

Inhibition of neurogenesis at the zebrafish midbrain-hindbrain boundary by the combined and dose-dependent activity of a new *hairy/E(spl)* gene pair

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Summary

The intervening zone (IZ) is a pool of progenitor cells located at the midbrain-hindbrain boundary (MHB) and important for MHB maintenance, midbrain-hindbrain growth and the generation of midbrain-hindbrain neurons. Recently, we implicated the *Hairy/E(spl)* transcription factor *Her5* in the formation of the medial (most basal) part of the IZ (MIZ) in zebrafish; the molecular bases for lateral IZ (LIZ) formation, however, remain unknown. We now demonstrate that *her5* is physically linked to a new family member, *him*, displaying an identical MHB expression pattern. Using single and double knockdowns of *him* and *her5*, as well as a *him+her5* deletion mutant background (*b404*), we demonstrate that *Him* and *Her5* are equally necessary for MIZ formation, and that they act redundantly in LIZ formation *in vivo*. We show that these processes do not involve cross-regulation

between *Him* and *Her5* expression or activities, although *Him* and *Her5* can heterodimerize with high affinity. Increasing the function of one factor when the other is depleted further shows that *Him* and *Her5* are functionally interchangeable. Together, our results demonstrate that patterning and neurogenesis are integrated by the *her5-him* gene pair to maintain a progenitor pool at the embryonic MHB. We propose a molecular mechanism for this process where the global 'Him+Her5' activity inhibits *ngn1* expression in a dose-dependent manner and through different sensitivity thresholds along the medio-lateral axis of the neural plate.

Key words: *Hairy*, *E(spl)*, *her5*, *him*, Midbrain-hindbrain, MHB, Neurogenesis, Zebrafish

Introduction

Development of the vertebrate central nervous system (CNS) is a complex process that needs to connect patterning and neurogenesis. Crucial to this process are local events of neurogenesis inhibition, which maintain pools of progenitors in defined locations of the neural tube. The delayed differentiation of these progenitor zones permits the generation of large as well as spatiotemporally patterned structures, such as the layered cortex in higher vertebrates, the polarized optic tectum in birds, or the retina. The molecular events controlling the generation or maintenance of neural progenitor pools remain largely hypothetical.

The embryonic midbrain-hindbrain domain (MH) is characterized by the maintenance of a zone of delayed differentiation at the midbrain-hindbrain boundary (MHB). This zone, called 'intervening zone' (IZ), separates midbrain from anterior hindbrain neuronal clusters and has been described in all vertebrates (Bally-Cuif et al., 1993; Geling et

al., 2003; Vaage, 1969). Its functional importance is attested by genetic ablation experiments. For instance, in mouse embryos lacking the function of the two bHLH *E(spl)*-like transcription factors *Hes1* and *Hes3*, premature differentiation of the IZ occurs, leading to the lack of several MH neuronal populations, and to the collapse of MH structures (Hirata et al., 2001). These defects may primarily result from a disruption of the isthmus organizer, an inducing cell population located at the MHB and involved in MH maintenance (Hirata et al., 2001; Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Recent results in zebrafish have permitted dissection of the mechanisms of IZ formation in more detail. There, expression of the *hairy/E(spl)* gene *her5* (Muller et al., 1996) precisely delineates the IZ at all embryonic stages (Geling et al., 2003). At the onset of neurogenesis (tail-bud stage), *her5* expression separates the early midbrain ventrocaudal proneural cluster (vcc) from the anterior hindbrain proneural clusters of rhombomere 2 (presumptive motoneurons –r2MN– and lateral

neurons –r2LN–). In the absence of Her5 function, ectopic neurogenesis occurs in the medial (future basal) part of the IZ, as revealed by the ectopic expression of the proneural genes *neurogenin1* (*ngn1*) and *coe2* and the later differentiation of neurons across the basal MHB, bridging the vcc and r2MN (Geling et al., 2003; Geling et al., 2004). Conversely, forced expression of *ngn1* within the MH domain leads to a partial downregulation of MHB markers' expression (Geling et al., 2003). These results have two implications. First, they confirm that the IZ is necessary to maintain MHB integrity. Second, they demonstrate that the IZ is composed of at least two domains along the mediolateral axis, which differ in their requirement for Her5 function: the medial IZ domain (MIZ), which crucially depends on Her5 for neurogenesis inhibition, and the lateral (future alar) IZ domain (LIZ), which forms even in the absence of Her5. Within the MIZ, Her5 acts as a prepattern factor that prevents the formation of a proneural cluster, and inhibits expression of *ngn1* and *coe2* upstream of Notch signaling (Geling et al., 2004).

Our study of Her5 function did not address the formation of the LIZ, in spite of its crucial role in controlling midbrain and anterior hindbrain alar neurogenesis. To now approach this issue, we reasoned that other Hairy/E(spl) factors might be expressed within this domain and act redundantly with Her5. Because there are examples of physically linked *E(spl)* genes in *Drosophila* (*E(spl)* complex) (Klamt et al., 1989; Knust et al., 1992) and zebrafish (*her1* and *her7*) (Henry et al., 2002), and because linked genes are more likely to share spatiotemporal characteristics of expression, we searched for new Hairy/E(spl) genes in the vicinity of the *her5* locus. Sequencing a *her5*-containing PAC revealed a new *her*-like gene, *him*, adjacent to *her5* and in opposite orientation (hence *him* for *her5* image), identically expressed across the IZ. We report here that Him is the hypothetical factor cooperating with Her5 to control LIZ formation in vivo, and that Him also crucially contributes to MIZ formation. Together, our results unravel the genetic combination preventing neurogenesis across the MHB.

Materials and methods

Cloning and phylogenetic analysis of *him*

Systematic sequencing of genomic DNA surrounding known *her* genes was performed using PAC clones obtained from RZPD. A new ORF was detected close to the *her5* gene locus in PAC BUSMP706H15152Q2. 5' RACE experiments were done to identify full-length cDNA, according to the manufacturer's recommendations (Invitrogen). The newly cloned gene, *him*, corresponds to ENSDARG00000002707 now predicted by The Wellcome Trust Sanger Institute, with the exception that in the prediction the first exon is missed and the last exon is truncated.

The phylogenetic tree of zebrafish Her family was done using the phylodendron software (www.es.emblnet.org/Doc/phylodendron/treeprint-form.html). To construct the phylogenetic tree, the VectorNTI software and full-length sequences were used. The accession numbers of compared proteins are: Her5: NP_571152, Her1: NP_571153, Her7: NP_571684, Her4: NP_571165, Her2: NP_571164, Her3: NP_571155, Her9: NP_571948 and Her6: NP_571154. Proteins predicted by The Wellcome Trust Sanger Institute are: Her11 (ENSDARP00000012990), Her13 (ENSDARP00000008307), Hes6 (ENSDARP00000021078) and Her12 (ENSDARP000000038100) (<http://www.ensembl.org>, release from 08-02-2004).

Zebrafish strains and transgenic lines

Embryos obtained from natural spawning of AB wild-type or transgenic fish, *her5*PAC::egfp (Tallafuss and Bally-Cuif, 2003) and –8.4ngn1::egfp (Blader et al., 2003), were raised and staged according to Kimmel et al. (Kimmel et al., 1995). *no isthmus* (*noi^{tu29a}*), *acerebellar* (*ace^{ti282a}*) (Brand et al., 1996) and *knypek* (*b404*, *kny^{m119}*) (Solnica-Krezel et al., 1996; Topczewski et al., 2001) mutants were obtained by pairwise mating of heterozygous adult carriers, as described previously.

Protein expression interference assays

Morpholino antisense oligonucleotides (*her5*MO^{ATG}) were purchased from Gene-Tools, Inc. (Oregon, USA). The morpholino was dissolved to a stock concentration of 5 mM in H₂O and injected into one-cell stage embryos at 1 mM. *him*MOs lead to non-specific cell death (not shown). Thus, *GripNA* antisense oligonucleotides preventing *him* translation, specific for the *him* ATG region (*himGripNA^{ATG}*) or acceptor site of the second *him* exon (*himGripNA^{SPL}*), were purchased from Active Motif (Belgium). *GripNA*s were dissolved to stock solution of 1 mM in H₂O and injected into one-cell stage embryos at 0.5 mM. At this dose, the effect of *himGripNA*s on *ngn1* expression was maximal. Sequences of antisense oligonucleotides were as follows: *her5*MO^{ATG}: 5'-TTGGTTCGCTCATTTTGTGTATTCC-3'; *himGripNA^{ATG}*: 5'-ATTCGGTGTGCTCTTCAT-3' and *himGripNA^{SPL}*: 5'-TACTCACAGTGTCTGCAG-3'. All injection experiments were repeated at least three times.

In situ hybridization and immunohistochemistry

Probe synthesis, in situ hybridization and immunohistochemistry were carried out as previously described (Hammerschmidt et al., 1996). The following in situ antisense RNA probes were used: *her5* (Muller et al., 1996), *him* (this paper), *ngn1* (Korzh et al., 1998), *pax2.1* (Lun and Brand, 1998) and *egfp* (Clontech). Primary antibodies used for immunohistochemistry were rabbit anti-GFP (ams biotechnology Europe, TP401) used at a final dilution of 1/500 and mouse anti-human neural protein HuC/HuD (MoBiTec A-21271) (1/300). They were revealed by using FITC-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, 111-095-003) or Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, 115-165-044) (1/200), as appropriate. Embryos were scored and photographed under a Zeiss SV 11 stereomicroscope or a Zeiss Axioplan photomicroscope.

RNA injections

knypek capped RNA was synthesized using Ambion mMessage mMachine kit following the recommended procedure. Capped RNA was injected at the concentration of 60 ng/μl into the embryos at the one-cell stage.

Protein interaction assays

For two-hybrid assays, The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used following procedures described by the manufacturer. The 'bait' and 'AD' plasmids were constructed by fusing in-frame the complete ORFs of *her5*, *him* and *ngn1* to either pGBKT7 (encoding the GAL4 DNA-binding domain) or pGADT7 (encoding the GAL4 activation domain). The relative stringency of Her5 homodimerization versus its heterodimerization with Him was quantified by β-galactosidase assay according to the manufacturer's recommendation (Clontech). The β-galactosidase activity was quantified according to Lazo et al. (Lazo et al., 1978).

Co-immunoprecipitation and western blot analysis

Transformed yeast cells expressing the two proteins of interest were lysed in 0.5 ml of lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP40, 0.1 mM DTT, 0.1 mg/ml pepstatin A, 0.03 mM leupeptin, 145 mM benzamidin, 0.37 mg/ml aprotinin, 1 mM

phenylmethylsulfonyl fluoride) with 0.25 g of glass beads (425-600 µl, Sigma) for one hour at 4°C with shaking. The extracts were then centrifuged at 15 000 *g* for 10 minutes at 4°C to eliminate cell debris, and the supernatant was collected. For each immunoprecipitation, 0.4 ml aliquots of lysate were pre-cleaned by incubation with 150 µl of pre-immune rabbit serum and 100 µl of 1:1 slurry of Protein A Sepharose™ CL-4B (Amersham Bioscience AB) for 30 minutes at 4°C. Pre-cleaned extracts were immunoprecipitated with 10 µl of rabbit anti-HA antibody (dilution 1/1000) (Sigma) and 100 µl of 1:1 slurry of Protein A Sepharose CL-4B (Amersham Bioscience AB) for 30 minutes at 4°C. The sepharose beads were washed three times with 1 ml of lysis buffer. The precipitates were fractionated on SDS-PAGE and subsequent western blot analysis was performed according to standard protocols, by using mouse anti-c-Myc antibodies (1/1000) (Sigma). The primary antibodies were revealed using HRP-coupled secondary antibodies (Jackson Laboratories) diluted to 1/200 and enhanced chemiluminescence (Amersham Bioscience AB).

