-amplified using the following primers: upstream, 5′ GCGAATTCGCACAAGTGTCAT 3′; downstream, 5′ CGCTCGAGATCAGGAGATTACACA 3′. It was then subcloned into the pXT7 vector (Dominguez et al., 1995) and verified by sequencing. *her5VP16* encodes a dominant form of Her5 and was described previously (Bally-Cuif et al., 2000). All capped RNAs were synthesised using Ambion mMessage mMachine kits following the recommended procedure. RNAs were injected at the following concentrations: 100 ng/μl *Notch-nicd-myc* (Takke et al., 1999); 100 ng/μl *XDelta*^{stu} (Haddon, 1998); with or without *nls-lacZ* (40 ng/μl) as lineage tracer; 100 ng/μl *Xcoe2ΔDBD* (Dubois et al., 1998); 100 ng/μl *coe2*; 5 ng/μl *her5VP16*. For capped RNA injections together with the MO^{her5} we used 100 ng/μl *NICD* or *XDelta*^{stu} RNAs together with 2 mM MO^{her5}.

DAPT treatment

DAPT treatment was performed as described (Geling et al., 2002) from 60% epiboly until the three-somite stage. After treatment, the embryos were fixed with 4% PFA overnight at 4°C and processed for in situ hybridisation.

In situ hybridisation and immunohistochemistry

Probe synthesis, in situ hybridisation and immunohistochemistry were carried out as previously described (Hammerschmidt et al., 1996). The following antibodies were used: rabbit anti-β-galactosidase (Cappel) (dilution 1:4000), mouse anti-Myc (Sigma 9E10) (dilution 1:1000), mouse anti-HNK1 (DSHB Zn12) (dilution 1:500) and rabbit anti-GFP (AMS TP401) (dilution 1:500). Secondary antibodies were goat anti-mouse-HRP, goat anti-rabbit-HRP, goat anti-mouse-Cy3 and goat anti-rabbit-FITC (Jackson ImmunoResearch Laboratories), all diluted to 1:200. The staining for HRP-conjugated antibodies was revealed with DAB following standard protocols.

Results

The expression and activity of Her5 are independent of Notch signalling in vivo

Because many E(spl) transcription factors are downstream effectors of Notch signalling, we first tested if *her5* expression and function is dependent on Notch signalling. Most *notch* family members cloned to date (like *notch1b*, *notch5* and *notch6*) (Westin and Lardelli, 1997) are not expressed in the MH territory at the end of gastrulation. However, an exception appears to be *notch1a*, which is weakly expressed in the ventral midline from the onset of neurogenesis until at least 24 hpf (Fig. 2A-C). In addition, upon injection of mRNA encoding the constitutively active form of Notch1a, NICD, *ngn1* expression was inhibited in all proneural clusters including the vcc and r2 motor- and lateral neurons, leading to an apparent enlargement of the IZ along the AP axis (Fig. 2F,G) (Haddon et al., 1998; Takke et al., 1999). These results suggest that medial IZ formation might result from Notch-mediated inhibition. To test this hypothesis, we analysed *her5* expression and, as a read-out of Her5 activity, measured the IZ size, at early neurogenesis stages, in embryos where Notch signalling is impaired. Surprisingly, we found that *her5* expression at the three-somite stage was severely downregulated upon forced expression of NICD (75% of cases, *n*=19) (Fig. 2D,E). Thus, *her5* expression is sensitive to Notch signalling, but, in striking contrast to other *her*-like genes, is inhibited rather than activated by NICD. This suggests that Notch does not act upstream of Her5 during IZ formation.

To further support this notion, we impaired Notch signalling in three different ways and asked whether this affects expression of *her5* and *ngn1*. First, we investigated *deadly-seven* (*des*) mutants (Kane et al., 1996), which carry a non-functional *notch1a* allele (Holley et al., 2002). *her5* expression and the lack of *ngn1* expression at the IZ were comparable in wild-type embryos and *deadly-seven* mutant embryos (*n*=25) (Fig. 2H-K), suggesting that Notch1a is not involved in controlling *her5* and *ngn1* transcription at the IZ. Second, we performed a conditional inhibition of Notch processing by applying a soluble gamma-secretase inhibitor to zebrafish embryos from stages immediately preceding *her5* expression in the neural plate (60% epiboly stage). This inhibitor (DAPT) prevents activity of the enzymatic complex cleaving Notch (De Strooper et al., 2001; Steiner and Haass, 2000) and induces faithful phenocopies of Notch signalling mutants when applied from blastula stages onwards (Geling et al., 2002). This conditional approach has the advantage that it avoids interfering with early Notch-dependent processes. We observed that DAPT treatments did also not trigger alteration

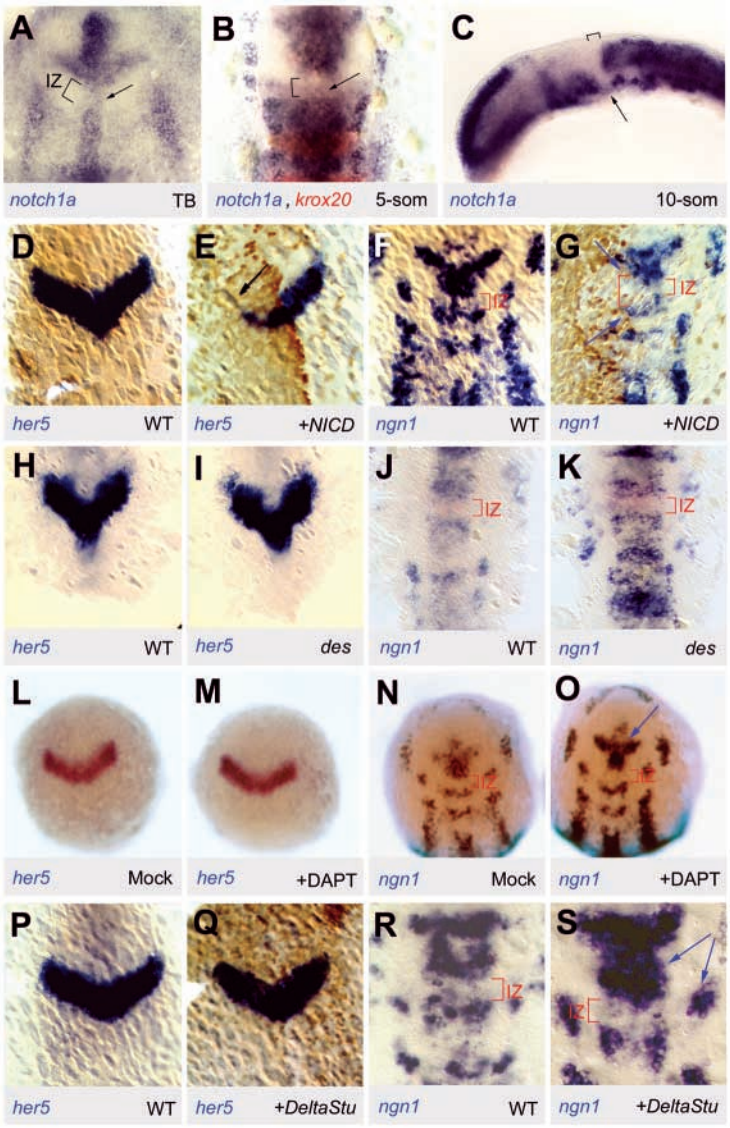

Fig. 2. *her5* expression and activity at the MH junction are independent of Notch signalling. (A-C) Expression of *notch1a* (Bierkamp and Campos-Ortega, 1993) revealed by whole-mount in situ hybridisation (blue staining) in wild-type embryos at the stages indicated (bottom right). (A,B) Flat-mounted views of the MH area, anterior towards the top; (C) lateral view, anterior leftwards. In B, double staining for *krox20* expression (red) identifies rhombomeres 3 and 5. Note the faint expression of *notch1a* (arrow) at the ventral midline of the IZ (bracket) at all stages. (D-S) Expression of *her5* and *ngn1* as indicated (bottom line) in three- to five-somite wild-type (D,F,H,J,P,R) or mock-treated embryos (L,N) versus: (E,G) embryos injected at the two-cell stage with *nicd-myc* RNA; (I,K) *deadly-seven* (*des*) *notch1a*-deficient mutants; (M,O) embryos treated with the gamma-secretase inhibitor DAPT; and (Q,S) embryos injected in at the two-cell stage with *Delta*^{Stu} mRNA. All views are dorsal, anterior towards the top; in D-G and Q lineage tracers (Myc and β-galactosidase, respectively) are revealed in brown by immunocytochemistry. NICD inhibits *her5* expression and decreases the number of neurons per proneural cluster (arrows in G). All other manipulated or mutant contexts increase this number (e.g. compare the intensity of *ngn1* staining between control and experimental embryo in the vcc in K,O,S with J,N,R, arrows); however, none of these manipulations affects *her5* expression or the presence and size of the IZ (I,M,Q). *des*, homozygote *deadly-seven* embryos; IZ, intervening zone; NICD, Notch intracellular domain; som, somite stage.

