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bHLH transcription factor Her5 links patterning to regional inhibition of neurogenesis at the midbrain-hindbrain boundary

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SUMMARY

The midbrain-hindbrain (MH) domain of the vertebrate embryonic neural plate displays a stereotypical profile of neuronal differentiation, organized around a neuron-free zone ('intervening zone', IZ) at the midbrain-hindbrain boundary (MHB). The mechanisms establishing this early pattern of neurogenesis are unknown. We demonstrate that the MHB is globally refractory to neurogenesis, and that forced neurogenesis in this area interferes with the continued expression of genes defining MHB identity. We further show that expression of the zebrafish bHLH Hairy/E(spl)-related factor Her5 prefigures and then precisely delineates the IZ throughout embryonic development. Using morpholino knock-down and conditional gain-of-function assays, we demonstrate that Her5 is essential to prevent neuronal differentiation and promote cell proliferation in a medial compartment of the IZ. We identify one probable target of this activity, the zebrafish Cdk inhibitor p27^{Xic1}. Finally, although the *her5* expression domain is determined by anteroposterior patterning cues, we show Her5 does not retroactively influence MH patterning. Together, our results highlight the existence of a mechanism that actively inhibits neurogenesis at the MHB, a process that shapes MH neurogenesis into a pattern of separate neuronal clusters and might ultimately be necessary to maintain MHB integrity. Her5 appears as a partially redundant component of this inhibitory process that helps translate early axial patterning information into a distinct spatiotemporal pattern of neurogenesis and cell proliferation within the MH domain.

Key words: Zebrafish, Midbrain-hindbrain boundary, MHB, Neurogenesis, Her5, bHLH, E(spl), Hairy, Proliferation, Cyclin-dependent kinase inhibitor, p27

INTRODUCTION

A conspicuous feature of the vertebrate embryonic CNS is the absence of a homogeneous gradient of neurogenesis across the neural tube, young post-mitotic neuroblasts arise at discrete patches of the neuroepithelium in a disjoined spatiotemporal pattern. Among the first neurons to differentiate in all species is a basal cluster located at the diencephalic-mesencephalic junction, which projects growth cones caudally to pioneer the medial longitudinal fascicle (MLF) (Puelles et al., 1987; Chitnis and Kuwada, 1990; Metcalfe et al., 1990; Wilson et al., 1990; Ross et al., 1992; Easter et al., 1994; Mastick and Easter, 1996). This neuronal group is known as the ventrocaudal cluster (vcc) or nucleus of the MLF (nMLF). Concomitantly in the hindbrain, motorneurons become identifiable in the center of each even-numbered rhombomere (Lumsden and Keynes, 1989). Molecular markers such as the Atonal-like bHLH transcription factors neurogenins (Gradwohl et al., 1996; Ma et al., 1996; Blader et al., 1997) confirmed these pioneering studies. These findings demonstrate that the early pattern of neuronal differentiation is established following a highly similar and stereotypical spatiotemporal sequence in all vertebrates, suggesting that it responds to precise and shared patterning cues. How positional identity information and the onset of neurogenesis versus proliferation are integrated in vertebrates is, however, not fully understood.

A crucial and extensively studied domain of the anterior neural plate is the midbrain-hindbrain (MH), which contains at the MH boundary (MHB) the isthmic organizer, a critical regulator of MH growth and patterning (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Strikingly, the MH is also characterized by a distinct pattern of neurogenesis at early stages: mesencephalic and anterior rhombencephalic neurons are separated by a neuron-free, transverse stripe of delayed differentiation (hereafter referred to as 'intervening zone', IZ), precisely located at the level of the MHB. In the

zebrafish, the IZ is identifiable from the onset of neurogenesis, when it separates two of the earliest neuronal clusters, the vcc and the presumptive motorneurons of rhombomere 2 (r2MN). The IZ is conspicuous during neurogenesis of all vertebrates examined (see Palmgren, 1921; Bally-Cuif et al., 1993). According to classical neuroanatomical studies (Vaage, 1969; Vaage, 1973), it corresponds in the chick to a caudal 'mesomere' which initially encompasses half the midbrain but upon regression during development forms a narrow, neuron-free stripe at the junction with the first rhombomere. Lineage analysis in the zebrafish (A.T. and L.B.-C., unpublished) demonstrate that this is a dynamic process where the IZ progressively contributes cells to adjacent territories upon cell divisions. The zebrafish IZ also maintains a large population of proliferating cells at larval stages, long past the time when proliferation has ceased in adjacent neural domains (Wullimann and Knipp, 2000). As such, the IZ has been proposed to play a crucial role in permitting the growth and regionalization of MH structures over a long period (Tallafuß and Bally-Cuif, 2002). Understanding its formation is thus an important issue.