Quantification of *him* mRNA in the *her5PAC::egfp* line

Total RNA was isolated from *her5PAC::egfp* embryos and WT siblings at the five-somite stage and reverse-transcribed before real-time PCR amplification. Real-time PCR was done by using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Germany) and Light Cycler Instrument (Roche, Germany). Quantitative values were obtained from the threshold cycle number at which the increase in the signal associated with exponential growth of the PCR products begins to be detected using the LightCycler Software, according to the manufacturer's recommendations. The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (lack of extensive degradation) are both difficult to assess precisely. We therefore also quantified the transcript of the *pax6* gene as the endogenous RNA control, and both samples were normalized to the basis of *pax6* content (*R*-value on the graph). The nucleotide sequences of the specific primers used are shown in Table 1. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes and 65 cycles at 95°C for 15 seconds, 55°C for 10 seconds and 72°C for 15 seconds. The quantifications were performed in triplicate on a pool of 50 embryos for each line and results represent the mean values $\pm$ s.e.m.

Results

A new *hairy/E(spl)* gene, *him*, is physically linked to *her5* in a head-to-head orientation

We searched for genes physically linked to *her5* by sequencing a *her5*-containing PAC (Tallafuss and Bally-Cuif, 2003). This revealed the presence of an open reading frame encoding a new Hairy/E(spl) factor located 3.3 kb upstream of *her5* and in a head-to-head orientation (Fig. 1A). RT-PCR experiments demonstrated the presence of the corresponding transcript in embryos (not shown), and blasting against the zebrafish genome (release from 08-02-04) confirmed the association of this gene with *her5*. We named this new gene *him* (for '*her5* image') (GenBank accession number AY705671). The genomic structure of *him* was determined by aligning cDNA and genomic sequences and shows several alterations compared to the zebrafish genome prediction, notably the presence of an additional 5' coding exon (true exon 1) as well as a different position and size of the last exon (exon 4), coding for 195 amino acids instead of the 22 predicted (Fig. 1A).

The head-to-head association of the gene pair *him-her5* is reminiscent of the genomic organization of *her7-her1* (Henry et al., 2002). Further analysis of the zebrafish genome suggests

Table 1. Sequences of primers used in real-time PCR

Gene	Oligonucleotide	Sequence*	Amplicon size (bp)
<i>him</i>	forward primer	5'-GTTATATCTCCTGCGGGT-3'	163
	reverse primer	5'-GGGATAAGAGGAAGCCTTT-3'	
<i>otx1</i>	forward primer	5'-GATACCCAGCAACACCG-3'	136
	reverse primer	5'-ATCTTCAGTGCCACCT-3'	
<i>pax6</i>	forward primer	5'-TTTGCTGGGAGATTC-3'	167
	reverse primer	5'-CTCTGCCCGTTGAGCATTCT-3'	

*Primers were chosen with the assistance of LightCycler Probe Design software.

at least another additional gene pair, *her4* and a new predicted *her* gene that we named *her12* (ENS DARG00000028110) (Fig. 1B). Therefore, we propose that a paired organization might be a conserved feature among zebrafish *her* genes.

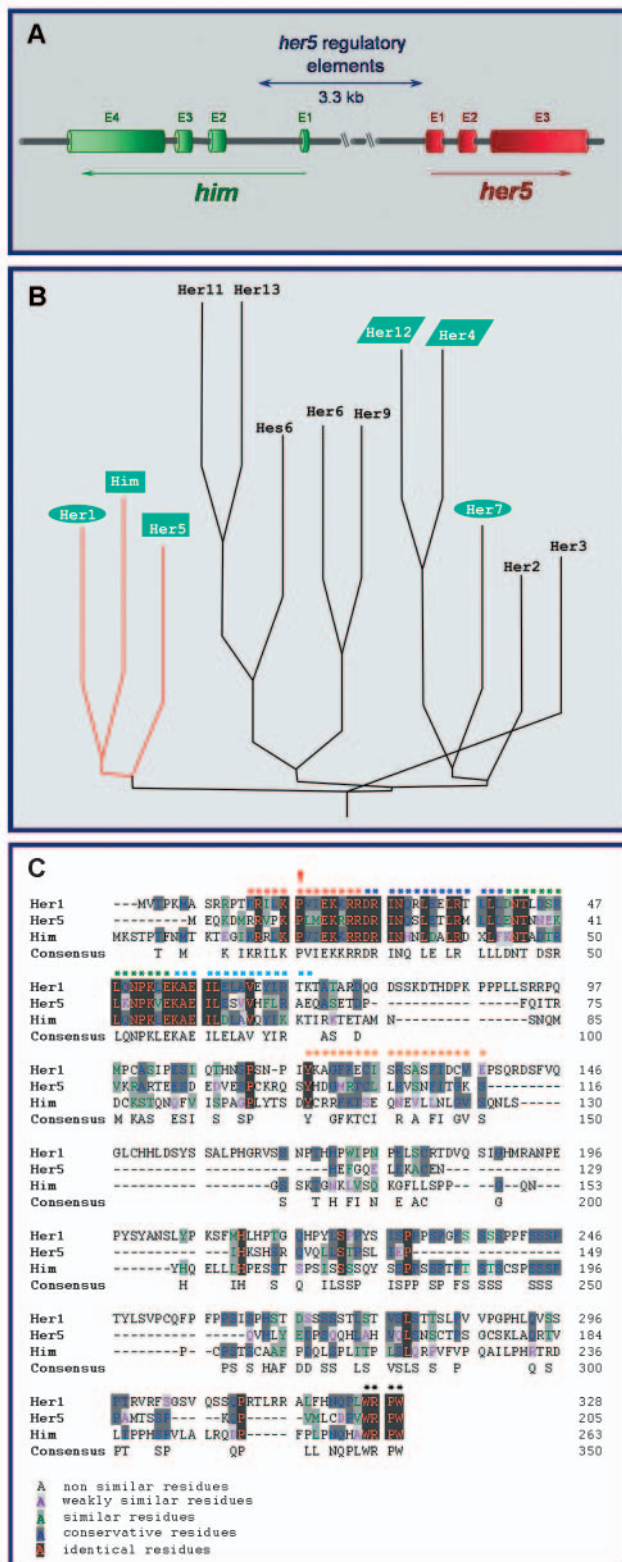
The Him protein, translated from our full-length cDNA sequence, consists of 297 amino acids and exhibits all structural features of an Hairy/E(spl) bHLH factor acting as transcriptional repressor (Davis and Turner, 2001): a conserved proline residue in the basic domain, an 'orange' domain (Dawson et al., 1995) and a WRPW tetrapeptide in the C-terminus (Fisher et al., 1996) (Fig. 1C). Within the zebrafish Her family, Him shows the highest similarity to Her1 with 28.6% identical and 35.2% conserved amino acid (aa) residues. Similarity between Him and Her5 is slightly weaker (20.1% identical and 29.2% conserved aa residues) (Fig. 1B, red lines, and Fig. 1C). Comparison restricted to the functional bHLH domain reveals 66% identity to Her1 and 50% identity to Her5.

him expression within the presumptive midbrain-hindbrain is identical to *her5* and marks the intervening zone

We analyzed *him* expression by RT-PCR and in situ hybridization. *him*, like *her5*, is maternally expressed (Fig. 2A). Early zygotic *him* expression is ubiquitous (data not shown) but rapidly resolves in a first, transient, profile within the presumptive dorsal endoderm and mesoderm (Fig. 2B) at 30% epiboly: *him* is expressed in deep scattered cells of the dorsal embryonic margin and in the deep layer of the dorsal mesendoderm (Fig. 2B and 2B', red arrows). From mid-gastrulation onwards (70% epiboly), *him* expression in the presumptive endo- and mesoderm becomes undetectable (Fig. 2C). At that stage, *him* becomes transcribed in the anterior neural plate, in a V-shaped domain interrupted at the midline (Fig. 2C, red arrowhead). Expression in the lateral aspects of this domain is slightly broader and stronger than medially. At the three-somite stage, this expression fuses medially and, by anatomical landmarks, is clearly located within the presumptive MH domain (Fig. 2E,F). *him* expression is maintained at the MHB later on until 36 hpf (Fig. 2H,I). Starting at late gastrulation, *him* is also expressed in the presomitic mesoderm (Fig. 2D-F, blue arrows). Expression in this territory is detectable until late somitogenesis (Fig. 2H).

To determine whether and to what extent *him* and *her5* share expressing cells within the presumptive MH, we compared their expression profiles by double ISH. At three somites (Fig. 2F), observation at high resolution of double-stained embryos showed that the MH expression of *him* and *her5* are exactly identical. Thus, *him* expression, like *her5*, precisely delineates

the MH primordium (Tallafuss and Bally-Cuif, 2003). Like *her5* (Geling et al., 2003), it is also a permanent marker of the IZ, separating the early midbrain proneural cluster *vcc* from rhombomere 2 presumptive motor- and sensory neurons at late gastrulation (Fig. 2J). *him* expression is detected in this domain slightly earlier than *her5* (not shown).



Taken together, our data suggest a more complex picture than expected from the genomic organization of *him* and *her5*. *him* and *her5* share expression within the MH domain, but differ elsewhere in *her5*-specific versus *him*-specific domains (pharyngeal precursors versus presomitic mesoderm at late gastrulation, respectively). In the neural plate however, Him is, together with Her5, the earliest marker of the MH and IZ, prompting us to analyze its function in this domain in more detail, in relation to MH patterning and Her5 activity.

Like Her5, and in contrast to most MH factors, Him does not control patterning events within the MH region

Refined regionalization and maintenance of the MH domain at somitogenesis stages depends on a positive cross-regulatory loop involving Fgf8 and Pax2.1 (Brand et al., 1996; Lun and Brand, 1998; Reifers et al., 1998; Tallafuss and Bally-Cuif, 2003). To determine whether *him* was part of this loop, we analyzed its expression in *ace/fgf8* and *noi/pax2.1* mutants (Brand et al., 1996; Lun and Brand, 1998; Reifers et al., 1998). Expression of *him* in *ace* mutant embryos is initiated normally (data not shown) but, from mid-somitogenesis stages onwards, gradually narrows to persist at the MHB only in a dorsal patch (Fig. 3A,B). At 24 hpf *him* expression in *ace* is undetectable (data not shown). Similarly, in *noi* mutants, a downregulation of *him* expression can be observed from mid-segmentation stages onwards. In contrast to *ace*, *him* expression in *noi* later remains restricted to the ventral MHB (Fig. 3C,D), like *her5* (Lun and Brand, 1998; Reifers et al., 1998). Together, these observations demonstrate that the maintenance of *him* expression is, like that of other MH genes and within a similar time-window, under control of the MH regulatory loop.