in *her5* expression at somitogenesis stages, and did not change the width of the IZ along the AP axis (*n*=20) (Fig. 2L-O). As expected, however, DAPT had a neurogenic effect and strongly increased the number of neurons within each proneural cluster (83% of cases, *n*=24) (Fig. 2N,O). Finally, to rule out an involvement of Notch signalling that does not require processing of Notch, we injected embryos with mRNA encoding the dominant-negative extracellular form of *delta*, Delta^{stu} (Haddon, 1998), which renders cells globally insensitive to Notch function. Although this manipulation also largely increased the number of *ngn1*-positive cells within proneural clusters, it did not affect *her5* expression and IZ formation (*n*=22) (Fig. 2P-S).

Together, these observations indicate that both *her5* expression and its activity, although inhibited by NICD in an artificial overexpression context, are independent of Notch signalling. This is in striking contrast to the E(Spl)-like bHLH factors that act downstream of Notch in lateral inhibition during neurogenesis (Bertrand et al., 2002; Fisher and Caudy, 1998).

Her5 activity is required to inhibit the establishment of a neurogenic field in the medial IZ

The above experiments indicate that Her5 does not act as a downstream effector of Notch to promote lateral inhibition. Thus, we examined whether Her5 might instead act upstream of Notch, by blocking the specification of a proneural field at the IZ. If this were the case, removing Her5 activity should reveal a neurogenic domain at the IZ, in which Notch controls the selection of neurons by lateral inhibition.

With the exception of *notch1a* (Fig. 2A-C), the other known components of the zebrafish lateral inhibition pathway are not expressed within the medial IZ. However, expression of these factors, such as the *deltaA* gene (*delA*), was induced upon injection of the morpholino antisense oligonucleotide MO^{her5} that was previously shown to antagonise *her5* selectively (Geling et al., 2003) (75% of cases, *n*=16) (Fig. 3A,B, and data not shown). Similarly, expression of *notch1a* was enhanced across the medial IZ in these conditions to reach levels comparable with those of adjacent anterior and posterior domains (78% of cases, *n*=18) (Fig. 3C,D).

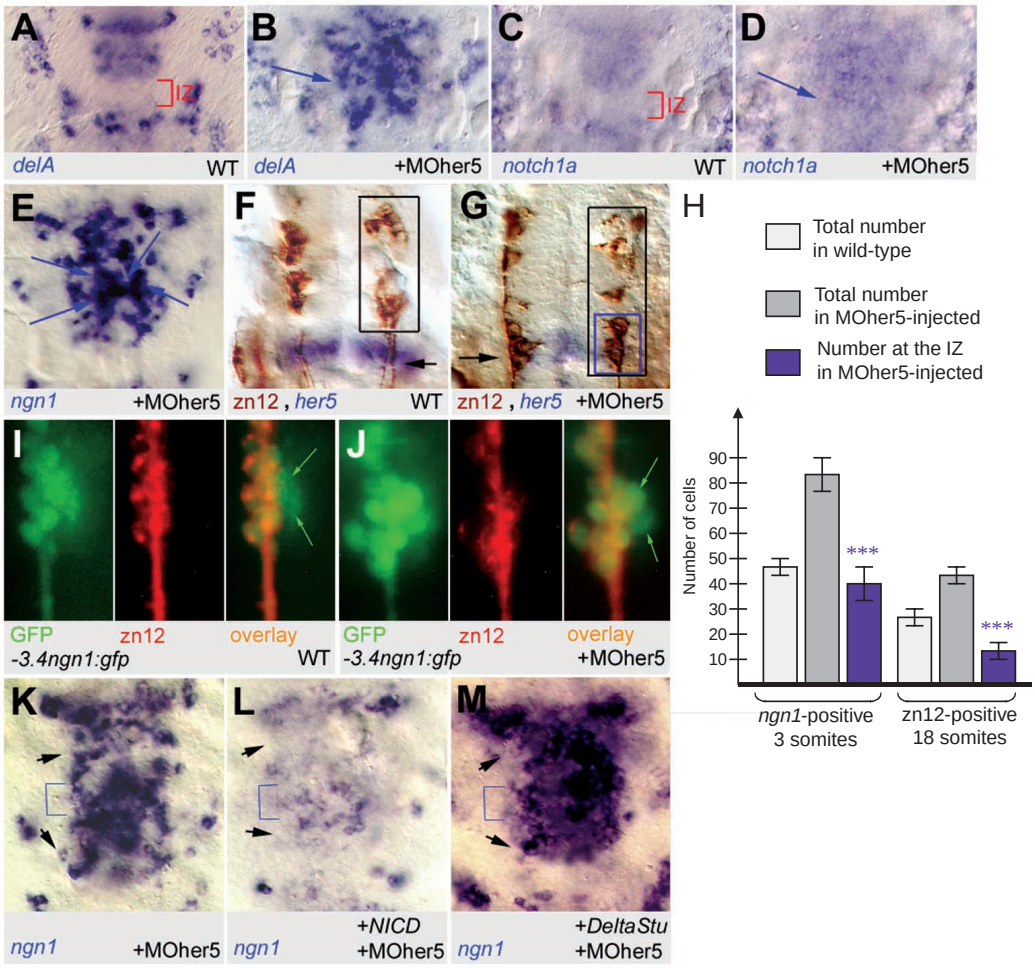

Fig. 3. Her5 acts by blocking the formation of a proneural cluster across the IZ. (A-D) Expression of components of the lateral inhibition machinery (e.g. *delA*, *notch1a*) is induced across the IZ upon injection of MO^{her5} (B,D, arrows) compared with non-injected controls (A,C) (brackets indicate IZ). All views are whole-mount in situ hybridisation for the markers indicated (bottom left), dorsal views of the MH area in flat-mounted embryos at the three-somite stage. (E-G) Induced expression of *ngn1* across the IZ upon injection of MO^{her5} (arrows in E) exhibits at the three-somite stage a salt-and-pepper pattern similar to that of anterior (vcc) or posterior (r2) proneural clusters. This is followed by the development, at the 18-somite stage, of zn12-positive differentiated neurons across the IZ (G, compare with F; brown immunocytochemistry staining). Arrow in F indicates the IZ, which is labelled in blue for *her5* expression. (Geling et al., 2003). (H) The number of ectopic zn12-positive neurons (right panel) differentiating across the IZ in the absence of Her5 activity [G, blue box (calculated as black box in G minus black box in F)] is lower than the number of *ngn1*-positive cells (left panel) initially induced across the IZ at the three-somite stage (Fig. 1C, blue box). (I,J) Fate of MH cells expressing *ngn1* at the onset of neurogenesis in wild-type embryos (I) and across the IZ in MO^{her5}-injected embryos (J). Descendants of early *ngn1*-positive cells are revealed by their retention of GFP protein at the 20-somites stage in the *–3.4ngn1:gfp* transgenic line (green staining), while differentiated neurons are positive for the zn12 antigen (red staining). Note in the overlay (right panels) that several green cells are negative for zn12 in both cases (green arrows). (K-M) The *ngn1*-positive domain induced across the IZ in the absence of Her5 activity is sensitive to lateral inhibition. The number of strongly *ngn1*-positive cells in the IZ (brackets) at the three-somite stage, induced by lack of Her5 expression, is reduced upon forced expression of NICD (L, compare with K) and increased upon expression of Delta^{Stu} (M, compare with K). It follows similar dynamics as *ngn1* expression in adjacent anterior (vcc) and posterior (r2) proneural clusters (arrowheads).