Several factors have been identified that positively define early neurogenesis competence domains and proneural clusters within the embryonic neural plate. Neuronal differentiation-promoting factors include members of the Achate-Scute, Atonal, Gli and Iroquois families (Allende and Weinberg, 1994; Fisher and Caudy, 1998; Cavodeassi et al., 2001; Davis and Turner, 2001). Neuroblasts that engage into the differentiation process are then selected following similar genetic cascades to those originally defined in *Drosophila*. In the zebrafish neurectoderm for example, Neurogenin1 (Ngn1) (Blader et al., 1997; Korzh et al., 1998) drives the expression of *Delta* homologues *delta A (delA)* and *delta D* (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). *delA*, *delD* and *ngn1* transcripts are expressed by engaged but probably still proliferating neuronal precursors. Delta then activates Notch in its neighboring cells, an inhibitory interaction that allows only a subset of precursors within each proneuronal cluster to become neurons. The selected neuronal precursors exit the cell cycle and begin expressing genes characteristic of differentiating neurons, such as *delB*, *zcoe2*, *neuroD* transcripts and Hu proteins, expressed by committed and no longer proliferating cells (Bally-Cuif et al., 1998; Haddon et al., 1998; Korzh et al., 1998; Mueller and Wullimann, 2002).

While a broad network of genes that positively instructs where neurons differentiate has been identified in vertebrates, mechanisms that define where neurons are not permitted to form remain less studied. To date, Hairy/Enhancer of split [E(spl)]-like proteins (Davis and Turner, 2001) such as *Xenopus* ESR6e (Chalmers et al., 2002), and *Xenopus* Zic2 (Brewster et al., 1998), have been identified as inhibitors but the role of their homologs during neural plate development in other species remain unexplored. In *Drosophila*, Hairy has a prominent role in inhibiting neurogenesis. Unlike most transcription factors encoded by the E(spl) Complex, Hairy is a Hairy/E(spl) transcription factor that is not driven by Notch activation, rather it acts as a prepattern gene to define domains in the notum where sensory bristles are not permitted to differentiate (Fischer and Caudy, 1998; Davis and Turner, 2001). A related Hairy/E(spl) factor, Hes1, was shown to be necessary, together with Hes3, for maintaining a neuron-free zone at the MHB at a relatively late stage (E10.5) in the mouse embryo (Hirata et al., 2001). However these genes did not have an early role in the establishment of the neuron-free zone. Thus, globally, the inhibitory processes regulating neurogenesis in the vertebrate neural plate remain poorly understood.

Using manipulated and mutant contexts in zebrafish, we first demonstrate that the establishment of the neuron-free zone (IZ) at the MHB is crucial to the maintenance of MHB integrity. We next report that expression of the zebrafish *Hairy/E(spl)*-like gene *her5* at late gastrulation precisely prefigures the IZ, separating the vcc from r2MN. By combining knock-down and conditional gain of Her5 function in zebrafish transgenics, we demonstrate that Her5 is essential in vivo for inhibiting neurogenesis and increasing cell proliferation in a medial domain of the IZ, without influencing other aspects of MH patterning. Our results demonstrate that Her5 is part of a key regulatory process that links early axial patterning mechanisms to the spatial pattern of neurogenesis and cell proliferation within the vertebrate anterior neural plate.

MATERIALS AND METHODS

Zebrafish strains

Wild-type embryos were obtained from natural spawning of AB adults, and raised according to Kimmel et al. (Kimmel et al., 1995). *headless* (*hdl*) embryos were obtained by pair-wise mating of heterozygous adult carriers, as described previously (Kim et al., 2000).