Because Him is one of the earliest selective markers of the MH primordium, we asked in turn whether Him activity was involved in controlling aspects of MH regionalization. Loss of Him function, performed by injection of an antisense *GripNA* oligonucleotide specific for the ATG region of *him* (*himGripNA*^{ATG}) into one-celled embryos (see below for results demonstrating the functionality and specificity of this *GripNA*), however affected neither the expression of MH

Fig. 1. Sequence analysis and phylogeny of the new bHLH transcription factor Him. (A) Genomic organization of the *him/her5* locus. The coding region is interrupted by three introns, the bHLH domain being encoded by exons E2 and E3. Note that the first exon of *him* is within the domain identified as necessary for MH expression of *her5* (Tallafuss and Bally-Cuif, 2003). (B) Phylogenetic tree of the zebrafish Her family, showing relationship between known and predicted Her proteins. The alignment used for tree construction was obtained using the VectorNTI software, and is based on full-length sequences. Paired genes are boxed in blue, and individual pairs are marked with the same symbol (oval, rectangle or diamond). Accession numbers for the proteins used in this tree are: Her5 (NP_571152), Her1 (NP_571153), Her7 (NP_571684), Her4 (NP_571165), Her2 (NP_571164), Her3 (NP_571155), Her9 (NP_571948) and Her6 (NP_571154). Proteins predicted by The Wellcome Trust Sanger Institute (<http://www.ensembl.org>) are: Her11, Her12, Her13 and Hes6. (C) Sequence alignment of Her1, Her5 and Him. Basic domains are overlaid with red asterisks, Helix 1 domains with dark blue, loop domains with green, Helix 2 domains with light blue, orange domains (H3/H4) with orange stars and WRPW tetrapeptides

patterning markers (e.g. *pax2.1*, *eng2*, *eng3*), nor that of IsO activity markers (e.g. *wnt1*, *fgf8*) at the 3- and 15-somite stages (data not shown).

Thus, although *him* expression depends on the MH maintenance loop, *Him* activity itself does not appear to impinge on this loop to influence MH patterning, like that of *Her5*.

Him activity is crucial for the formation of the medial IZ at early neurogenesis stages

We demonstrated previously that *Her5* is crucially necessary to prevent neurogenesis across the MIZ (Geling et al., 2003). Because of the similar expression of *him*, and its encoding a related Hairy/E(Spl) factor, we explored a potential involvement of *Him* in IZ formation. At early neurogenesis stages, the IZ is also the major site of *pax2.1* expression, which we used as landmark in future experiments (see Fig. 4D).

We tested a requirement for *Him* in IZ formation in loss-of-function experiments where *him* mRNA translation was blocked by specific antisense *Grip*NAs. Two *Grip*NAs were used: *himGrip*NA^{ATG} (as above), and a second *Grip*NA targeting the acceptor splice site of *him* exon2 (*himGrip*NA^{SPL}). Undistinguishable phenotypes (see below) were obtained with the two *Grip*NAs, but not with a control *Grip*NA of unrelated sequence, indicating that the phenotypes observed are a specific consequence of *Him* dysfunction. Specifically, *himGrip*NA-injected embryos assayed at the three-somite stage for *ngn1* expression displayed a complete lack of the MIZ, with bridging of the vcc and r2MN clusters by ectopic *ngn1*-expressing cells (76% of cases, *n*=21 for *himGrip*NA^{ATG}, 83% of cases, *n*=24 for *himGrip*NA^{SPL}) (Fig. 4B,C, compare to 4D). This phenotype is followed by the development of ectopic differentiated neurons across the basal MHB at later stages (data not shown). It is in all respects

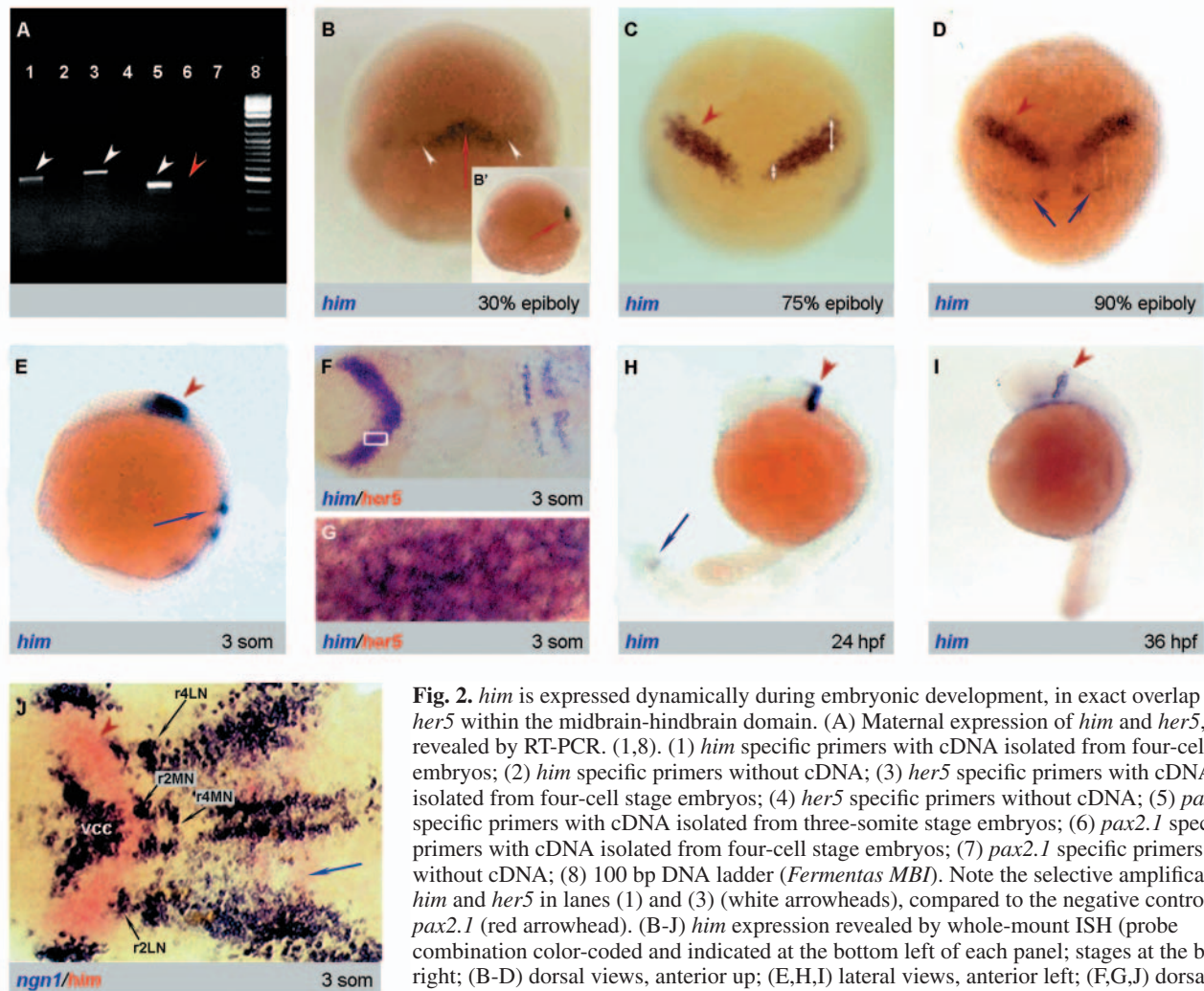


Fig. 2. *him* is expressed dynamically during embryonic development, in exact overlap with *her5* within the midbrain-hindbrain domain. (A) Maternal expression of *him* and *her5*, revealed by RT-PCR. (1,8). (1) *him* specific primers with cDNA isolated from four-cell stage embryos; (2) *him* specific primers without cDNA; (3) *her5* specific primers with cDNA isolated from four-cell stage embryos; (4) *her5* specific primers without cDNA; (5) *pax2.1* specific primers with cDNA isolated from three-somite stage embryos; (6) *pax2.1* specific primers with cDNA isolated from four-cell stage embryos; (7) *pax2.1* specific primers without cDNA; (8) 100 bp DNA ladder (Fermentas MBI). Note the selective amplification of *him* and *her5* in lanes (1) and (3) (white arrowheads), compared to the negative control *pax2.1* (red arrowhead). (B-J) *him* expression revealed by whole-mount ISH (probe combination color-coded and indicated at the bottom left of each panel; stages at the bottom right; (B-D) dorsal views, anterior up; (E,H,I) lateral views, anterior left; (F,G,J) dorsal views of flat-mounted embryos, anterior left). At 30% epiboly (B), *him* is transcribed in the

deep layer of the mesoderm (red arrows, see sagittal view in B') and in scattered cells of the dorsal embryonic margin (white arrowheads). *him* expression within the MH domain (red arrowheads) is initiated at 75% epiboly (C) (note the difference in expression in medial and lateral parts of the IZ is indicated with white arrows) and maintained until 36 hpf (I). Note in F (and see higher magnification of the boxed area in G) that *him* and *her5* expression in this domain are exactly coincident. *him* expression in the presomitic mesoderm starts at 90% epiboly (D) (blue arrows) and is maintained until 24 hpf (H). *him* and *ngn1* are complementarily expressed in the MH region (J). Red arrowheads indicate *him* expression at the MHB and blue arrows expression in the presomitic mesoderm. IZ, intervening zone; vcc, ventrocaudal cluster, r2M, presumptive motorneurons of rhombomere 2; r2L, presumptive lateral neurons of rhombomere 2; r4M, presumptive motorneurons of rhombomere 4; r4L, presumptive lateral of rhombomere 4.

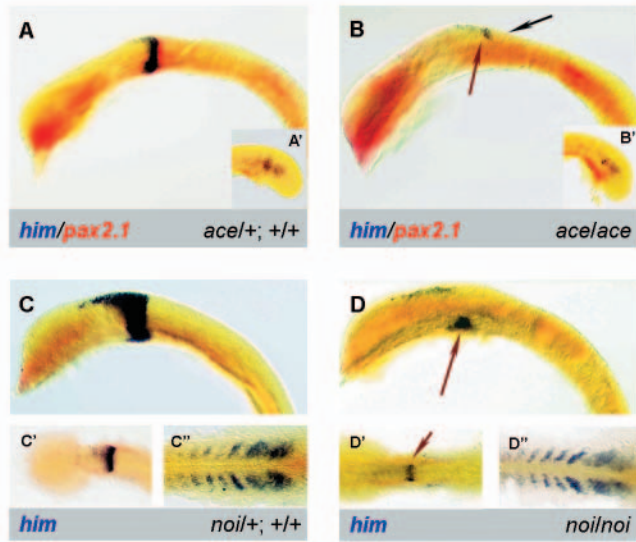


Fig. 3. *him* expression is controlled by Pax2.1 and Fgf8 during the MH maintenance loop. (A,B) *him* (blue) and *pax2.1* (red) expression in *ace* mutants (B) and WT siblings (A) at the 21-somite stage (all embryos deyolked, lateral view, anterior left). Note that both genes are coincidentally switched off at the MHB, except for a common dorsal patch (arrows), while *him* expression in the presomitic mesoderm is intact (A',B', insets). (C,D) *him* expression (blue) in *noi* mutants (D) and WT siblings (C) at the 17-somite stage (C,D: deyolked embryos, lateral views, anterior left; C'-D': dorsal views of flat-mounted heads (C',D') and tails (C'',D'')). *him* expression at the MHB is restricted to a ventral patch in *noi* (arrow), while presomitic expression is unaffected (C'',D'').

similar to that triggered by loss of Her5 function in *her5MO^{ATG}* morphants (Fig. 4A) (Geling et al., 2003). Thus, loss-of-function of either Her5 or Him results in the same failure to form and maintain the MIZ.