The outcome of lateral inhibition is the reinforcement of neurogenic gene expression in only a subset of neuronal precursors, which will commit to differentiation. In agreement, *ngn1* expression that was induced at the medial IZ upon removal of Her5 activity displays a salt-and-pepper pattern of expression (Fig. 3E). This juxtaposition of strongly and weakly *ngn1*-positive cells is similar to that observed in the vcc and r2M neurogenic fields and in other proneural clusters of the zebrafish neural plate (Blader et al., 1997; Haddon, 1998; Takke et al., 1999). Next, we compared the number of induced *ngn1*-positive cells at early somitogenesis with the number of neurons differentiating around the MHB in MO^{her5} embryos at later stages. Although on average 30(±4) *ngn1*-positive cells were induced across the medial IZ at the three-somite stage in MO^{her5}-injected embryos (Fig. 1C, blue box) (n=4), only 14(±2.4) differentiated neurons were detectable in this area at 20 somites (n=4) (Fig. 3G, blue box; Fig. 3H, blue bars). vcc cells expressing *ngn1* at the three-somite stage can also be traced until 20 somites using the stability of GFP protein in the *–3.4ngn1:gfp* transgenic line (Blader et al., 2003). At 20

somites, differentiated neurons (Fig. 3I, red label) constitute only a subset of these GFP-positive cells (green label) in the basal midbrain of wild-type embryos. We made a similar observation in the cluster of neurons induced at the MHB by MO^{her5} injections (Fig. 3J). Thus, only a subset of early *ngn1*-positive cells is driven to neuronal differentiation in the ectopic area of *ngn1* expression, suggesting that these cells are subjected to lateral inhibition.

To corroborate this notion further, we monitored *ngn1* expression upon the concomitant block of Her5 activity and impairment of Notch-Delta signalling. When MO^{her5} and *NICD* RNA were co-injected into one-cell stage embryos, the level of *ngn1* expression induced across the medial IZ was much reduced compared with injections of MO^{her5} alone (80% of cases, *n*=21) (compare Fig. 3K,L), and this level was comparable with the downregulated expression of *ngn1* in the vcc and r2 territories (Fig. 3L). Conversely, co-injection of MO^{her5} and RNA encoding $Delta^{Stu}$ led to increased levels of *ngn1* expression across the medial IZ compared with injection of MO^{her5} alone (85% of cases, *n*=20) (compare Fig. 3K,M). Again, the intensity of *ngn1* expression achieved within the medial IZ matched that of more anterior and posterior domains (Fig. 3M). We conclude that blocking Her5 activity generates a neurogenic domain at the medial IZ, in which committed neuronal precursors are selected by Delta/Notch signalling.

Together, the above experiments demonstrate that Her5 acts upstream of Notch signalling, by blocking the differentiation of a proneural field within the medial IZ. Thus, Her5 can be regarded as a prepattern factor that is involved in the spatial control of neurogenesis in the anterior neural plate.

The non-basic HLH transcription factor gene *coe2* is also target of Her5 activity

We next aimed at determining the targets of Her5 activity in neurogenesis inhibition. Her5 acts at an early step in the neurogenic cascade; we thus investigated whether expression of early proneural genes other than *ngn1* were also regulated by Her5.

In addition to *ngn1*, at least three other related bHLH genes with putative proneural function are expressed in territories adjacent to the IZ at the end of gastrulation: the *achaete-scute* homologues *asha* and *ashb* (formerly *zash1a*, *zash1b*) (Allende and Weinberg, 2002) and the *atonal*-related gene *neurod4* (previously *zath3* and *atonal3*) (Park et al., 2003; Wang et al., 2003). A comparative expression analysis of these proneural markers with precisely staged embryos showed that expression of *asha*, *ashb* and *neurod4* within the MH area was initiated slightly later than *ngn1*. *asha* is expressed at the three-somite stage mostly anterior to the IZ (Fig. 4A), whereas *ashb* expression lies posterior of the IZ in the presumptive hindbrain (Fig.4C). *neurod4* flanks the IZ like *ngn1* (Fig. 4E) (Park et al., 2003; Wang et al., 2003). In striking contrast to *ngn1*, we found that removal of Her5 activity did not cause ectopic expression of these genes (*n*=20) (Fig. 4B,D,F). Thus, these genes are not involved in the establishment of the ectopic neurogenic field in the IZ of Her5-blocked embryos. It furthermore suggests that the ectopic activation of *ngn1* by removal of Her5 is a specific effect on *ngn1*.

In *Xenopus*, the non-basic HLH transcription factor Xcoe2

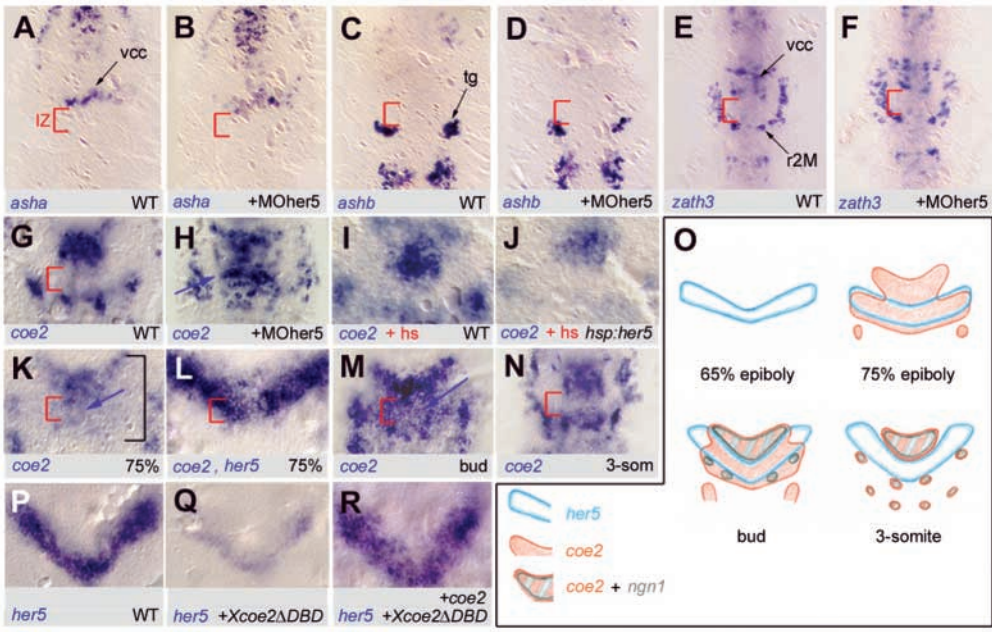

Fig. 4. *coe2* expression, but not that of *asha*, *ashb* and *neurod4*, is an additional target of Her5 activity at the IZ. Whole-mount in situ hybridisation (except O); dorsal views of the MH area in flat-mounted embryos at the three-somite stage (A-J,N-R), tail bud (M) or 75% epiboly (K,L); anterior towards the top; the markers indicated bottom left; red bracket indicates the IZ. (A-H) Comparison of *asha* (A,B), *ashb* (C,D), *neurod4* (*zath3* in figure) (E,F) and *coe2* (G,H) expression upon loss of Her5 activity (B,D,F,H) compared with wild-type siblings (A,C,E,G). *coe2* expression is the only target solely repressed by Her5 across the medial IZ (H, blue arrow indicates *coe2* induction). (I,J) Comparison of *coe2* expression in a *pzhsp70:her5* transgenic embryo (J) compared with non-transgenic sibling (I) upon heat-shock (hs) of both embryos at late gastrulation. Note that *coe2* expression is repressed upon ectopic *her5* expression (weaker staining in J). (K-O) Time-course of *her5* and *coe2* expression. *coe2* expression is initiated at 75% epiboly, thus following *her5*, across the entire MH area (K, black bracket). Double staining for *coe2* and *her5* at 75% epiboly (L, both in blue) demonstrates that expression of *coe2* and *her5* are overlapping across the IZ. *coe2* expression is maintained at the IZ until the bud stage (M, blue arrow). Later on, *coe2* expression is cleared from the IZ and becomes similar to *ngn1* (see O). (P-R) *her5* expression at the tail-bud stage in embryos injected with capped RNA encoding a dominant-negative form of Coe2 (*Xcoe2ΔDBD*) (Dubois et al., 1998) (Q) compared with non-injected siblings (P) demonstrates strong downregulation of *her5* expression when Coe2 activity is impaired. This phenotype is rescued upon coinjection of wild-type *coe2* RNA (R). IZ, intervening zone; hs, embryo submitted to a 1 hour heat-shock pulse at late gastrulation; r2M, presumptive motorneurons of rhombomere 2; tg, presumptive trigeminal ganglia; vcc, ventrocaudal cluster.