hsp-her5 transgenic lines

To construct *hsp-her5* (Fig. 2D), the published coding sequence of *her5* (Müller et al., 1996) flanked by the 5′ and 3′ UTR of *Xenopus β-globin* was extracted from *pXT7-her5Δ3′* (Bally-Cuif et al., 2000) and cloned downstream of *pzhsp70* (Shoji et al., 1996) in pBluescript SK(+). Wild-type *her5* encodes 9 additional N-terminal amino acids (Fig. 2D) (A.T. and L. B-C., unpublished) but both proteins are intact in their bHLH and further C-terminal sequence. The *hsp-5′βglob-her5-3′βglob* insert (2.5 kb) was extracted from the vector backbone by *Sma*I + *Apa*I digestion, resuspended in water and injected at 50 ng/μl into freshly laid AB embryos. Injected embryos were raised to sexual maturity and pair-wise crossed to AB fish. DNA was extracted from pools of 1- to 2-day-old embryos by incubating for 3 hours at 60°C in 250 μl lysis buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 3% Tween-20, 3% NP40; 1.5 mg/ml proteinase K). The samples, complemented with 750 μl H_2O, were heated at 95°C for 10 minutes and PCR reactions were carried out using an upstream primer from the zebrafish *hsp70* promoter sequence (5′ GTGGACTGCCT-ATGTTCATCT 3′) and a downstream primer within the *her5* sequence (*her5#2*: 5′ TTTCTCCATGAGAGGCTTGG 3′) that yielded a 900 bp PCR product. For genomic DNA control the following primers were used, which amplified the endogenous *her5* cDNA (*her5#6*: 5′ AGTTCTTGGCACTCAAGCTCAA 3′ and *her5#4AP*: 5′ GCTCTCCAAAGACTGAAAAGAC 3′). PCR was performed with 5 μl of the diluted genomic DNA in 1× PCR buffer with 2.5 mM $MgCl_2$, 2.5 mM of each primer and 0.2 mM dNTPs, for 35 cycles at an annealing temperature of 56°C. Carrier G0 fish were re-crossed to wild-type fish to test for expression of the transgene upon heat-shock: the resulting embryos were submitted to a 1-hour heat-shock pulse before the 24 hpf stage and tested in whole-mount using situ hybridisation for ubiquitous *her5* expression. G0 carriers transmitting inducible *hsp-her5* were then crossed to wild-type fish and the F_1 generation was raised. F_1 carriers were identified by PCR

on tail genomic DNA. From more than 100 injected embryos, the integration rate in the G0 generation was 15%, of which 50% transmitted the transgene to their progeny. The transgene was inducible in a ubiquitous fashion upon heat-shock in 50% of these families.

Heat-shock induction and time course experiments

50-100 embryos originating from a cross between F_1, F_2 or F_3 heterozygote carriers (to generate both wild-type and transgenic embryos within each pool) were immersed in a 38°C water bath for 1-2 hours from 80% epiboly to the 3-somite stage. The embryos were then fixed in 4% PFA or further incubated at the normal temperature of 28°C before being processed for analysis. All embryos were processed together in blind experiments, the transgenic embryos being identified a posteriori using *her5* in situ hybridization or PCR genotyping as described above. The amount of transgenic *her5* mRNA in the time-course experiment on Fig. 3C was estimated as follows: following heat-shock, embryos were fixed every 0.5 hour and *her5* expression was revealed by whole-mount in situ hybridization using the fluorescent Fast-Red substrate. All embryos were processed in parallel and the color reaction was stopped at the same time. Fluorescence intensities were compared using the linear amplification system of a 3CCD Color Video Camera (Sony MC3255) and the Axiovision Software (Carl Zeiss GmbH).

Antisense experiments

Morpholino antisense oligonucleotides (MOs) were purchased from Gene-Tools, Inc. (Oregon, USA). MOs were dissolved to a stock concentration of 2 mM in H_2O and injected into 1-cell stage embryos at 1 or 2 mM. Sequences were as follows (see also Fig. 2D): MO^{tg}: 5′ CCTTCTCATGTCTTTTTGCTCCATT 3′; MO^{her5}: 5′ TTGGTT-CGCTCATTTTGTGTATTCC 3′. Both MOs were tested for their blocking efficiency by injection into a transgenic line *her5PAC-GFP* (A.T. and L.B.-C., unpublished) which carries an in-frame fusion of Her5 and GFP 3′ to the basic domain of Her5 (thus where endogenous *her5* ATG is used) and more than 40 kb of upstream regulatory sequences; this line faithfully reproduces endogenous *her5* expression. In this line, MO^{her5} fully inhibited the expression of GFP, demonstrating that, in the conditions used, this MO fully blocks the translation of endogenous *her5*. In the same context, MO^{tg} was inefficient at blocking GFP expression.

RNA injections

Capped RNAs were synthesized using Ambion mMessage mMachine kits following the recommended procedure. RNAs were injected at the following concentrations: 25 ng/μl (low dose) or 125 ng/μl (high dose) *ngn1* (Blader et al., 1997), with or without *nls-lacZ* (40 ng/μl) as lineage tracer.

In situ hybridization and immunohistochemistry

Probe synthesis, in situ hybridization and immunohistochemistry were carried out as previously described (Hammerschmidt et al., 1996). The following antibodies were used: mouse anti-myc (Sigma M 5546) (dilution 1:1000), rabbit anti-β-galactosidase (Cappel 55976) (dilution 1:4000), rabbit anti-phosphohistone H3 (Upstate Biotechnology, no. 06-570) (dilution 1:200), mouse anti-HNK1 (DSHB Zn12) (dilution 1:500), mouse anti-human neuronal protein HuC/HuD (MoBiTec A-21271) (dilution 1:300). Secondary antibodies HRP-conjugated goat anti-mouse or goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) diluted to 1:200. The staining was revealed with DAB following standard protocols.