Him and Her5 are independently required for medial IZ formation

The above results are compatible with a simple model where Him and/or Her5 would act in a common regulatory cascade, one factor positively regulating expression of the other gene. Thus, loss of Him function would cause loss of *her5* expression, or the reverse. Alternatively, Him and Her5 might be independently necessary for MIZ formation. To address this question, we studied *him* and *her5* expression in embryos where Her5 or Him activity, respectively, was blocked. We observed that *him* expression was unchanged in Her5 morphants, in both the MH region and the presomitic mesoderm, under conditions where *ngn1* expression was strongly induced in place of the MIZ (Fig. 4E,F, and data not shown). Thus *him* expression is not under immediate control of Her5. Likewise, injection of *GripNA^{ATG}* into wild-type embryos did not produce alterations in *her5* transcription, although the MIZ was lost (Fig. 4I compared to 4K, and data not shown). Furthermore, injection of *GripNA^{ATG}* did not affect the production of the fusion Her5-GFP protein, driven under control of all *her5* regulatory elements in *her5PAC::egfp* transgenics (Tallafuss and Bally-Cuif, 2003) (Fig. 4J,L). Thus, Him does not influence *her5* transcription or translation.

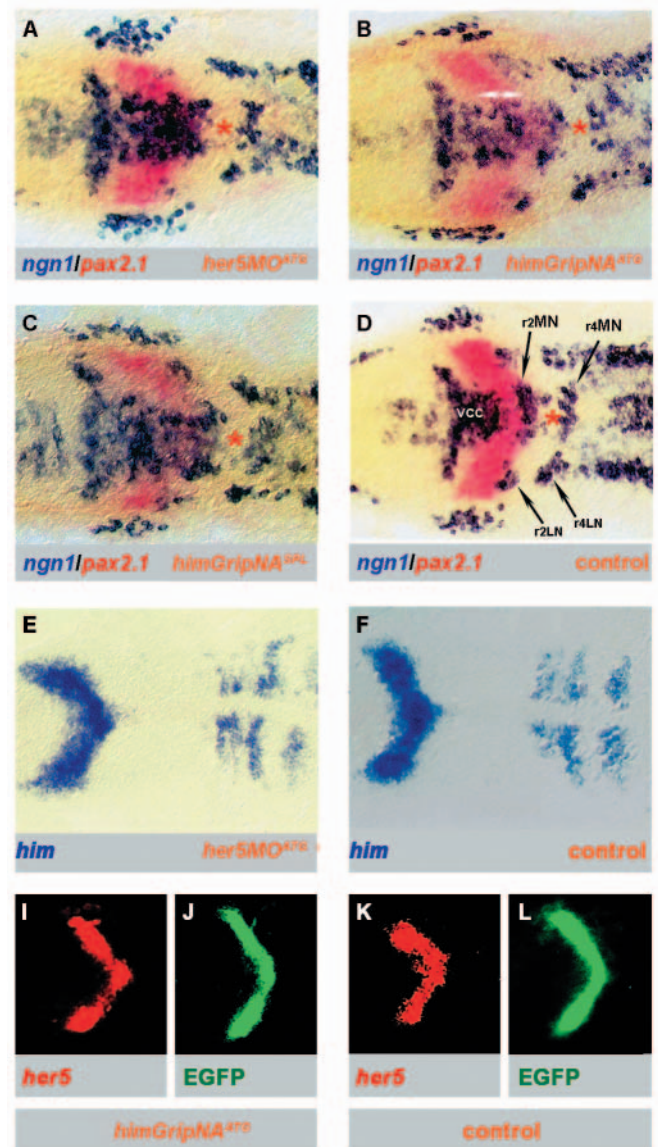


Fig. 4. The activity of both Him and Her5 is necessary to prevent neurogenesis across the medial IZ in vivo. (A-D) The inhibition of either Him or Her5 function triggers ectopic neurogenesis in place of the MIZ (dorsal views of the MH region in flat-mounted embryos at the four-somite stage, anterior to the left). Embryos are probed for *ngn1* (blue) and *pax2.1* (red) expression following injection of *her5MO^{ATG}* (A), *himGripNA^{ATG}* (B), *himGripNA^{SPL}* (C) (orange labels), compared to a non-injected WT control embryo (D). Note that the vcc and r2MN are bridged by ectopic *ngn1*-positive cells (double arrows) after blocking Her5 or Him activity, while other undifferentiated areas are not affected (e.g. area between r2MN and r4MN, asterisk). (G-L) *him* and *her5* expression are not successive and interdependent steps of the anti-neurogenic cascade acting in the MIZ (dorsal views of the MH area in flat-mounted embryos at the three-somite stage, anterior to the left, used markers are color-coded). (G,H) *him* expression in wild-type embryos (H) or after injection with *her5MO^{ATG}* (G). Note that *him* expression is not modified. (I-L) Expression of *her5* (I,K) and GFP (J,L) in *her5PAC::egfp* embryos injected (K,L) or not (I,J) with *himGripNA^{ATG}*. Note that *her5* and GFP expression are unaffected. vcc: ventro-caudal cluster, r2MN: prospective motoneurons of rhombomere 2, r4MN: prospective motoneurons of rhombomere 4.

We conclude that Him and Her5 do not act in a simple cascade of cross-regulation of expression. Rather, the two genes are expressed independently of each other and are both essential to MIZ formation.

The crucial determinant of MIZ formation is the total dose of Him + Her5 inhibitory activities

Several hypotheses could account for the above results. First, Him and Her5 might both be required for MIZ formation because they need to heterodimerize with each other to be active. Alternatively, these factors do not have unique essential activities, but rather are required to reach together a threshold level of Hairy/E(spl) activity necessary to prevent proneural gene expression. Finally, both factors might exert distinct and/or complementary functions necessary for MIZ formation.

To unravel the relevance of each hypothesis in vivo, we first tested whether Him and Her5 could interact in a yeast two-hybrid system. bHLH factors have the capacity to dimerize via their HLH domain, however their affinity for hetero- versus homodimerization cannot be a priori predicted, and some instances of DNA binding as oligomers have also been reported (Firulli et al., 2000; Iso et al., 2001; Wainwright and Ish-Horowicz, 1992). We observed that Her5 can homodimerize as well as bind Ngn1, while Him and Ngn1 failed to interact. In addition, heterodimers of Him and Her5 were produced. Whether Him is also able to homodimerize could not be tested due to unexplained toxicity of the him-expressing constructs (Table 2). All these interactions were confirmed by coimmunoprecipitation (Fig. 5A). Moreover, the affinity for heterodimerization between Him and Her5, based on beta-galactosidase activity (Lazo et al., 1978), appeared six-fold higher than the affinity of Her5 for homodimerization (Fig. 5B), suggesting that the Him-Her5 configuration predominates in vivo if the amount of proteins is equal. Thus, the requirement for Him and Her5 for MIZ formation in vivo might indeed be explained by the necessity for these factors to heterodimerize.

Our finding that Her5 can homodimerize, however, as well as reports of functional bHLH oligomers (Firulli et al., 2000; Iso et al., 2001; Wainwright and Ish-Horowicz, 1992), suggest that Her5 and/or Him alone could permit MIZ formation, provided the dose of this factor is sufficient. To test this hypothesis we studied neurogenesis in *her5PAC::egfp* transgenic embryos (Tallafuss and Bally-Cuif, 2003), where Him is in excess. This transgenic line was obtained by

recombination of *egfp* into the second exon of *her5* in a *her5*-containing PAC. Thus, in this transgene *her5* is not functional. However, because of the small genetic distance separating the *her5* and *him* locus, and because the entire recombinant PAC was used for germ line transformation (Tallafuss and Bally-Cuif, 2003), these transgenics carry an additional copy of *him* together with the *her5-egfp* fusion (see Fig. 5C for a scheme of the transgene). Real-time PCR confirmed a 1.5-fold increase in the amount of *him* messenger in embryos heterozygous for the *her5PAC::egfp* transgene, while the amount of *otx1* mRNA, used as a control, was unchanged (Fig. 5D). Because *him* regulatory elements are also contained within the recombinant PAC, additional *him* transcripts produced from the transgene display the endogenous *him* profile, restricted to the IZ within the neural plate (not shown). Therefore, *her5PAC::egfp* transgenics provide an ideal background to measure MIZ formation in a context where *her5* expression is normal but the dose of Him is increased across the IZ. Most interestingly, we observed that *her5PAC::egfp* transgenic embryos where Her5 activity was blocked by *her5MO^{ATG}* formed a normal MIZ (Fig. 5E, compare to Fig. 4D). In all cases, we verified that Her5 activity was completely abolished, by monitoring the lack of EGFP protein expression (data not shown). Thus Him, when present in sufficient amount (in a minimum of three doses, as in *her5PAC::egfp/+* heterozygote embryos), is capable of replacing Her5 activity to prevent neurogenesis across the MIZ.

We conclude from these observations that the crucial component of MIZ formation and maintenance is a threshold level of 'Him + Her5' inhibitory activity. In the normal embryo, this level is probably achieved by Her5-Him heterodimers, although a possible contribution of homodimers and/or oligomers from each factor separately cannot be excluded.

Formation of the lateral IZ also relies on the level of 'Him + Her5 activity' but with a lower threshold than the medial IZ

The LIZ is preserved in both *her5* and *him* single knockdown embryos, suggesting that it might require other factors than Him and Her5 for its formation. Alternatively, the LIZ might primarily differ from the MIZ in requiring a lower threshold of 'Him + Her5' activity, the endogenous level of one factor alone (two doses) being sufficient to block neurogenesis in this location. To address these hypotheses we assayed for lateral *ngn1* expression in double knockdown embryos obtained by the co-injection of *her5MO^{ATG}* and *himGripNA^{ATG}*. Strikingly, we observed that the simultaneous interference with both Her5 and Him activities results in ectopic *ngn1* expression in place of the entire IZ, i.e. including the LIZ (88% of cases, $n=25$) (Fig. 6A,A' compared to 6D,D'), in striking contrast to single knockdowns (0% of cases for *her5* knockdowns, $n=21$, 0% of cases for *him* knockdowns, $n=24$) (Fig. 6B,C).

To confirm these results we analyzed *b404* mutants (Topczewski et al., 2001), which we found to carry a deletion encompassing the *her5* and *him* locus in addition to *knypek* (*kny*). As expected, expression of both *him* and *her5* in these mutants is completely absent at all stages (Fig. 7A-D). Mutations in zebrafish *kny* impair gastrulation movements of convergence and extension that normally narrow the embryonic body and elongate it from head to tail, resulting in shorter and broader embryos (see Fig. 7B,D) (Henry et al.,

Table 2. Possible interactions of the proteins relevant to IZ formation, revealed in a yeast two-hybrid assay

pGADT7	pGBKT7		
	Her5	Him	T-antigen
Her5	+	+	+
Ngn1	+	–	+
p53	+	+	+
Lam	–	–	–

The assay was performed with full-length Her5, Him and Ngn1 proteins expressed from pGBKT7 and pGADT7 expression vectors. p53 expressed from pGADT7 and T-antigen expressed from pGBKT7 were used as positive controls. Lam protein from pGADT7 was used as a negative control for interactions.

+, interaction between the proteins tested, revealed by activation of *ade*, *his* and *mel* genes.

–, absence of interaction.