plays a role in primary neurogenesis downstream of Neurogenin-related 1 in the stabilisation of a determined neuroblast state (Dubois et al., 1998). In zebrafish, *coe2* (formerly *zcoe2*) (Bally-Cuif et al., 1998; Dubois et al., 1998), expression is initiated before *ngn1* in the MH territory, suggesting that it might play an early role in neurogenesis in this territory (Blader et al., 1997; Bally-Cuif et al., 1998). At the three-somite stage, *coe2* is expressed with a profile reminiscent of *ngn1* (Fig. 4G). In striking contrast to *asha*, *ashb* and *neurod4*, we found that injection of MO^{her5} led to a strong induction of *coe2* expression across the medial IZ (Fig. 4H) (90% of cases, *n*=20). In addition, as for *ngn1* (Geling et al., 2003), ectopic activation of *her5* expression from late gastrulation onwards (by applying a heat-shock pulse to *pzhsp70:her5* transgenic embryos) strongly downregulated *coe2* expression in the MH domain (80% of cases, *n*=20) (Fig. 4I,J).

Thus, Her5 activity is crucially involved in the selective repression of *ngn1* and *coe2*, both of which have proneural activity and may thus be involved in the establishment of the ectopic neurogenic domain at the IZ of embryos that lack Her5 activity.

Crossregulatory interactions between *her5* and *coe2* expression at the IZ

In contrast to *ngn1*, *coe2* exhibits an early expression phase, which precedes *ngn1* expression and straddles the whole MH area (Bally-Cuif et al., 1998). These observations prompted us to analyse in more detail a potential connection between Her5 and Coe2 activities. Precise comparison of *her5* and *coe2* expression on exactly staged embryos showed that *her5* transcription, detectable from 65-70% epiboly, precedes *coe2*, initiated at 75% epiboly in the anterior neural plate over a broad domain that covers the presumptive mes- and anterior rhombencephalon (Fig. 4K, black bracket). Until the tail-bud stage, *coe2* and *her5* expression overlap across the entire mediolateral extent of the IZ (Fig. 4L,M). Then, *coe2* expression is cleared from the IZ at early somitogenesis (Fig. 4N; schematised in Fig. 4O). We tested a possible crossregulation between her5 and coe2 by monitoring her5 expression in embryos injected with RNA encoding a dominant-negative form of Xcoe2, Xcoe2ΔDBD (Dubois et al., 1998). The Xcoe2ΔDBD protein harbours a deletion in its DNA-binding domain but has an intact dimerisation domain, and was previously used to inhibit the function of endogenous Xcoe2 protein via the formation of non DNA-binding Xcoe2-Xcoe2ΔDBD heterodimers (Dubois et al., 1998). We reasoned that the high sequence identity between Xcoe2 and Coe2 HLH domains (89%) would permit Xcoe2ΔDBD to act dominant-negatively on zebrafish Coe2 as well. Indeed, we could show that injection of *Xcoe2ΔDBD* RNA into one-cell stage zebrafish embryos downregulated *ngn1* expression strongly, as reported for Xcoe2ΔDBD in *Xenopus* (Dubois et al., 1998) (see Fig. 5E) (78% of cases, *n*=15). This effect was suppressed by co-injection of *coe2* RNA (not shown, 75% of cases, *n*=16), underscoring its selectivity. Injections of *Xcoe2ΔDBD* RNA inhibited *her5* expression at tail-bud stages (Fig. 4P,Q) (73% of cases, *n*=19), a phenotype also rescued by the co-injection of *coe2* RNA (Fig. 4R) (73% of cases, *n*=20). Given that the onset of *coe2* expression in vivo follows *her5* induction, we conclude that Coe2 is necessary for the early maintenance of *her5* expression. Together, our results point to a loop of crossregulation where Coe2 initially maintains *her5* expression, and Her5 in turn clears *coe2* expression from the IZ at early somitogenesis stages.

coe2 and *ngn1* expression are separately targeted by Her5 activity

Because *Xenopus neurogenin1* and *Xcoe2* expression are functionally linked (Dubois et al., 1998), we wondered whether the regulation of *ngn1* and *coe2* by Her5 might reflect a linear cascade, where only one of these genes would be a primary target of Her5 activity. To address this issue, we first tested whether *coe2* expression was responsive to Her5 in the absence of Ngn1 function. $ngn1^{-/-}$ mutants ($neurod3^{hi1059}$) (Golling et al., 2002) or embryos where Ngn1 expression is knocked down (Cornell and Eisen, 2002; Park et al., 2003) probably represent a full loss of Ngn1 activity. They display a severe reduction of cranial ganglia and of the number of spinal sensory neurons, while spinal motor neurons are less affected (Cornell and Eisen, 2002; Golling et al., 2002; Park et al., 2003). We found that *coe2* expression was severely reduced but not completely abolished in the MH area at three somites (Fig. 5A,B). Thus, Ngn1 is necessary for the maintenance of high levels of *coe2* expression in this location. Importantly, upon injection of MO^{her5} into one-cell stage $ngn1^{-/-}$ embryos, *coe2* expression was still induced at the medial IZ, at levels comparable with adjacent domains (65% of cases, *n*=14) (Fig. 5C). Thus, Ngn1 expression is not required for the ectopic induction of *coe2* across the medial IZ in the absence of Her5 activity. Hence, *ngn1* is targeted in parallel by Her5 or acts downstream of Coe2.

We tested next whether *ngn1* expression was responsive to Her5 in the absence of Coe2 function. Embryos injected with RNA encoding Xcoe2ΔDBD display downregulated expression of *ngn1* in the MH area (82% of cases, *n*=19) (Fig. 5D,E), demonstrating that Coe2 is necessary for the maintenance of high levels of *ngn1* expression in this location. Furthermore, upon co-injection of *Xcoe2ΔDBD* RNA and MO^{her5}, *ngn1* was still induced across the medial IZ at levels comparable with those found in the vcc and r2M clusters (Fig. 5F) (85% of cases, *n*=20). Thus, Coe2 activity is not necessary for the induction of *ngn1* expression across the medial IZ in the absence of Her5, and is unlikely to be an intermediate step in the inhibition of *ngn1* expression by Her5 in that location.

We conclude from these experiments that *ngn1* and *coe2* expression positively crossregulate each other in the MH area to maintain reciprocal high levels of transcription. However, they are also independent targets of Her5 in its repression of the formation of a neurogenic domain in the medial IZ.

