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

Andrea Geling1, Charles Plessy2, Sepand Rastegar2, Uwe Strähle2,* and Laure Bally-Cuif1,†

1Zebrafish Neurogenetics Junior Research Group, Institute of Virology, Technical University-Munich, Trogerstrasse 4b, D-81675 Munich, Germany and GSF-National Research Center for Environment and Health, Institute of Developmental Genetics, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany
2Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1 rue Laurent Fries, BP 10142, F-67404 Illkirch Cedex, C.U. de Strasbourg, France
*Present address: Institute of Toxicology and Genetics, Forschungszentrum Karlsruhe, PO 3640, D-76021 Karlsruhe, Germany
†Author for correspondence (e-mail: bally@gsf.de)

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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 dorsosventral (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 epithilium (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 (MOher5) 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, motoneurons 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 17α) and ngn1 1– (neurod 11059) 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 (−4.4ngn1: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 pcksp70/her5 (homo or heterozygote) transgenic embryos a heat-shock pulse between 80% epiboly and tailbud stage, as described (Geling et al., 2003). pcksp70/her5 transgenic embryos were identified by PCR following in situ hybridisation (Geling et al., 2003).

Generation of the −3.3ngn1:gfp mutated construct (−3.3Eboxngn1: 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 −3.3Eboxngn1:gfp, the E-box located in the ANPE element (CAGTTG) was selectively replaced by an unrelated sequence (CTCTAGA), using standard procedures. Details of these constructs are available upon request. Both constructs were then flanked by I SceI restriction sites, which allow efficient integration in the Medaka genome in co-injection with the I SceI meganuclease enzyme (Thermes et al., 2002). For transient reporter assays, 50 ng/µl of −3.3ngn1:gfp or −3.3Eboxngn1:gfp circular plasmid DNAs were injected together with 1 U/µl I SceI 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 MÖher5 (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 H2O 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′ GGCAATTCGCAACAGTGTCA T 3′; downstream, 5′ GCCTCGAGTTTCAAGATGAC 3′. It was then subcloned into the pXT7 vector (Dominguez et al., 1995) and verified by sequencing. her5VP16 encodes a dominant form of Her5 (Gene-Tools Inc., Oregon, USA) as lineage tracer; 100 ng/µl her5VP16 (Hollie et al., 2002) was dissolved to a stock concentration of 2 mM in H2O and injected into one-cell stage wild-type or transgenic embryos at 2 mM.

DNA injection
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′ GGCAATTCGCAACAGTGTCA T 3′; downstream, 5′ GCCTCGAGTTTCAAGATGAC 3′. It was then subcloned into the pXT7 vector (Dominguez et al., 1995) and verified by sequencing. her5VP16 encodes a dominant form of Her5 (Gene-Tools Inc., Oregon, USA) as lineage tracer; 100 ng/µl her5VP16 (Hollie et al., 2002) was dissolved to a stock concentration of 2 mM in H2O and injected into one-cell stage wild-type or transgenic embryos at 2 mM.

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.

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 vce 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
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 δA, 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(Sp)l-like bHLH factors that act downstream of Notch in lateral inhibition during neurogenesis (Bertrand et al., 2002; Fisher and Caudy, 1998).

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 DeltaStu 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; however, none of these manipulations affects her5 expression or the presence and size of the IZ (L,M,Q); des, homozygote deadly-seven embryos; IZ, intervening zone; NICD, Notch intracellular domain; som, somite stage.

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 (delta), was induced upon injection of the morpholino antisense oligonucleotide MOher5 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).
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 MOher5 embryos at later stages. Although on average 30(±4) ngn1-positive cells were induced across the medial IZ at the three-somite stage in MOher5-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\text{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\text{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\text{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\text{her5} and RNA encoding Delta\text{Stk} led to increased levels of ngn1 expression across the medial IZ compared with injection of MO\text{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 (previously 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). (IJ) Comparison of coe2 expression in a pshsp70: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 (Xcoe2\text{DBD}) (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 MOher5 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 phsp70:her5 transgenic embryos) strongly downregulated coe2 expression in the MH domain (80% of cases, n=20) (Fig. 4I).

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 dominantly-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. 4PQ) (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 (neurod3hi1059) (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 MOher5 into one-cell stage ngn1−/− embryos, coe2 expression was still induced at the medial IZ, at levels comparable with those found in the vcc and r2M clusters (Fig. 5F) (85% of cases, n=20). 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 MOher5, 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...
zn12 immunoreactivity in this location at the 18-somite stage (Fig. 5L,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 MOher5 was injected into ngn1−/− mutants (100% of cases, n=19) (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 MOher5 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. 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,L), ngn1−/− mutants (B,E,H,J,K) or embryos injected with capped RNA encoding a dominant-negative form of Coe2 (Xcoe2ΔDBD) (E,F,M,N). Embryos injected with MOher5 (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–C), 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 sufficient to drive correct reporter expression in neuronal clusters of the anterior neural plate and sensory precursors of the spinal cord, which are largely preserved (Fig. 5H, 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). (I–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).
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 MOher5 (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 SceI 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<sup>her5</sup> 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 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 SceI, 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 in anterior clusters, including the vcc and r2M.

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<sup>Sma</sup> 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 zebrash (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. Sgp/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, ashaB, 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.
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 ephiphysial 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 Coe2 (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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