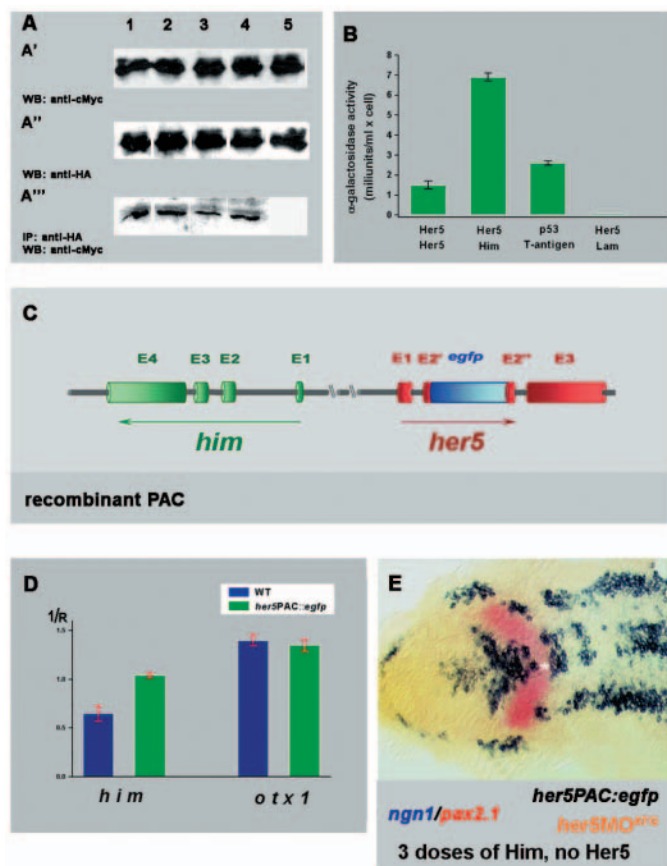


Fig. 5. The crucial determinant of medial IZ formation is the level of Him + Her5 inhibitory activity, probably achieved in vivo by Him/Her5 heterodimers but replaceable by a higher level of either factor alone. (A) Co-immunoprecipitation assays reveal possible interactions between the bHLH transcription factors important to prevent/promote neurogenesis at the MHB. Crude protein extracts were isolated from yeasts transformed with the following constructs combinations: (1) *her5pGBKT7* + *her5pGADT7*, (2) *her5pGBKT7* + *ngn1pGADT7*, (3) *himpGBKT7* + *her5pGADT7*, (4) *T-antigen pGBKT7* + *p53pGADT7*; (5) *her5pGADT7* + *lampGADT7*. Isolated extracts were either probed with anti-cMyc antibodies (A') and anti-HA antibodies (A'') or immunoprecipitated with anti-HA antibodies and then probed with anti-cMyc antibodies (A'''). (B) Stringency of Her5 homodimerization and Her5/Him heterodimerization, based on β -galactosidase activity of yeast cells expressing appropriate construct combinations (Lazo et al., 1978). Note that the interaction between Him and Her5 is significantly stronger than Her5 homodimerization. (C-E) A higher dose of Him alone can compensate for the loss of Her5 activity and maintain the MIZ. (C) Schematic representation of the transgene integrated to generate *her5PAC::egfp* embryos (Tallafuss and Bally-Cuif, 2003): the *egfp* cDNA (blue cylinder) is inserted into the *her5* region coding for the bHLH domain, resulting in a dysfunctional protein unable to bind both DNA and other bHLH factors. However, the *him* gene, contained in the PAC, is intact. (D) Quantification of *him* and *otx1* (control) mRNAs in *her5PAC::egfp* transgenic compared to wild-type embryos using real-time RT-PCR. We do not know the number of recombined PAC copies integrated into the genome in our transgenic lines; however, note that the amount of *him* mRNA is 1.5-fold higher in the *her5PAC::egfp* transgenic embryo than in wild-type siblings. The change in threshold-crossing cycle ($1/R$) is shown for each mRNA relative to that for *pax6* (assumed as a housekeeping gene) (a decrease in threshold-crossing corresponds to increase in mRNA level). The increase in *him* expression in the transgenic line is significant ($P < 0.02$ by Student's *t*-test). Standard deviations are indicated with red lines. (E) Blocking Her5 activity (by injecting *her5MO^{ATG}*) in *her5PAC::egfp* transgenic embryos fails to trigger ectopic expression of *ngn1* across the MIZ (white asterisk) (flat-mounted embryo at three somites, anterior left, used markers color-coded).

2000; Topczewski et al., 2001). In addition to this phenotype, assaying *b404* mutants for neurogenesis revealed ectopic *ngn1*-positive cells in place of both the MIZ (Fig. 7F, asterisk) and LIZ (Fig. 7F, arrow, enlarged in F'), a phenotype never observed in control siblings (Fig. 7E, enlarged in E') or in embryos for a null point mutation in the *knypek* gene (*kny^{m119}* allele, not shown) (Solnica-Krezel et al., 1996). The number of *ngn1*-positive cells in the lateral aspect of the MH domain (white box in Fig. 7E-G) is increased by 40% in *b404* embryos compared to control siblings ($n=13$ mutants and 10 wild-type embryos), while other populations of neuronal precursors, such as trigeminal ganglia neurons, are unaffected (black box in Fig. 7E-G), further supporting the specificity of this phenotype. Further, this phenotype was maintained upon rescuing Kny function in *b404* homozygous embryos injected with *kny* mRNA at the one-cell stage (Fig. 7H,H').

The above results demonstrate that ectopic neurogenesis in *b404* is selective of the IZ and unrelated to the lack of Kny activity itself. It remains possible however that the *b404* deletion encompasses other genes than *him* and *her5* that contribute to this phenotype. To address this issue, we rescued Him activity in *b404* homozygotes (Fig. 7J) by crossing them into the transgenic *her5PAC::egfp* background. *b404/b404;her5PAC::egfp* embryos display a normal expression of *him* in time and space (Fig. 7J), but no expression of *her5* (not shown). We observed that, under these conditions, the LIZ was preserved (Fig. 7I, white box, enlarged in I'). Thus, restoring Him activity is sufficient to rescue formation of the LIZ in *b404* mutants, strongly arguing that the neurogenic phenotype in the lateral MH of these mutants results from the

lack of Her5 and Him function. In addition, these results demonstrate that one dose of Him activity provides a level of inhibition sufficient for LIZ formation. In contrast, loss of the MIZ was maintained in *b404/b404;her5PAC::egfp* embryos (Fig. 7I), in keeping with our finding that MIZ formation requires more than one or two copies of *him* or *her5* (Fig. 4A-C, 5E).

Him and Her5 are equally potent neurogenesis inhibitors in the lateral IZ

We demonstrated above that the total amount of Him + Her5 inhibitory activity is the crucial determinant for IZ formation, and that increased levels of Him could compensate for loss of Her5 both in the medial and lateral IZ. These experiments however did not address the relative contribution of Her5 to the total inhibitory activity required for IZ formation. To determine whether Him and Her5 contribute equally to this activity, we analyzed LIZ formation in *b404/+; kny^{m119}/+* embryos where the function of either Her5 (Fig. 7K) or Him (Fig. 7L) was abolished. This background, obtained by crossing *b404/+* with *kny^{m119}/+* heterozygote adults, allows immediate identification of the embryos carrying one single copy of each gene *him* and *her5*, since such embryos display the *knypek* phenotype. Blocking the activity of Him or Her5 *b404/+; kny^{m119}/+*

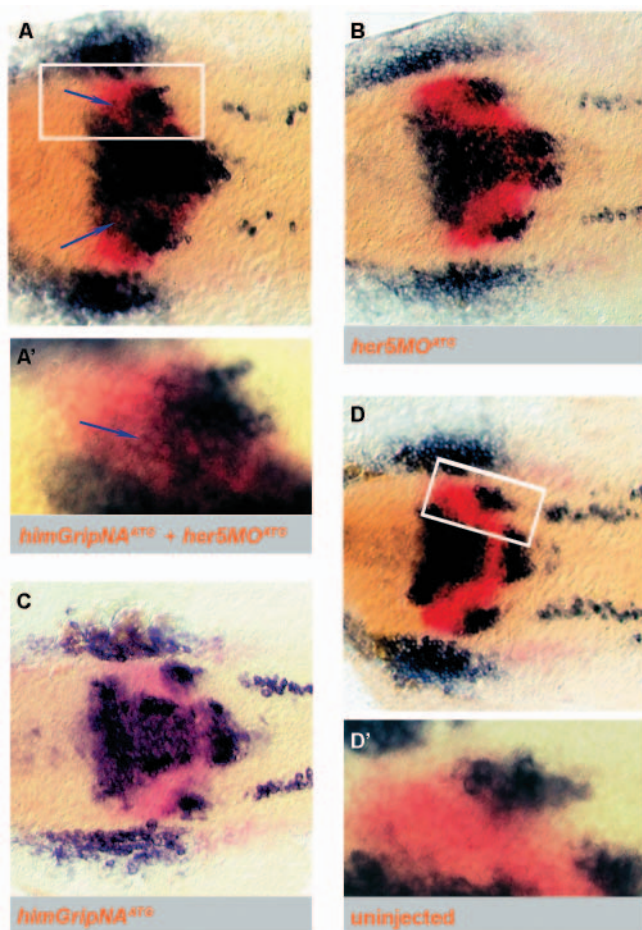


Fig. 6. The activity of both Her5 and Him is necessary to prevent neurogenesis in the lateral IZ. Dorsal views of flat-mounted $-8.4ngn1::egfp$ transgenic embryos (Blader et al., 2003) probed at the three-somite stage for *egfp* expression (blue) after injection of 1 mM *her5MO*^{ATG} + 0.25 mM *himGripNA*^{ATG} (A), 1 mM *her5MO*^{ATG} (B), 0.25 mM *himGripNA*^{ATG} (C), compared to an uninjected control (D). A' and D' are enlargements of the boxed areas in A and D, respectively. The IZ is indicated by *pax2.1* expression (red staining). Note that the LIZ is undergoing ectopic neurogenesis only in the embryo injected with both *her5MO*^{ATG} and *himGripNA*^{ATG} (blue arrows).

embryos leaves only one functional copy of either *her5* or *him*, respectively. Assaying for *ngn1* expression revealed that the LIZ forms normally in such embryos (Fig. 7K,K' and 7L,L' compared to 7M,M', white arrowhead) (while, as expected, the MIZ is lost, Fig. 7K, and 7L, compared to 7M, white arrow). These results are in agreement with an equal potency of Him and Her5 to inhibit lateral neurogenesis at the MHB and, like for the MIZ, we propose that a crucial determinant of LIZ formation is the threshold of Him + Her5 activities rather than the specific presence of both factors.

Discussion

Maintaining a progenitor pool at the embryonic MHB is crucial to MH growth and IsO maintenance, and we report here a new molecular player and its associated mechanism preventing neurogenesis in this territory. Looking for genes physically

linked to *her5*, we unraveled a previously unknown, paired and divergently transcribed *hairy/E(spl)* gene, *him*, which shares with *her5* expression across the IZ. We demonstrate that blocking either Her5 or Him function results in the same failure to form and maintain the MIZ, and that interfering concomitantly with the function of both factors prevents formation of the LIZ. In both domains, we demonstrate that sufficient levels of one factor alone are sufficient to compensate for the lack of the other. Together, our results are most compatible with a model where the molecular basis of IZ formation is the total Him + Her5 inhibitory activity, at different thresholds along the mediolateral axis. They highlight a new mechanism, relying on paired Hairy/E(spl) factors, for the maintenance of a non-differentiating signaling boundary during embryonic development.

him and *her5* are a new co-functional gene pair

him and *her5* are located 3 kb apart in a head-to-head, in a manner reminiscent of the *her7/her1* gene pair (Henry et al., 2002). Our search through the zebrafish genome revealed an additional similarly organized pair of *her* genes, *her4/her12*, located on chromosome fragment ctg10516. ESTs BM023698 and AL716753 match with 100% to the cDNA sequence deduced from *ENSDARG00000028110*, suggesting that *her12* is a real transcribed gene. Thus, our results suggest that a paired and divergently transcribed configuration is a frequent organization of zebrafish *her* genes. Our search through other vertebrate genomes, however, including mouse and *Fugu rubripes* (*Fugu* genome, The Wellcome Trust Sanger Institute, release 08-02-04), failed to reveal a similar organization of *hairy/E(spl)*-like genes in these species, suggesting that the molecular process(es) generating *her* pairs took place along the lineage leading to zebrafish.