Inhibition of *coe2* or *ngn1* expression by Her5 is sufficient to prevent neuronal differentiation across the medial IZ

Because *ngn1* and *coe2* are both targets of Her5, we asked next to which extent the inhibition of either gene's expression contributed to the absence of neuronal differentiation across the medial IZ. In spite of remaining levels of *coe2* expression in $ngn1^{-/-}$ mutants (Fig. 5B), we found that the progression of neurogenesis was fully impaired at later stages in these mutants in the MH area, as revealed by the absence of *deltaB* (*delB*) expression in eight-somite stage embryos (Fig. 5G-H′) and of

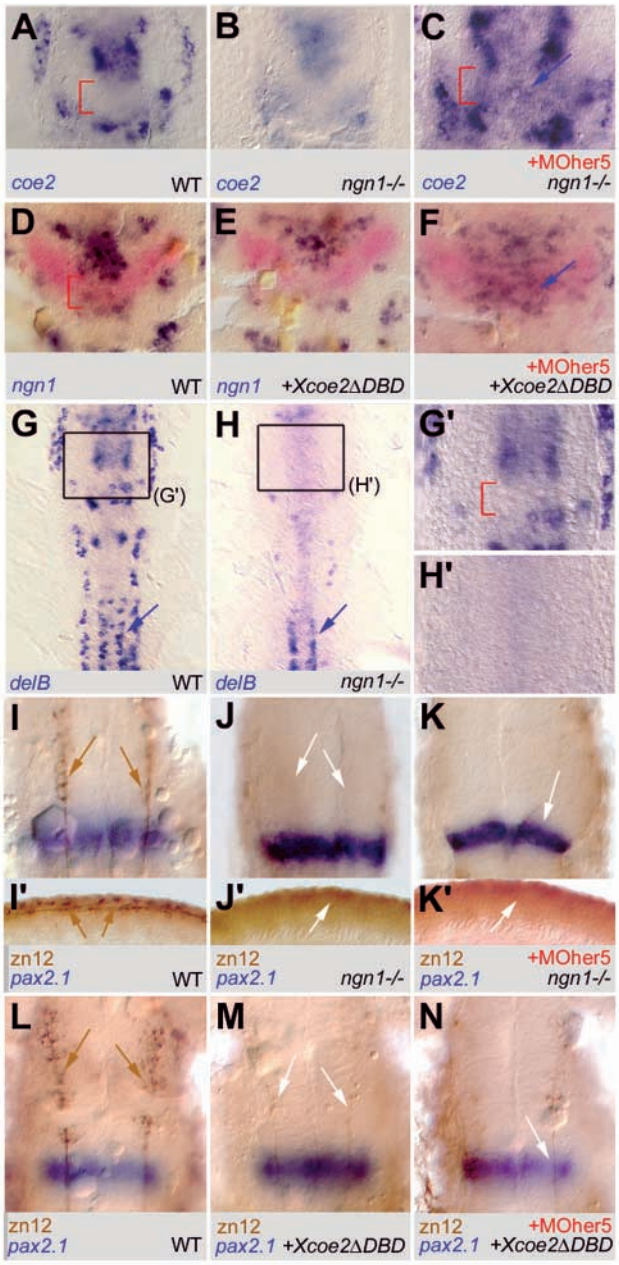

Fig. 5. *ngn1* and *coe2* expression are independently inhibited by Her5, but downregulation of one of these targets is sufficient to prevent neuronal differentiation at the IZ. Expression of *coe2* (A-C), *ngn1* (D-F), *delB* (G-H′), *pax2.1* and zn12 (I-N) in wild type (A,D,G,I,I′,L), *ngn1*$^{-/-}$ mutants (B,C,H,H′,J,K) or embryos injected with capped RNA encoding a dominant-negative form of Coe2 (*Xcoe2ΔDBD*) (E,F,M,N). Embryos injected with MO^{her5} (C,F,K,N) are compared with non-injected controls. All views (except I′-K′) are flat-mounted embryos, anterior towards the top, at three somites (A-F), eight somites (G-H′) or 18 somites (I-N). (I′-K′) Lateral views of the tail area of embryos in I-N, anterior leftwards; (G′,H′) high-magnification views of the areas boxed in G,H, respectively; red brackets indicate the IZ. (A-C) *coe2* expression in the vcc and r2 is lower in *ngn1*$^{-/-}$ mutants (B) but still induced at the IZ in the absence of Her5 (C, blue arrow). C is a higher magnification of the IZ area compared with A and B. (D-F) *ngn1* expression is lower in the vcc and r2 when Coe2 activity is reduced, but still induced at the IZ (labelled in red by *pax2.1*) in the absence of Her5 (F, blue arrow). (G-H′) Progression of neurogenesis, as revealed by the commitment marker *delB*, is fully impaired in the MH area in the absence of Ngn1 (see G′,H′). This contrasts with the maintenance of neurogenesis in spinal motorneurons (blue arrows) (Cornell and Eisen, 2002). (I-K′) In *ngn1*$^{-/-}$ mutants (identified by their lack of sensory neurons in the spinal cord, compare J′, K′ and I′, arrows), neuronal differentiation in the MH, revealed by zn12 immunocytochemistry, is fully blocked (brown staining and brown arrows in I, white arrows to the absence of staining in J). In addition, in the absence of Her5, neuronal differentiation at the IZ (blue *pax2.1* staining) does not take place (white arrow in K). (L-N) Neuronal differentiation within the MH (brown staining and brown arrows in L) is also impaired in the absence of Coe2 function (white arrows in M), and does not take place at the IZ when Her5 activity is blocked (white arrow in N).

zn12 immunoreactivity in this location at the 18-somite stage (Fig. 5I,J). This is in striking contrast to the development of basal neuronal populations in the spinal cord, which are largely preserved (Fig. 5H, blue arrows) (Cornell and Eisen, 2002). Thus, Ngn1 function is strictly necessary for the progression of neurogenesis to neuronal commitment and differentiation of basal MH populations. Furthermore, we found that no neurons differentiated across the medial IZ when MO^{her5} was injected into *ngn1*$^{-/-}$ mutants (100% of cases, *n*=18) (Fig. 5K, compare with 3G). Thus, the block of *ngn1* expression by Her5 is sufficient to ensure the absence of neuronal differentiation across the medial IZ.

In striking parallel, blocking Coe2 function by injection of *Xcoe2ΔDBD* RNA lead to a dramatic decrease in neuronal differentiation within the MH domain (82% of cases, *n*=18) (Fig. 5L,M), identifying Coe2 as another factor crucially necessary for progression of neurogenesis in this area. Furthermore, absence of Coe2 function prevented neuronal differentiation induced by removing Her5 activity across the medial IZ (85% of cases, *n*=19) (Fig. 5N, compare with Fig. 3G). Thus, the downregulation of *coe2* expression by Her5 at the medial IZ, like inhibition of *ngn1* expression, is sufficient to prevent neuronal differentiation in this area.

We conclude that, as both *ngn1* and *coe2* are required for ectopic neurogenesis at the IZ, Her5 acts redundantly on these two genes to prevent neuronal differentiation in this location.

An E-box in the anterior neural plate enhancer of the *ngn1* gene is necessary for repression by Her5

We next investigated whether the inhibition of *ngn1* expression by Her5 could be tracked down to specific enhancer regions in the *ngn1* upstream sequence. Previous characterisation of the *ngn1* locus demonstrated that an 8.4 kb upstream fragment was sufficient to drive correct reporter expression in neuronal clusters of the anterior neural plate and sensory precursors of the spinal cord (*–8.4ngn1:gfp*) (Blader et al., 2003) (Fig. 6A,B). We found that injection of MO^{her5} into this transgenic line induced strongly *gfp* transcription across the medial IZ (Fig. 6B, blue arrow) (77% of cases, *n*=18). Conversely, ectopic expression of Her5 within this line (obtained by crossing into the *pzhsp70:her5* transgenic background and heat-shock at the onset of neurogenesis) severely reduced *gfp* expression (not shown). Thus, the element(s) of response to Her5 are contained within the 8.4 kb fragment of the *ngn1* enhancer.