Cloning of zebrafish *Cdk* inhibitor-encoding cDNAs

Random-primed cDNA prepared from 15-somite AB zebrafish RNA was amplified using oligonucleotides directed against cDNA AF398516 (forward primer: 5′ TCCGCTTGTCTAATGGCAGCC 3′; reverse primer: 5′ CACTTCATCCACACAGATGTGC 3′), and EST BI887574 (forward primer: 5′ CAAGCATCT GGAGCGTCATGTTG 3′; reverse primer: 5′ TAACGGCGTTCATCCTGCTCCG 3′). PCR products were subcloned and sequenced according to standard protocols. EST fx62e01.y1 was obtained from the rzpd (Berlin). All subclones were used for the generation of in situ hybridization probes following standard procedures. Sequence analyses revealed that the three clones encode CDI domain-containing proteins, characteristic of Cdk inhibitors. The CDI domains of BI887574 and fx62e01.y1 are 60% identical to each other and most related to that of *Xenopus* $p27^{XIC1}$ (53-56% identity). They are equally distant from the CDI domain of AF398516 (45% identity). The CDI domain of AF398516 is itself is more related to that of mammalian $p27^{Kip1}$ (56-59% identity) than to *Xenopus* $p27^{XIC1}$ (46% identity). Based on these findings, and on the fact that BI887574 is expressed earlier than fx62e01.y1 (see text), we re-named BI887574 zebrafish $p27^{Xic1}$-a.

Aphidicolin treatments

Embryos were incubated for 2 hours (from 70% epiboly to the 3-somite stage) [compared to an estimated 4-hour cell cycle length at this stage in the neural plate (Kimmel et al., 1994)] in embryo medium containing 1 or 10 μg/ml aphidicolin (Sigma A-9914) at 28.5°C (Marheineke and Hyrien, 2001). The embryos were then washed in embryo medium, fixed and processed for in situ hybridization and immunodetection.

RESULTS

The IZ displays distinct mediolateral restriction to neurogenesis, and results from active inhibition of neuronal differentiation at the MHB

In the zebrafish embryo, the IZ can be visualized as a gap in the expression of markers identifying the first proneural clusters and differentiating neurons. At the tail-bud stage, shortly after the onset of *ngn1* expression, the IZ is clearly visible as a V-shaped *ngn1*-negative area of 6-8 rows of cells that separates the vcc from the early motor neurons of r2 (r2MN) (Fig. 1A). Laterally, in the future alar plate, the IZ abuts the presumptive early sensory neurons of r2 (r2S) (Fig. 1A). This area roughly corresponds to the domain expressing *pax2.1* (red in Fig. 1A), which covers most of the presumptive MH territory at that stage (Lun and Brand, 1998; Picker et al., 2002). By 24 hours post-fertilization (hpf), the IZ appears as a stripe of 3-6 cells wide between neuronal precursors of the basal midbrain and rostral hindbrain (Fig. 1B, bracket). It encompasses the domain of expression of *pax2.1* (Fig. 1C), *wnt1* and *eng1* (not shown), which have narrowed to the MHB at 24 hpf.

Absence of neurons in the MHB domain might reflect the local absence of proneural gene expression at or around the MH junction, the presence of intrinsic or extrinsic cues that actively inhibit proneural function in that location, or both. To discriminate between these possibilities, we determined how the MH domain responds to ectopic expression of the proneural gene *ngn1* within the IZ. One-celled wild-type embryos were injected with capped mRNA encoding Ngn1, and probed at the tail-bud stage for *delA* expression to reveal induction of Ngn1-responsive genes (Fig. 1D-F). A dose of *ngn1* (25 pg) that was sufficient to trigger neurogenesis throughout the neural plate induced *delA* expression within the medial part of the IZ, but not in the lateral or dorsal IZ (88%, *n*=17) (Fig. 1D,E). *delA* expression was effectively induced within the presumptive lateral and dorsal parts of the IZ only upon injection of higher