Our results do not suggest a simple evolutionary model leading to the generation of zebrafish *her* pairs. Indeed, the six *her* genes involved belong in sequence to two groups of orthologs (group 1: *him/her5/her1* versus group 2: *her7/her4/her12*), but only the *her7/her1* pair contains one gene from each group. Thus the situation is not comparable to *dlx* gene pairs, interpreted to result from a tandem duplication followed by a cluster duplication. Because *her4* and *her12* have very similar coding sequences, it is possible that these two genes underwent a recent event of gene conversion, facilitated by the formation of intrachromosomal hairpins (Hickey et al., 1991). We failed to detect indications supporting gene conversion within the *him/her5* gene pair, but other recombination events might have occurred (D. Chourrout, J.N. and L.B.-C., unpublished observations).

Linked genes sharing sequence similarity have been reported for a variety of genetic functions in several organisms (Akam, 1989; Alonso and Cabrera, 1988; Bober et al., 1994; Coleman et al., 1987; Kmita and Duboule, 2003; Knust et al., 1992; Stein et al., 1996). Duplication events resulting in linked arrays of related genes generate copies that often share cis-acting regulatory sequences. Whether *him* and *her5* expression across the IZ are coregulated remains to be directly demonstrated but is highly likely, given that the enhancer driving MH expression of *her5* extends into the *him* locus (Tallafuss and Bally-Cuif, 2003). In addition, *him* and *her5* differ in some aspects of their expression profiles (in the shield and presomitic mesoderm, versus presumptive pharyngeal endoderm, respectively). The

Fig. 7. Ectopic neurogenesis in both the medial and lateral IZ in *b404* deletion mutants results from the deletion of *him* and *her5*. (A-D) *b404* mutants lack *her5* and *him* expression. Lateral views of whole-mount embryos assayed for *him* or *her5* expression at the 17-somite stage (anterior left, probes indicated bottom left, genotype bottom right). The position of the MHB in mutant embryos is indicated with a red arrowhead. The position of the head, reflecting the delayed convergence and extension problems in the mutant embryo, is indicated with a black arrowhead. (E-G) Ectopic neurogenesis across the LIZ revealed by *ngn1* expression (blue) in *b404* mutants (F,F') compared to non-mutant siblings (E,E'). In *b404* embryos, ectopic *ngn1*-positive cells are present both in the lateral (blue arrow) and medial (asterisk) IZ (E' and F' are high magnification of the areas boxed in white in E and F, respectively). (G) The number of *ngn1*-positive cells in the future alar MH (area indicated with white box in E and F) is 40% higher in mutant embryos compared to WT siblings, while other neural plate areas are not affected (e.g. trigeminal ganglia, area boxed in black in E and F). (H) Reintroducing Knypek function in *b404* mutants does not alter the IZ neurogenesis phenotype. Expression of *ngn1* (blue) in three-somite *b404* mutants where Kny function has been restored by *kny* RNA injection (dorsal views of flat-mounted embryos, anterior left, A' is a high magnification of the area boxed in A). Note that ectopic *ngn1* expression both across the MIZ (asterisk) and LIZ (blue arrows) is not altered compared to uninjected *b404* mutants (Fig. 7F). (I-J) Restoring Him function at endogenous levels in *b404* mutants is sufficient to rescue the LIZ. (J) Crossing the *b404* mutation into the *her5PAC::egfp* background generates *b404/b404;her5PAC::egfp* embryos where *him* expression is recovered with endogenous levels and expression pattern (lateral view of a 17-somite embryo, anterior left). (I,I') Expression of *ngn1* (blue) in three-somite *b404/b404;her5PAC::egfp* embryos (dorsal views of flat-mounted embryos, anterior left, I' is a high magnification of the area boxed in I). Note that no ectopic neurogenesis is detectable any longer across the LIZ (white arrowheads), while the MIZ remains bridged by ectopic *ngn1*-positive cells (asterisk). (K-M). Him and Her5 equally contribute to the total inhibitory activity and one copy of either Him or Her5 is sufficient for formation of the LIZ. Formation of the LIZ in the *b404/+; m119/+* embryos is indistinguishable in Him morphants (K,K'), Her5 morphants (L,L') and uninjected embryos (M,M') (white arrowheads). Note that after blocking either Him or Her5 activity ectopic neurogenesis occurs in the MIZ (white asterisk). K', L' and M' are enlargements of boxed area in K, L and M respectively (25 embryos was analyzed for *b404/+; m119/+* injected with *her5MO^{ATG}*, 27 for *b404/+; m119/+* injected with *himGrip^{ATG}* and 20 uninjected *b404/+; m119/+*).

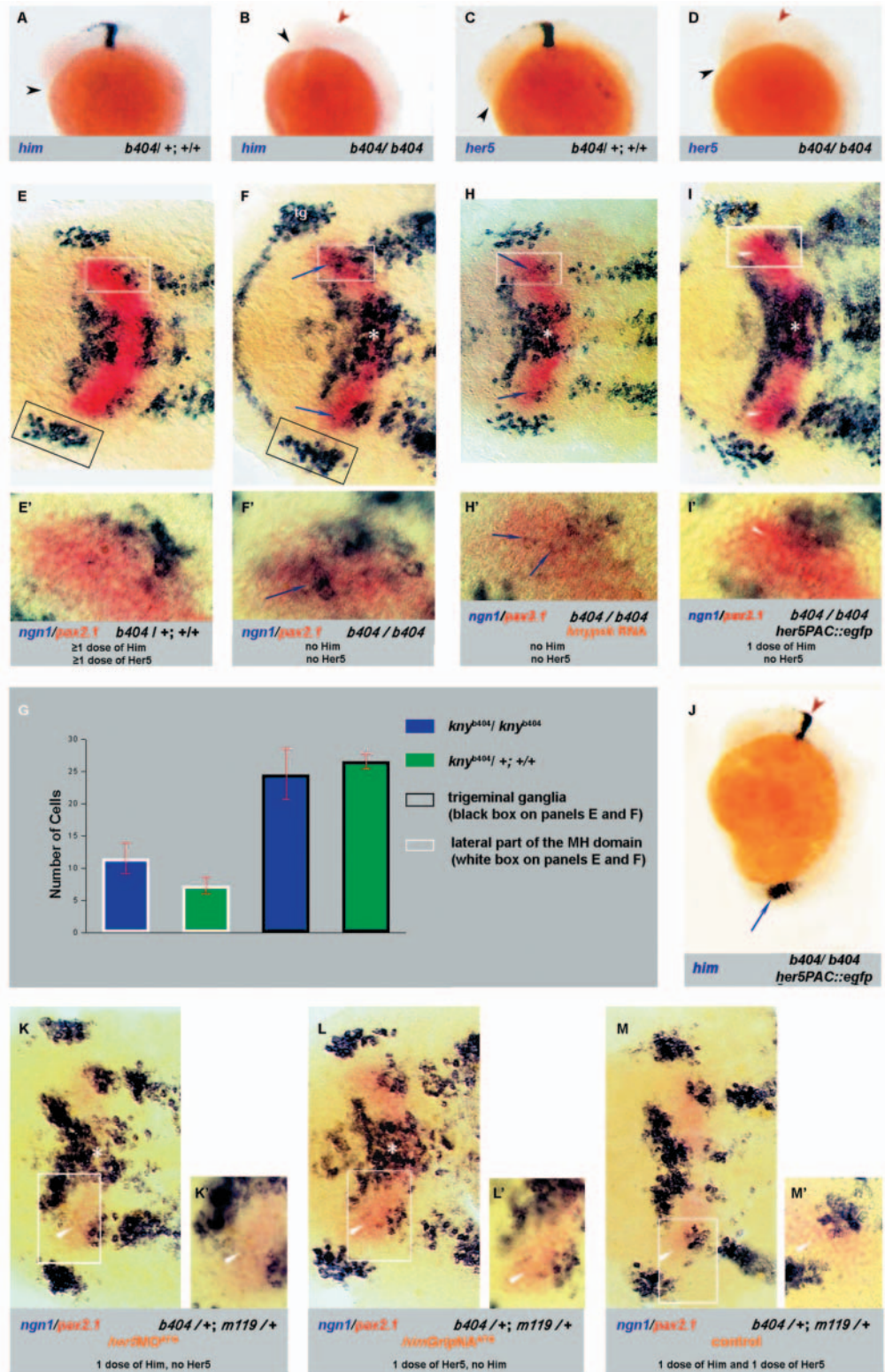
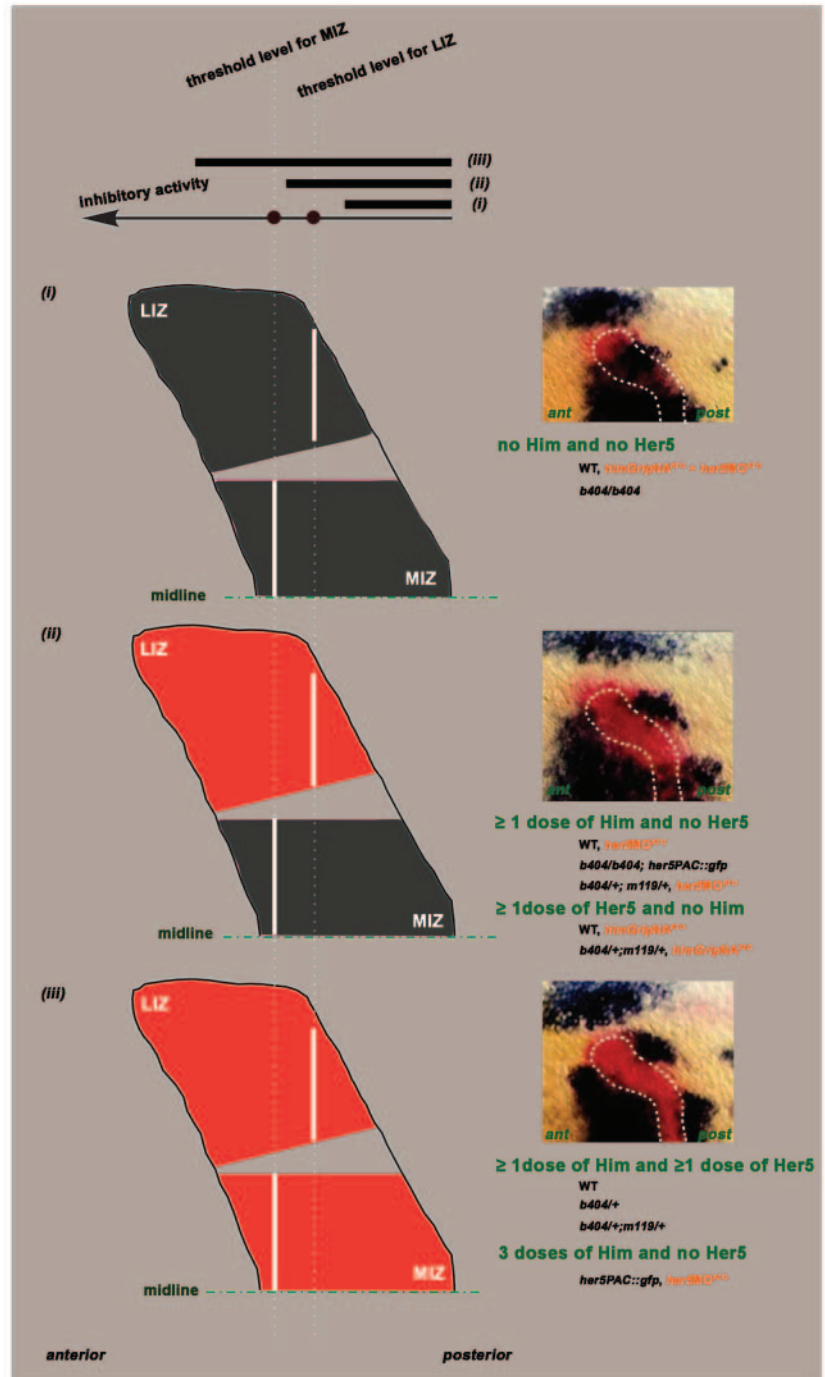