The 8.4 fragment contains two elements, the lateral stripe

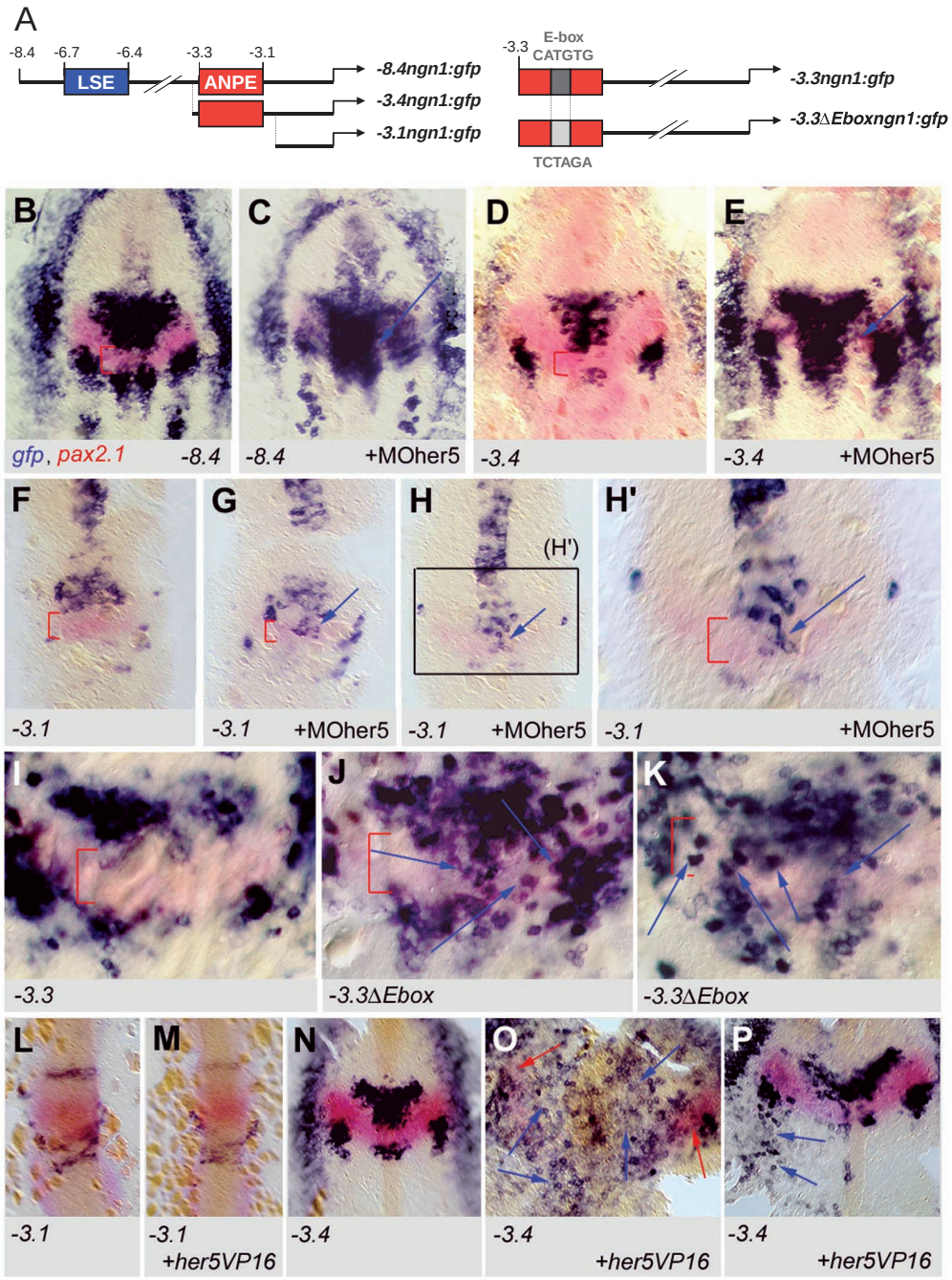

Fig. 6. An E-box contained within the ANPE element of the *ngn1* gene is the major Her5 response element. (A) *ngn1* transgenic reporter lines (left panel) (Blader et al., 2003) and reporter constructs used in transient assays (right panel) used to locate the response elements to Her5 within the *ngn1* enhancer. (B-H′) Expression of *gfp* (revealed by in situ hybridisation, blue staining) and *pax2.1* (red staining, used to located the IZ) in the following transgenic lines: *–8.4ngn1:gfp* (B,C), *–3.4ngn1:gfp* (D,E), *–3.1ngn1:gfp* (F-H′) upon injection of MO^{her5} (C,E,G-H′) or in non-injected siblings (B,D,F). All panels are flat-mounted embryos, anterior towards the top, at the three-somite (B-E) and eight-somite (F-H′) stages; red brackets indicate the IZ. Two different embryos are shown for injection in the *–3.1* line (G,H); H′ is a highly magnified view of the area boxed in H. Note that *gfp* expression is strongly induced across the IZ upon block of Her5 activity in the *–8.4* and *–3.4* lines, in a manner similar to endogenous *ngn1* expression, but that the response of the *–3.1* transgene is minor and restricted to a few cells at the ventral midline. (I-K) Expression of *gfp* (blue) and *pax2.1* (red) in founder embryos injected with *–3.3ngn1:gfp* (I) and *–3.3ΔEboxngn1:gfp* (J,K, two different embryos are shown). Both constructs carry *Sce*I sites at their extremities and were co-injected with the meganuclease enzyme to trigger early integration (Thermes et al., 2002). Note the large number of ectopic *gfp*-positive cells in the entire medial IZ domain in embryos expressing the mutated construct without blocking Her5 activity, demonstrating that the E-box located within the ANPE is the major element mediating *ngn1* repression at the IZ in vivo. (L-P) Expression of *gfp* (blue) and *pax2.1* (red) in transgenic embryos (lines indicated bottom left). Uninjected embryos (L,N); embryos injected with *her5VP16* capped RNA (M,O,P). Embryos are observed at the eight-somite (L,M) and one-somite (N-P) stage. Note that *gfp* expression in *–3.1ngn1:gfp* embryos is unperturbed by Her5VP16 (M), while ectopic expression is evident in *–3.4ngn1:gfp* embryos (two examples shown in O,P, blue arrows indicate ectopic *gfp*-positive cells, red arrows indicate *pax2.1* expression in O).

element (LSE) driving expression in sensory spinal clusters, and the anterior neural plate element (ANPE) driving expression in anterior clusters, including the vcc and r2M (Blader et al., 2003) (Fig. 6A,B). To determine whether the Her5 response was confined to one of these elements, we monitored *gfp* expression upon injection of MO^{her5} into the *–3.4ngn1:gfp* transgenic line (which lacks the LSE but maintains the ANPE) and *–3.1ngn1:gfp* line (where both elements are deleted) (Fig. 6A). Strong *gfp* induction across the medial IZ was observed when Her5 activity was blocked in the *–3.4ngn1:gfp* background, in a manner indistinguishable from that observed in *–8.4ngn1:gfp* transgenics (Fig. 6D,E) (78% of cases, *n*=14). Thus, the response element to Her5 activity is contained within the 3.4 kb of upstream *ngn1* sequence, thus is excluded from the LSE. By contrast, *gfp* expression was only marginally induced in the *–3.1ngn1:gfp* line generally in a few cells that are located close to the ventral midline (Fig. 6F-H′, blue arrows) (66% of cases, *n*=15). We conclude that the *ngn1* transgene contains partially redundant Her5 response elements. The major repressor element resides between –3.4 and –3.1 kb upstream of the *ngn1* start site while a weaker element is located proximal to the ANPE.