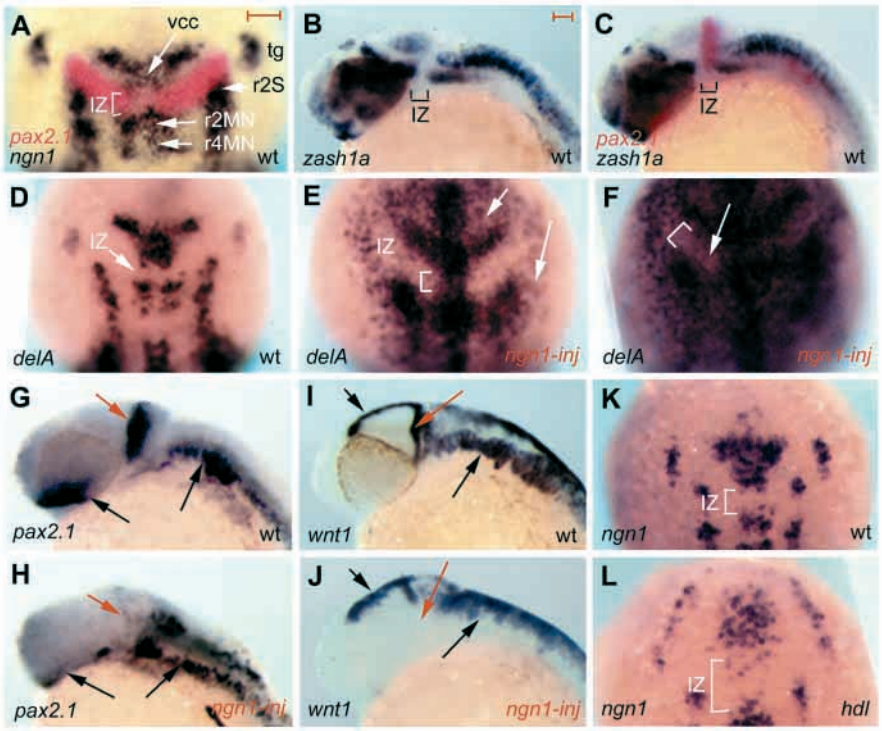

Fig. 1. The intervening zone (IZ) displays intrinsic mediolateral differences and is shaped by antagonistic activities from neurogenesis-promoting signals and the IsO. Whole-mount in situ hybridization at the 3-somite stage (A,D-F,K,L) (dorsal views, anterior to the top) and 24 hours post-fertilization (hpf) (B,C,G-J) (sagittal views, anterior to the left) with the markers indicated (bottom left, color-coded). (A-C) Intervening zone (IZ) location in wild-type (wt) embryos. At the 3-somite stage, the IZ separates the ventro-caudal cluster (vcc) from the r2 motor (r2MN) and sensory neurons (r2S), and encompasses most of the MH primordium, as revealed by *pax2.1* expression. By 24 hpf, the IZ (bracket in B,C) has narrowed to a stripe at the MHB. (D-F) Intrinsic differences between the neurogenic capacities of lateral versus medial domains of the IZ. Upon injection of 25 pg *ngn1* mRNA at the 1-cell stage (*ngn1-inj*, E), ectopic neurogenesis is induced within the neural plate outside proneural clusters (arrows in E) including the basal domain of the IZ (bracket), while the IZ remains neurons-free in lateral regions. 125 pg *ngn1* (F) are necessary to force neurogenesis within the IZ alar domain (arrow in F; location of the IZ in F is indicated by the bracket). This phenotype is correlated with the loss of expression of the MH markers *pax2.1* and *wnt1* at 24 hpf (G-J, red arrows). Note that the profile of *pax2.1* and *wnt1* expression is otherwise unaltered (*pax2.1*: optic chiasm, hindbrain interneurons: G,H, black arrows. *wnt1*: midbrain dorsal midline, rhombic lips: I,J, black arrows). (K,L) The anterior-to-posterior extent of the IZ correlates with IsO activity, and is enlarged in *hdl* mutants, which overactivate Wnt signaling (L compared with K, bracket). Scale bars: 0.1 mm. IZ, intervening zone; vcc, ventrocaudal cluster; r2MN, rhombomere 2 motorneurons; r4MN, rhombomere 4 motorneurons; r2S, rhombomere 2 sensory neurons; *delA*, *delta A*; *ngn1*, *neurogenin 1*.

ngn1 doses (125 pg) (Fig. 1F). Even in this case, ectopic *delA* remained mosaic rather than ubiquitous and the IZ could still be distinguished (77%, *n*=22) (Fig. 1F, bracket). Similar results were obtained when probing for the expression of the neuronal differentiation marker *huC* (*n*=42, data not shown). Thus, although the IZ is globally non-neurogenic in vivo, there appears to be intrinsic mediolateral differences in the mediation of its non-neurogenic character within this domain. In particular, the lack of expression of neuronal determination factors such as Ngn1 in the basal IZ domain might solely account for this region remaining neuron-free, while additional intrinsic or extrinsic blocks acting downstream or in parallel to Ngn1 activity are likely involved within the lateral and dorsal IZ domains.