Fig. 8. Models for IZ formation based on dose dependence for Him + Her5 inhibitory activity. In each panel, a half IZ is represented and color-coded (red: MIZ or LIZ, light grey: intermediate field, dark grey: *ngn1*-positive areas), and the level of Him + Her5 inhibitory activity is indicated by black horizontal bars. The threshold required for *ngn1* inhibition within the LIZ is lower than the MIZ threshold (white bars). Thus, if Him + Her5 activity is below the LIZ threshold level, ectopic neurogenesis will occur both laterally and medially, and the MIZ and LIZ are lost (i). This situation corresponds to the absence of activity of both Her5 and Him. When Him + Her5 activity reaches the LIZ threshold level (one dose of Her5 or Him is sufficient), *ngn1* expression is prevented laterally but not medially, and the LIZ forms but not the MIZ (ii). Finally, if Him + Her5 activity is above the MIZ threshold (≥ 1 dose of Him and ≥ 1 dose of Her5 or three doses of Him), the whole IZ is formed properly (normal development of wild-type embryos, iii). The persisting absence of *ngn1* expression within the intermediate field located between the MIZ and LIZ (light grey triangle) could result from additional inhibitor(s) expressed along the AP axis, in register with longitudinal non-differentiation stripes of the rhombomere and spinal cord. Flat-mounted embryos at three-somite stage, with *ngn1* expression in dark and *pax 2.1* in red, corresponding to each situation are shown adjacent to the scheme. The combinations of inhibitory factors producing the appropriate level of inhibitory activity for each situation are listed (green letters), as well as the genetic ways to obtain this particular inhibitory activity (black letters for genotype and orange for interfering agents).

regulatory elements controlling endodermal expression of *her5* are located closer to the *her5* ATG than the MH expression elements (Tallafuss and Bally-Cuif, 2003). Thus expression of the *him/her5* pair may be controlled by a combination of proximal and gene-specific elements (accounting for the differential expression sites of the two genes) and distal and probably common elements (driving IZ expression). It is possible that the proximal elements are new modifications in the evolution of the gene pair, extending genetic functions by the acquisition of new expression domains (Ohta, 2000). It will be interesting to determine whether such cis-regulatory organization is involved in generating different expression sites within other gene pairs.

The combined activities of Her5 and Him determine LIZ formation

We previously identified Her5 as the first determinant of MIZ formation in zebrafish (Geling et al., 2003). However, although *her5* expression covers the whole IZ, and ectopic *her5* expression can inhibit *ngn1* in the lateral MH area, we failed to implicate Her5 alone in LIZ formation in vivo. A main advance of our present work is to provide an interpretation for this finding, by identifying a new Hairy/E(spl) factor, Him, as the partner for Her5 in LIZ formation. Our arguments rely on the phenotype of embryos where the functions of Her5 and Him are concomitantly blocked in a non-genetic interference



approach, and of embryos carrying the *b404* deletion, where both *him* and *her5* genes are absent. In both cases, ectopic *ngn1*-positive cells replace the LIZ. This phenotype is not found by blocking the function of either Him or Her5 alone, and is rescued by selectively reintroducing endogenous levels and profile of Him function into the *b404* background, arguing for its specificity. Further, we show that one copy of either *him* or *her5* (as in *b404/+*; *kny^{m119}/+* heterozygote embryos where Her5 or Him function is blocked) similarly preserves the LIZ, demonstrating that Him and Her5 are equally potent at inhibiting *ngn1* expression in that location. Thus, our results identify Him and Her5 as truly redundant factors that play

equivalent roles, and are together the only determinant, in LIZ formation.

The molecular cascade downstream of Him remains unknown. Because Him is sufficient for LIZ formation in the absence of Her5, we can exclude a mechanism where Him would primarily promote Her5 activity. Rather, because of the similar sequences of Him and Her5, it is more likely that both factors act together on common targets controlling neurogenesis. Within the MIZ, Her5 and Him (J.N. and L.B.-C., unpublished) act upstream of Notch to inhibit expression of *ngn1* and *coe2*, but not other early MH proneural genes such as *asha*, *ashb* and *ato3* (Geling et al., 2003). It will be important to determine whether the molecular cascade(s) and mechanisms downstream of Her5 and Him are conserved in the MIZ and LIZ. Compared to the MIZ, the LIZ exhibits an additional block, still molecularly unknown, that prevents neurogenesis downstream of Ngn1 activity (Geling et al., 2003). Whether Him and Her5 also take part in this second block remains to be tested.

A unified model for IZ formation along the entire mediolateral extent of the neural plate

The absence of Her5 leads to disappearance of the entire MIZ and its replacement by *ngn1*-expressing cells, which later differentiate into Hu-, HNK1- and acetylated-tubulin-positive neurons (Geling et al., 2003). Surprisingly, our results now demonstrate that Him plays an equally important role in MIZ formation, since an exactly identical phenotype is triggered by lack of Him activity (this paper, and data not shown). We further rule out an interdependent regulation of *him* and *her5* expression (Fig. 4E-L). Thus, another important implication of our work is that MIZ formation relies on prepatternning by both Him and Her5.

A priori, the finding that loss of Him or Her5 function result in identical phenotypes can have three different molecular interpretations: first, Him and Her5 might act in distinct pathways that converge on and are both necessary for neurogenesis control at the MIZ; second, the activities of Him and Her5 might be interdependent; third, Him and Her5 might have equivalent functions, a minimal dose of 'Him + Her5' activity being required for MIZ formation. The first two mechanisms are unlikely, given the observation that increased levels of Him alone to three doses (as in *her5PAC::egfp* *l*+ heterozygote transgenic embryos injected with *her5*MO, Fig. 5E) can compensate for the lack of Her5 function within the MIZ. We do not have genetic means of assessing whether a high dose of Her5 alone would also suffice for MIZ formation. However, our findings that Him and Her5 are equally potent to prevent lateral neurogenesis strongly suggest that this is the case. Thus, we propose that the crucial determinant of MIZ formation, is a total level of 'Him + Her5' inhibitory activity. Hence, above a threshold of Him + Her5, *ngn1* expression is prevented medially and the MIZ is formed, while *ngn1* expression is induced below this threshold (Fig. 8). As discussed above, our results indicate that three doses of one factor alone is the minimum level of inhibitory activity required for MIZ formation. Interestingly, however, two doses are sufficient when both Him and Her5 are present, as in *b404*+ heterozygote embryos. This result might be related to the higher propensity of Him and Her5 to hetero- than homodimerize, or to an increased activity of heterodimers versus

homodimers or oligomers. Because the same factors Him and Her5 account for LIZ formation, and can functionally replace each other in this domain as well, a parsimonious interpretation of our findings is to implicate the same dose-dependent mechanism within the LIZ, albeit with a lower threshold level (Fig. 8). The LIZ minimal level of inhibition would be achieved with one dose of Him or Her5 alone. Together, our results thus lead to a unified model where the maintenance of a pool of progenitor cells at the MHB is orchestrated by a variable dose-dependency to the Him/Her5 pair.

Even in the absence of Him and Her5, we failed to induce *ngn1* expression within a small intermediate field located between the MIZ and LIZ (see Fig. 7F, and grey triangle in Fig. 8). In this domain, an additional (as yet unknown) factor might increase the total inhibitory activity and/or prevent neurogenesis in addition to Him and Her5. We failed to recover additional IZ-expressed *E(spl)* genes following our search through the zebrafish genome and expression studies (J.N., C.L. and L.B.-C., unpublished). Because the intermediate field is aligned with the longitudinal domains of non-differentiation in the hindbrain and spinal cord, it is perhaps more likely that this factor is expressed along the AP axis of the neural plate, like other known neurogenesis inhibitors (Bally-Cuif and Hammerschmidt, 2003).

An interesting open question remains to identify the cues controlling the differential sensitivity of the MIZ versus LIZ to Him + Her5, and their functional significance. The MIZ and LIZ differ in their proliferation rates: the MIZ exhibits more cells in M phase than the LIZ at late gastrulation, based on anti-phosphoH3 immunostaining (Geling et al., 2003). It will be crucial to investigate the possible relationship between MIZ and LIZ cell cycle properties and their response to Him + Her5. Also, several morphogens acting in this region are expressed following a mediolateral gradient. For instance, *wnt1* is expressed in a spatio-temporal pattern similar to *her5* and *him* at late gastrulation, thus with initially higher levels laterally than medially, and might enhance cell sensitivity to neurogenesis inhibitors. This might be related to the delay of dorsal differentiation proposed to result from the gradient of Wnt signaling from the spinal cord roof plate (Megason and McMahon, 2002). Conversely Shh signaling from the ventral midline and specifically active at the MHB (Carl and Wittbrodt, 1999; Koster et al., 1997) could increase 'neurogenic competence'. These hypotheses will be important to test experimentally to gain insight into the prepatternning of IZ formation.

Biological significance of a redundant process for IZ formation

Redundant factors are generally viewed as 'safety' locks, and the biological significance of the Him/Her5 couple might be to secure IZ formation. This case of redundancy is more extreme than observed for Her1/Her7, where the disruption of each gene alone produced distinct (although moderate) somitic defects, indicating partially different activities (Henry et al., 2002). The embryonic MHB progenitor pool serves several vital functions. It generates the large majority of MH neurons and glia, as demonstrated in lineage tracing experiments (Tallafuss and Bally-Cuif, 2003) and genetic or surgical ablation (Cowan and Finger, 1982; Hirata et al., 2001). MH neurons form crucial integration centers involved

in visual, auditory and motor control and social behavior. By its long-lasting proliferative activity, the IZ also permits the expansion of MH tissue over time. Although the relative importance of MH derivatives varies between species, most vertebrates are characterized by highly developed visual, auditory or locomotor functions, which are paired with enlarged mesencephalic derivatives or cerebellum. Finally, and importantly, the IZ coincides in space with the isthmus organizer, necessary for patterning the entire MH domain and for the subdivision of mid-versus hindbrain structures (Bally-Cuif et al., 2000; Liu and Joyner, 2001; Rhinn and Brand, 2001). In the mouse, IZ formation also relies on the two redundant bHLH factors *Hes1* and *Hes3* (Hirata et al., 2001). In that case however *Hes1* and *Hes3* are not genetically linked and their expression profiles are clearly distinct, overlapping only at the MHB (Allen and Lobe, 1999; Lobe, 1997), suggesting that mouse and zebrafish have independently evolved a strategy for the redundant expression and function of Hairy/E(spl) factors at the MHB. A dose dependency and the spatial details of IZ formation in the mouse have not been explored. The fact that one dose of each factor *Him* and *Her5* suffices to maintain the MIZ in zebrafish, while two doses of each single factor do not, probably explains the maintenance of the two genes *him* and *her5* in zebrafish. Whether *Hes1* and *Hes3*, or *Him* and *Her5*, exert in addition other and perhaps distinct activities at the MHB remains to be explored.