Remarkably, the ANPE contains a CATGTG sequence (in position –3187 to –3182), which fits the canonical 'E-box' (CANNTG). E-boxes are known binding sites for bHLH proneural factors, and can also be bound by Hairy/E(Spl) proteins (Davis and Turner, 2001; Fisher and Caudy, 1998). We thus analysed whether this E-box might be part of the element(s) mediating Her5 repression. To this end, the E-box was replaced by a cluster of point mutations (CATGTG to TCTAGA). The mutation was placed into *–3.3ngn1:gfp* that has a 5′ deletion of 100 bp terminating immediately upstream of the ANPE (generating construct *–3.3ΔEboxngn1:gfp)* (Fig. 6A). Both constructs were flanked by the restriction site for the meganuclease *Sce*I, and were injected into wild-type embryos together with the meganuclease enzyme. As described in Medaka (Thermes et al., 2002), this procedure favoured early integration of the transgene, leading to the production of very moderately mosaic embryos that display remarkably low ectopic expression (Fig. 6I-K). These embryos are thus suitable for a founder analysis, and we studied expression of *gfp* mRNA at and around the IZ. Although the non-mutated *–3.3ngn1:gfp* construct never gave rise to *gfp* expression across the medial IZ (Fig. 6I) (100% of cases, *n*=20), we found that most embryos injected with *–3.3ΔEboxngn1:gfp* displayed prominent ectopic expression of *gfp* in this location (67% of cases, *n*=18) (Fig. 6J,K), as expected for a negatively acting element

Together, these results suggest that a major element mediating the active repression of *ngn1* expression at the medial IZ is the E-box contained within the ANPE. To test by a different experimental approach whether Her5 acts through the ANPE, we next examined whether it behaved as a repressor or an activator in the E-box-dependent process inhibiting *ngn1* expression. To this aim we tested the response of *–3.4ngn1:gfp* and *–3.1ngn1:gfp* to the fusion protein Her5VP16, which behaves as a dominant activator of Her5 targets (Bally-Cuif et al., 2000). Although *–3.1ngn1:gfp* failed to respond to Her5VP16 (Fig. 6L,M) (0% of cases, *n*=21), we found that *gfp* expression was induced ectopically by Her5VP16 in the *–3.4ngn1:gfp* line (Fig. 6N-P) (63% of cases, *n*=53). Thus, to prevent *ngn1* expression across the IZ, Her5 functions as a transcriptional inhibitor that might either bind directly the ANPE E-box or inhibit expression of an activator normally binding this site.

Discussion

We have here analysed the molecular mechanisms underlying the inhibition of neurogenesis by Her5 at the MHB. We demonstrated that Her5 does not act as a downstream effector of Notch signalling but rather as a prepattern factor, linking positional cues with the spatial control of proneural gene expression, in a manner reminiscent of *Drosophila* Hairy. We identified two downstream targets of Her5 in this process, *ngn1* and *coe2*, and showed that both are crucial for neuronal differentiation in the MH domain. Finally, we demonstrated that repression of *ngn1* expression by Her5 involves an E-box located in the ANPE that was shown previously to drive *ngn1* expression in the anterior neural plate, including the vcc and r2M.

her5 expression is not a target of Notch signalling at the MHB

Most E(spl) factors act as Notch effectors in cell fate decisions, including the control of somitogenesis and neurogenesis in vertebrates (Artavanis-Tsakonas et al., 1999; Davis and Turner, 2001). We found, however, that Her5, although belonging to the E(spl) class and inhibiting neurogenesis, is not a target of Notch signalling and lateral inhibition. Three independent experimental findings support this conclusion: blocking or lowering Notch signalling using either DAPT treatment, *notch1a*-deficient *des* mutant embryos or overexpression of $Delta^{Stu}$ does not perturb *her5* expression and does not cause ectopic neurogenesis in the IZ. Moreover, quite in contrast to what one would expect from a Notch effector, *her5* expression was inhibited rather than activated by ectopic activation of the Notch pathway in NICD-expressing embryos. Similar observations were previously made for *her5* expression in endodermal progenitors at early gastrulation (Bally-Cuif et al., 2000). These observations suggest that Notch signalling is not involved in controlling *her5* expression at the MHB. Moreover, upon induction of a proneural cluster in place of the IZ (by blocking Her5 function), the activation of lateral inhibition did not affect *her5* expression in this location (A.G. and L.B.-C., unpublished). Thus, the regulation of *her5* by ectopic NICD does not play a role in the control of MH neurogenesis, and Her5 does not, in contrast to most other E(spl) factors, act as a Notch effector in the control of neurogenesis at the IZ.

Her5 acts as a prepattern factor

Prepattern factors act at the interface of patterning and neurogenesis to control the location and extent of neuronal differentiation sites without influencing the overall structure of the neural plate/tube. This definition is based on the pre-patterning systems controlling neurogenesis in the *Drosophila* peripheral nervous system (Davis and Turner, 2001; Fisher and Caudy, 1998). Her5 meets these requirements as its expression is regulated by the embryonic patterning machinery including Wnt and Fgf signalling at the MHB (Geling et al., 2003; Reifers et al., 1998), its activity does not impinge on patterning (Geling et al., 2003), and it controls expression of the proneural

genes *ngn1* and *coe2* (Geling et al., 2003) (this paper). To date, only few factors have been identified in vertebrates that fulfil these strict criteria. These include the inhibitors of neurogenesis Anf, BF1 and Xrx1 in the anterior neural plate, Zic2 and Xiro3 in the spinal cord, and Hes1 in the mouse olfactory epithelium (for reviews, see Bally-Cuif and Hammerschmidt, 2003; Sasai, 1998), as well as some positive factors, such as Iro1 and Iro7 in *Xenopus* and zebrafish (Cavodeassi et al., 2001; de la Calle-Mustienes et al., 2002; Itoh et al., 2002) and Flh/Not1 in the zebrafish epiphysis (Cau and Wilson, 2003). All these factors control primarily expression of proneural genes rather than the patterning machinery. Moreover, like for Her5, their activity was in some cases directly shown to be independent of lateral inhibition (Bellefroid et al., 1998; Bourguignon et al., 1998; Andreazzoli et al., 2003). As previously mentioned (Andreazzoli et al., 2003), these observations suggest that independence of Notch signalling is a common theme of inhibitory prepatterning in the vertebrate neural plate.

The mode of action of prepatterning inhibitors at the molecular level remains mostly hypothetical. Our results demonstrate that Her5 acts by blocking expression of the proneural genes *ngn1* and *coe2* and preventing the specification of a neurogenic cluster at the level of the MHB, thereby generating the neuron-free IZ. Removal of Her5 activity creates a neurogenic domain at the medial IZ that is sensitive to Notch/Delta signalling, and where lateral inhibition operates to select and commit progenitors within a pool of precursors. A similar activity was reported for mouse Hes1 in the olfactory neuroepithelium (Cau et al., 2000). Our data suggest that inhibitory prepatterning in vertebrates might, at least in part, function by restricting the size of proneural fields within neurogenesis-competent areas of the neuroepithelium. The major response element to Her5 is an E-box located in the ANPE of the *ngn1* upstream region, which is the principal enhancer driving *ngn1* expression in anterior proneural clusters of the vcc and r2 (Blader et al., 2003). These results suggest that MH neuronal precursors belong to a single proneural cluster within which *ngn1* expression is locally repressed at the MHB to generate the IZ. A very similar situation has been reported for the control of *achaete* in *Drosophila*, where Hairy binds an element located close to the enhancer driving *achaete* expression in the notum (Ohsako et al., 1994; Van Doren et al., 1994). Hairy and Her5, however, diverge in two respects. First, Hairy establishes the distinction between non-neural and neural ectoderm within the fly notum, while Her5, like mouse Hes1, controls neurogenesis within an already neuralised tissue. Second, Her5 belongs in sequence to the E(spl), rather than the Hairy, subclass, suggesting that the distinction made in *Drosophila* between E(spl) and Hairy functions (Notch effectors versus Notch-independent prepatterning inhibitors, respectively) has not been conserved during evolution (Fisher and Caudy, 1998).

The factors that control the local induction of *her5* expression remain to be defined. Spg/Pou2 is required for the specification of a large portion of the anterior neural plate that includes the *her5* domain but also the entire hindbrain (Belting et al., 2001; Burgess et al., 2002; Hauptmann et al., 2002; Reim and Brand, 2002). MH factors such as Pax2.1, Eng2/3 and Fgf8 are only necessary for *her5* maintenance (Lun and Brand, 1998; Reifers et al., 1998; Scholpp and Brand, 2001). Finally, *her5* expression is transiently controlled by Coe2, but this interaction affects *her5* maintenance rather than *her5* induction, and is unlikely to be direct, as we failed to identify Coe2-binding sites (Dubois and Vincent, 2001) in a *her5* enhancer fragment sufficient to recapitulate *her5* expression at all stages (Tallafuss and Bally-Cuif, 2003).