Because the IZ develops at the MHB, we explored whether and to what extent IZ formation relates to and/or is required for isthmic organizer activity. We observed that ectopic expression of *ngn1* does not generally impair the establishment of MH identity (as revealed by *eng2*, *her5* or *pax2.1* expression) at early somitogenesis stages (not shown). At 24 hpf however, the expression of MHB markers such as *wnt1* and *pax2.1* was abolished upon injection of *ngn1* mRNA (Fig. 1G-J). Thus forced neurogenesis within the IZ eventually interfered with maintenance of genes that define MHB identity, suggesting that inhibition of Ngn1 expression and function may be necessary to maintain MHB identity and/or continued function of the isthmic organizer.

headless (*hdl*) mutants, characterized by reduced repression of Wnt target genes by Tcf3, have expanded expression of genes that define MHB identity (Kim et al., 2000). We observed that this phenotype correlates with an expansion of the *ngn1*-free domain in the MH (Fig. 1K,L).

Together, these results suggest that IZ formation depends on the combination of two antagonistic cues: positive neuronal differentiation signals, and an opposite inhibitory activity that is spatially associated with the isthmic organizer. In addition, they demonstrate that suppression of neurogenesis at the MHB is crucial to the maintenance of MHB integrity.

her5 expression at the onset of neurogenesis is sufficient to prevent neurogenesis around the MHB

The above results suggest that factors expressed at the MHB in response to early anteroposterior patterning cues may actively contribute to the local suppression of neurogenesis. Among those factors, Her5 appeared to be a good candidate to encode the anti-neurogenic influence spatially associated with the MHB. First, it is the earliest selective marker of the MH domain, and its expression precedes the onset of neurogenesis (Müller et al., 1996; Bally-Cuif et al., 2000). Second, it belongs to the Hairy/E(spl) family of bHLH transcription factors, which generally orient cell fate decisions during development (Kageyama et al., 1997; Fisher and Caudy, 1998; Guillemot, 1999). In support of our hypothesis, we found that *her5* expression faithfully outlines the IZ from the onset of neurogenesis at late gastrulation (Fig. 2A) until at least 24 hpf (compare Fig. 2B and C).

To examine the potential role of Her5 in IZ formation, we first used a gain-of-function approach. Ectopic expression of