The IZ is not an isolated case of maintenance of a non-differentiation zone at embryonic signaling boundaries. Such events have been reported, e.g. at the *Drosophila* wing margin, along the dorsal and ventral midlines of the neural tube (Alexandre and Wassef, 2003), as well as between rhombomeres (Cheng et al., 2004). Like the IZ, these boundaries are involved in the progressive building and patterning of their adjacent territories, and the maintenance of their integrity necessitates their remaining undifferentiated. This process is achieved by Notch signaling at the wing margin and inter-rhombomeric boundaries, and *Shh* signaling along the neural tube ventral midline, while the factors involved along the dorsal midline probably involve Wnt and BMP signaling. Our work demonstrates that a distinct molecular mechanism accounts for non-differentiation at the MHB, namely the differential response of MHB cells to the combined inhibitory activity of two twin and co-regulated Hairy/E(spl)-like factors, independently of Notch. Our findings add to the panel of identified developmental strategies used to build and maintain signaling centers.

Note added in proof

him is identical to *her11*, which has been recently reported for its role in zebrafish somitogenesis (Sieger et al., 2004). The gene referred to as *her11* in the present manuscript (Fig. 1B, ENSDARP00000012990), named before publication by Sieger et al., is a different coding sequence and corresponds to *her13* of Sieger et al. The gene referred to as *her13* in the present manuscript (Fig. 1B, ENSDARP00000008307) is a new gene, not reported by Sieger et al. We suggest that the latter gene be renamed *her16* and that the nomenclature of Sieger et al. be used for all other genes in future work.

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References

- Akam, M. (1989). Hox and HOM: homologous gene clusters in insects and vertebrates. *Cell* **57**, 347-349.
- Alexandre, P. and Wassef, M. (2003). The isthmus organizer links anteroposterior and dorsoventral patterning in the mid/hindbrain by generating roof plate structures. *Development* **130**, 5331-5338.
- Allen, T. and Lobe, C. G. (1999). A comparison of Notch, Hes and Grg expression during murine embryonic and post-natal development. *Cell Mol. Biol. (Noisy-le-grand)* **45**, 687-708.
- Alonso, M. C. and Cabrera, C. V. (1988). The achaete-scute gene complex of *Drosophila melanogaster* comprises four homologous genes. *EMBO J.* **7**, 2585-2591.
- Bally-Cuif, L. and Hammerschmidt, M. (2003). Induction and patterning of neuronal development, and its connection to cell cycle control. *Curr. Opin. Neurobiol.* **13**, 16-25.
- Bally-Cuif, L., Goridis, C. and Santoni, M. J. (1993). The mouse NCAM gene displays a biphasic expression pattern during neural tube development. *Development* **117**, 543-552.
- Bally-Cuif, L., Goutel, C., Wassef, M., Wurst, W. and Rosa, F. (2000). Coregulation of anterior and posterior mesendodermal development by a hairy-related transcriptional repressor. *Genes Dev.* **14**, 1664-1677.
- Blader, P., Plessy, C. and Strähle, U. (2003). Multiple regulatory elements with spatially and temporally distinct activities control neurogenin1 expression in primary neurons of the zebrafish embryo. *Mech. Dev.* **120**, 211-218.
- Bober, E., Baum, C., Braun, T. and Arnold, H. H. (1994). A novel NK-related mouse homeobox gene: expression in central and peripheral nervous structures during embryonic development. *Dev. Biol.* **162**, 288-303.
- Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Lun, K., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A. et al. (1996). Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. *Development* **123**, 179-190.
- Carl, M. and Wittbrodt, J. (1999). Graded interference with FGF signalling reveals its dorsoventral asymmetry at the mid-hindbrain boundary. *Development* **126**, 5659-5667.
- Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev. Cell* **6**, 539-550.
- Coleman, K. G., Poole, S. J., Weir, M. P., Soeller, W. C. and Kornberg, T. (1987). The inverted gene of *Drosophila*: sequence analysis and expression studies reveal a close kinship to the engrailed gene. *Genes Dev.* **1**, 19-28.
- Cowan, W. and Finger, T. (1982). Regeneration and regulation in the developing central nervous system, with special reference to the reconstitution of the optic tectum of the chick following removal of the mesencephalic alar plate. *Curr. Top. Neurobiol.* **11**, 377-415.
- Davis, R. L. and Turner, D. L. (2001). Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. *Oncogene* **20**, 8342-8357.
- Dawson, S. R., Turner, D. L., Weintraub, H. and Parkhurst, S. M. (1995). Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol. Cell. Biol.* **15**, 6923-6931.
- Firulli, B. A., Hadzic, D. B., McDaid, J. R. and Firulli, A. B. (2000). The basic helix-loop-helix transcription factors dHAND and eHAND exhibit dimerization characteristics that suggest complex regulation of function. *J. Biol. Chem.* **275**, 33567-33573.
- Fisher, A. L., Ohsako, S. and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* **16**, 2670-2677.
- Geling, A., Itoh, M., Tallafuss, A., Chapouton, P., Tannhäuser, B., Kuwada, J. Y., Chitnis, A. B. and Bally-Cuif, L. (2003). bHLH

- transcription factor Her5 links patterning to regional inhibition of neurogenesis at the midbrain-hindbrain boundary. *Development* **130**, 1591-1604.
- Geling, A., Plessy, C., Rastegar, S., Strahle, U. and Bally-Cuif, L. (2004). Her5 acts as a prepattern factor that blocks neurogenin1 and coe2 expression upstream of Notch to inhibit neurogenesis at the midbrain-hindbrain boundary. *Development* **131**, 1993-2006.
- Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., van Eeden, F. J., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C. P. et al. (1996). dino and mercedes, two genes regulating dorsal development in the zebrafish embryo. *Development* **123**, 95-102.
- Henry, C. A., Hall, L. A., Burr Hille, M., Solnica-Krezel, L. and Cooper, M. S. (2000). Somites in zebrafish doubly mutant for knypek and trilobite form without internal mesenchymal cells or compaction. *Curr Biol* **10**, 1063-1066.
- Henry, C. A., Urban, M. K., Dill, K. K., Merlie, J. P., Page, M. F., Kimmel, C. B. and Amacher, S. L. (2002). Two linked hairy/Enhancer of split-related zebrafish genes, her1 and her7, function together to refine alternating somite boundaries. *Development* **129**, 3693-3704.
- Hickey, D. A., Bally-Cuif, L., Abukashawa, S., Payant, V. and Benkel, B. F. (1991). Concerted evolution of duplicated protein-coding genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 1611-1615.
- Hirata, H., Tomita, K., Bessho, Y. and Kageyama, R. (2001). Hes1 and Hes3 regulate maintenance of the isthmus organizer and development of the mid/hindbrain. *EMBO J.* **20**, 4454-4466.
- Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H. Y., Chung, G., Kedes, L. and Hamamori, Y. (2001). HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol. Cell. Biol.* **21**, 6080-6089.
- Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
- Klamt, C., Knust, E., Tietze, K. and Campos-Ortega, J. A. (1989). Closely related transcripts encoded by the neurogenic gene complex enhancer of split of *Drosophila melanogaster*. *EMBO J.* **8**, 203-210.
- Kmita, M. and Duboule, D. (2003). Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301**, 331-333.
- Knust, E., Schrons, H., Grawe, F. and Campos-Ortega, J. A. (1992). Seven genes of the Enhancer of split complex of *Drosophila melanogaster* encode helix-loop-helix proteins. *Genetics* **132**, 505-518.
- Korzh, V., Sleptsova, I., Liao, J., He, J. and Gong, Z. (1998). Expression of zebrafish bHLH genes ngn1 and nrd defines distinct stages of neural differentiation. *Dev. Dyn.* **213**, 92-104.
- Koster, R., Stick, R., Loosli, F. and Wittbrodt, J. (1997). Medaka spalt acts as a target gene of hedgehog signaling. *Development* **124**, 3147-3156.
- Lazo, P. S., Ochoa, A. G. and Gascon, S. (1978). alpha-Galactosidase (melibiase) from *Saccharomyces carlsbergensis*: structural and kinetic properties. *Arch. Biochem. Biophys.* **191**, 316-324.
- Liu, A. and Joyner, A. L. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. *Annu. Rev. Neurosci.* **24**, 869-896.
- Lobe, C. G. (1997). Expression of the helix-loop-helix factor, Hes3, during embryo development suggests a role in early midbrain-hindbrain patterning. *Mech. Dev.* **62**, 227-237.
- Lun, K. and Brand, M. (1998). A series of no isthmus (noi) alleles of the zebrafish pax2.1 gene reveals multiple signaling events in development of the midbrain-hindbrain boundary. *Development* **125**, 3049-3062.
- Martinez, S. (2001). The isthmus organizer and brain regionalization. *Int. J. Dev. Biol.* **45**, 367-371.
- Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.
- Muller, M., v Weizsacker, E. and Campos-Ortega, J. A. (1996). Expression domains of a zebrafish homologue of the *Drosophila* pair-rule gene hairy correspond to primordia of alternating somites. *Development* **122**, 2071-2078.
- Ohta, T. (2000). Evolution of gene families. *Gene* **259**, 45-52.
- Reifers, F., Bohli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. and Brand, M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.
- Rhinn, M. and Brand, M. (2001). The midbrain-hindbrain boundary organizer. *Curr. Opin. Neurobiol.* **11**, 34-42.
- Sieger, D., Tautz, D. and Gajewski, M. (2004). her11 is involved in the somitogenesis clock in zebrafish. *Dev. Genes Evol.* **214**, 393-406.
- Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C., Malicki, J., Schier, A. F., Stainier, D. Y., Zwartkruis, F., Abdelilah, S. et al. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* **123**, 67-80.
- Stein, S., Niss, K. and Kessel, M. (1996). Differential activation of the clustered homeobox genes CNOT2 and CNOT1 during notogenesis in the chick. *Dev. Biol.* **180**, 519-533.
- Tallafuss, A. and Bally-Cuif, L. (2003). Tracing of her5 progeny in zebrafish transgenics reveals the dynamics of midbrain-hindbrain neurogenesis and maintenance. *Development* **130**, 4307-4323.
- Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001). The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell.* **1**, 251-264.
- Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (*Gallus domesticus*). A morphological, histochemical and autoradiographical investigation. *Ergeb. Anat. Entwicklungsgesch* **41**, 3-87.
- Wainwright, S. M. and Ish-Horowitz, D. (1992). Point mutations in the *Drosophila* hairy gene demonstrate in vivo requirements for basic, helix-loop-helix, and WRPW domains. *Mol. Cell. Biol.* **12**, 2475-2483.
- Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmus organizer. *Nat. Rev. Neurosci.* **2**, 99-108.