Molecular mode of Her5 action

We demonstrate that a number of early proneural genes (*asha*, *ashb*, *neurod4*, *ngn1* and *coe2*) are expressed in domains flanking the IZ, but that Her5 selectively inhibits expression of only two of them, *ngn1* and *coe2*. These two genes are probably independent targets of Her5 repression. This is surprising given that Ngn1 and Coe2, possibly because of their positive crossregulation, appear to play identical roles: blocking expression of either one of these genes is sufficient to prevent neurogenesis in the IZ. Several interpretations might account for the regulation of both *ngn1* and *coe2* by Her5. Given the crucial importance of the IZ in maintaining a pool of progenitors at the MHB, which is necessary both for the maintenance of MHB integrity (Geling et al., 2003; Hirata et al., 2001) and for MH growth (Cowan and Finger, 1982), it is possible that this dual inhibitory mechanism has been evolutionarily selected to efficiently prevent neurogenesis at the MHB. In addition, it is possible that Ngn1 and Coe2 control other and distinct processes in addition to neurogenesis. We demonstrated previously that Her5 is also necessary to enhance cell proliferation in the medial IZ, independently of its suppression of *ngn1* expression (Geling et al., 2003). Coe2 might impinge on the control of proliferation. In addition, other cellular processes could be regulated by Coe factors, such as neuronal specification, differentiation, migration and axonal pathfinding (Dubois and Vincent, 2001).

At the molecular level, several mechanisms appear to be used by Hairy/E(spl) factors to restrict neurogenesis. These include direct binding to the enhancer and transcriptional inhibition of proneural target genes, competition with activator bHLH proteins for the same DNA-binding sites, and functional inhibition by the formation of inactive heterodimers with proneural factors (Davis and Turner, 2001). *Drosophila* Hairy acts by direct binding and repression of the *achaete* enhancer (Ohsako et al., 1994; Van Doren et al., 1994). Her5 acts at a very early stage on the expression of *ngn1* and *coe2*, suggesting that its main early activity at the IZ is transcriptional inhibition of these targets. Whether the action of Her5 on *ngn1* and *coe2* expression is direct, however, remains to be shown. The regulatory regions controlling *coe2* expression have not been characterised. Our analysis of the *ngn1* enhancer identifies an E-box within the ANPE domain as the major element mediating transcriptional inhibition of *ngn1* at the medial IZ. Although E(spl) factors are generally considered to bind N boxes with higher affinity in vitro, interaction with E-boxes has also been reported (Davis and Turner, 2001). It is thus possible that Her5 binds to this element and directly inhibits *ngn1* transcription. Chromatin immunoprecipitation experiments will be required to resolve this issue. In addition, we observed that the proximal region of the *ngn1* upstream sequence (3.1 kb) also exhibits a moderate response to Her5 activity, restricted to the ventral midline of the IZ. A repetition of two N boxes is present in positions –235/–230 and –225/–220 upstream of the *ngn1* translation start site (C.P., P. Blader and U.S., unpublished), which might be involved in this regulation.

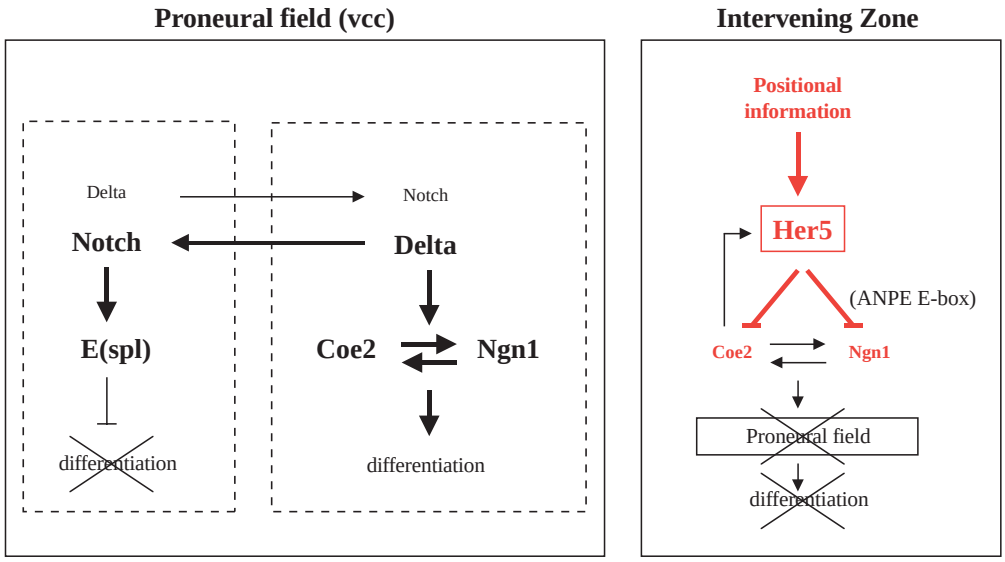

Fig. 7. Model for the establishment of the MH neurogenesis pattern. The entire MH territory is competent to become a proneural cluster. Within this domain (left), the activities of Ngn1 and Coe2, their positive crossregulation of expression and their sensitivity to the lateral inhibition machinery are crucial elements controlling the commitment of progenitors towards neuronal differentiation. At the MHB, however, Her5 exerts an early inhibition on the expression of *ngn1* and *coe2*, preventing the specification of a proneural cluster and the initiation of neurogenesis in this location, generating the IZ (right panel). As a result, neurogenesis is spatially restricted to the vcc and r2 (left panel).

However, our results with the –3.3 kb fragment suggest that, in the presence of the ANPE, these elements do not play a major role. The 3.1 kb fragment is also capable of driving reporter expression that excludes the IZ, but it is initiated with a delay within the vcc and r2M (Blader et al., 2003). Thus, elements contained within this fragment might be involved in controlling *ngn1* expression in the MH domain and its repression from the ventral midline of the IZ at a later, possibly maintenance stage.

Neurogenesis in the MH area requires Ngn1 and Coe2

We demonstrate here that both Ngn1 and Coe2 functions are necessary for the progression of neurogenesis and for the early events of neuronal differentiation in the MH domain. Blocking Coe2 activity downregulates *ngn1* expression throughout the neural plate (A.G. and L.B-C., unpublished), suggesting a requirement for Coe2 in all primary neurons. The absence of *ngn1* function prevents *delB* expression in the anterior proneural clusters, including the presumptive motorneurons of rhombomeres 2 and 4, and the vcc, and is also necessary for neuronal differentiation of vcc derivatives, which comprise at least the first differentiating populations of the reticulospinal nMLF neurons (Easter et al., 1994; Wilson et al., 1990). This, together with previous reports, indicates a strict requirement for Ngn1 in spinal sensory neurons (Cornell and Eisen, 2002; Golling et al., 2002) and the MH area (this paper) of the embryonic zebrafish CNS. By contrast, Ngn1 is not essential for motor- and interneuron development in the trunk and spinal cord (Cornell and Eisen, 2002; Golling et al., 2002; Park et al., 2003), and for epiphysial neurons (Cau and Wilson, 2003). Differential requirements for Ngn in CNS neuronal differentiation was also observed in other vertebrates, a typical example being the complementary requirements for Ngn2 and Mash1 in the mouse embryonic neural tube (see Bertrand et al., 2002). Other bHLH factors, such as Achaete-scute or Olig, may play redundant or prominent roles in neurogenic areas that differentiate normally in *ngn1*-deficient embryos.

Our results point to synergistic roles of Ngn1 and Coe2 in MH neurogenesis, possibly reflecting the positive cross-regulation of their expression, and a parallel activity of these factors rather than their action in a linear cascade. It is possible that the crossregulation of *ngn1* and *coe2* expression helps stabilise the committed state of neuronal progenitors, as described for *Xenopus* Xcoe2 (Dubois et al., 1998).

Together, our results lead to a model for the spatial control of MH neurogenesis (Fig. 7). In this process, *ngn1* and *coe2* expression are crucial elements that permit neurogenesis throughout the MH, which is initially identified as a single territory competent to form neurons. At the MHB, *ngn1* and *coe2* expression are the targets of Her5 inhibition. This inhibition prevents the specification of a proneural cluster in this location and permits the generation of the IZ.

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