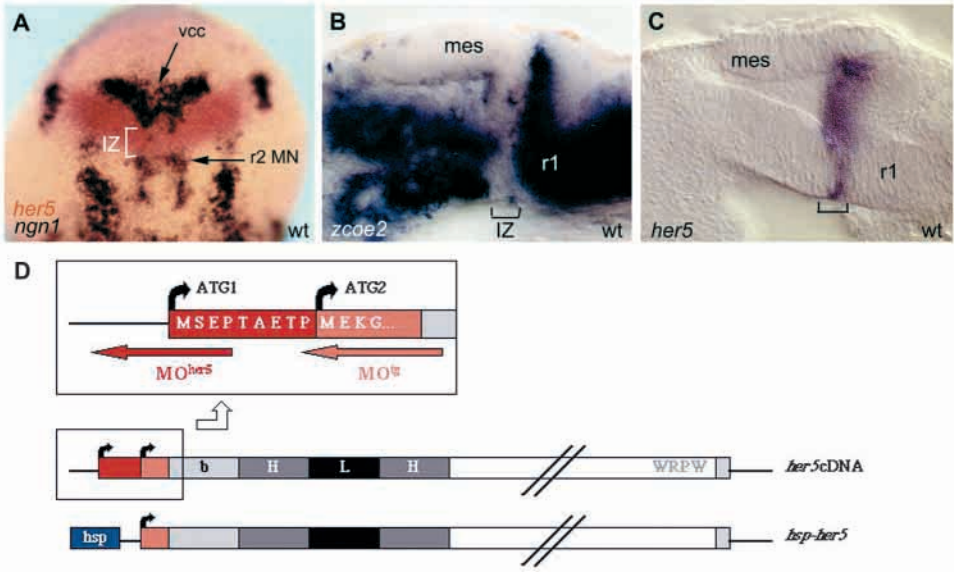

Fig. 2. Her5 as a candidate to control IZ formation. (A-C) Whole-mount in situ hybridization at the 3-somite stage (A, dorsal view, anterior to the top) and at 24 hpf (B,C, sagittal views, anterior to the left) with the markers indicated (bottom left, color-coded in A). Note that *her5* expression in wild-type embryos delineates the IZ (bracket) from the onset of neurogenesis (A) until at least 24 hpf (IZ identified by the gap in *zcoe2* staining in B). (D) Structures of the wild-type and mutant forms of *her5* cDNA and their encoded proteins used for functional assays. Top: full-length *her5* cDNA as determined from our genomic analyses (A.T. and L.B.-C., unpublished), which starts at ATG1 and encodes nine additional N-terminal amino acids compared to the published sequence (Müller et al., 1996) (see box for protein sequence). Bottom: *hsp-her5* construct used to generate transgenic lines for conditional misexpression; this construct is built from the clone of Müller et al. (Müller et al., 1996) such that the first ATG is deleted and the second ATG is used for the generation of an otherwise fully functional Her5 protein (see Materials and Methods). As a control, a morpholino directed against ATG2 (MO^{tg}, inset) inhibits translation of the transgene mRNA but not that of the endogenous *her5* (data not shown, see Materials and Methods). For loss-of-function experiments, a morpholino directed against ATG1 (MO^{her5}, inset) was used, which inhibits the function of the endogenous Her5 mRNA. Abbreviations as Fig. 1 plus, b, basic DNA-binding motif; HLH, helix-loop-helix dimerization motif; IZ, intervening zone; mes, mesencephalon; r1, rhombomere 1.

her5 severely perturbs gastrulation (Bally-Cuif et al., 2000), precluding an unambiguous interpretation of a neural phenotype at late stages. To overcome this problem we constructed *hsp-her5* transgenic lines carrying the *her5* cDNA (Müller et al., 1996) under control of the zebrafish heat-shock promoter *zhsp70* (Shoji et al., 1996; Halloran et al., 2000) (Fig. 2D). Three independent *hsp-her5* lines were generated. Because they produced similar results, they are considered together below.

We tested the reliability of *hsp*-driven transcription in these lines by monitoring *her5* expression in transgenic embryos immediately before and after heat-shock. At all stages examined, all embryos originating from a cross between a *hsp-her5* heterozygote and a wild-type fish displayed the endogenous *her5* expression profile (Fig. 3A). Upon heat-shock, strong and ubiquitous expression of *her5* was observed in 50% of the embryos (Fig. 3B), thus *hsp*-driven transcription is only induced upon heat-shock in our lines. In a time-course assay, transgenic *her5* mRNA, revealed by whole-mount in situ hybridization, was detectable as soon as 15 minutes after the beginning of the heat-shock but was gradually lost within the 1.5 hours following its end (Fig. 3C). These results are comparable to those of Scheer et al. (Scheer et al., 2002) and indicate that a heat-shock pulse translates into a narrow time-window when transgene *her5* mRNAs are available for translation.

Heat-shock pulses between 80% epiboly and tail-bud stages resulted in severe defects of *ngn1* expression in most *hsp-her5* transgenic embryos by the 3-somite stage (85% of cases, *n*=30) (Fig. 3E). Strikingly, *ngn1* expression was strongly diminished – in some cases abolished – in territories normally giving rise to the vcc and r2MN, located immediately adjacent to the domain of endogenous *her5* expression (Fig. 2A) (compare Fig. 3E with D). Other sites of neurogenesis, such as the motor, sensory and interneurons of the developing spinal cord or the trigeminal ganglia, were only marginally affected, if at all. In contrast, *ngn1* expression was not affected when transgenic embryos were injected, prior to heat-shock, with a morpholino selective for the *hsp-her5* transgene (MO^{tg}) (Fig. 2D). This morpholino has no effect on the translation of endogenous *her5* and does not affect embryonic development (see Materials and Methods). Thus, heat-shocked *hsp-her5* MO^{tg}-injected transgenic embryos (80% of cases, *n*=20) showed normal *ngn1* expression (Fig. 3F, compare with Fig. 3D and E), demonstrating that the inhibition of *ngn1* expression in the vcc and r2MN areas upon *her5* misexpression (Fig. 3E) is a selective consequence of ectopic Her5 activity.

To test whether ectopic *her5* mRNA provided at late gastrulation is sufficient to permanently inhibit *ngn1* expression in domains adjacent to the IZ, we heat-shocked embryos under the conditions described above, then resumed development at normal temperature and analyzed *ngn1* expression at the 20-somite stage. As *hsp*-driven *her5* mRNA is no longer detectable at this stage, transgenic embryos were identified a posteriori by PCR genotyping (Fig. 3I). We observed long-lasting inhibition of *ngn1* expression, which was still downregulated at the 20-somite stage around and within the MH (83% of cases, *n*=28) (bar in Fig. 3H, compare with G). Later, this phenotype was followed by a lack of neuronal differentiation: at 24 hpf, *hsp-her5* transgenic embryos harbored a significantly reduced number of differentiated vcc-derived nMLF neurons (identified by their HNK1 immunoreactivity) compared to non-transgenic heat-shocked siblings (73% of cases, *n*=15) (brown arrows in Fig. 3O, compare with N). Thus ectopic Her5 activity at the onset of neurogenesis is sufficient to inhibit *ngn1* expression and the subsequent steps of neuronal differentiation around and within the MH domain.

Her5 activity is necessary for IZ formation at early neurogenesis stages

To test whether Her5 activity was necessary for IZ formation, we 'knocked-down' *her5* translation by injecting a morpholino selective for endogenous *her5* (MO^{her5}) into wild-type embryos (Fig. 2D, see Materials and Methods). Strikingly, when MO^{her5}-injected embryos were assayed at the 3-somite stage for *ngn1* expression, no IZ was discernible in the medial MH domain: the vcc and r2MN clusters were bridged (84% of cases, *n*=19) (compare Fig. 3K with J). TUNEL assays performed between the normal onset of *her5* expression (70% epiboly) and the 3-somite stage consistently failed to reveal a significant difference in the number of apoptotic cells at any site between wild-type and MO^{her5}-injected embryos (Fig. 3L,M, and data not shown) (92% of cases, *n*=25). In contrast, cell counts indicated a large increase in the number of *ngn1*-expressing cells within the medial MH territory in MO^{her5}-injected embryos (91 cells ±5) compared to wild-type siblings (48 cells ±4) (90% of cases, *n*=10). Thus, lack of Her5 activity results in the generation of ectopic *ngn1*-positive cells in the territory located between the vcc and r2MN clusters. Importantly, this phenotype was followed by the development

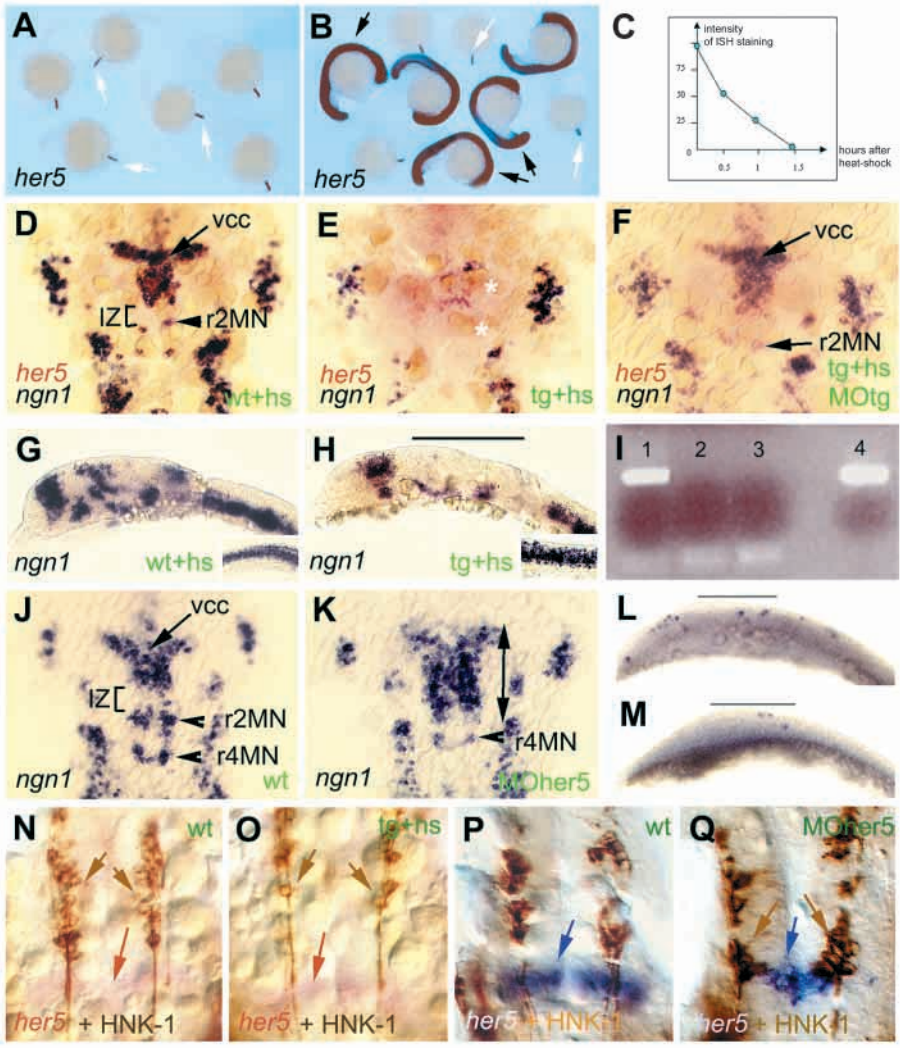

Fig. 3. Her5 is necessary and sufficient to control IZ formation. (A-C) Reliability of the *hsp*-dependent expression system. (A,B) Embryos from a cross between parents heterozygous for the *hsp-her5* transgene probed for *her5* expression (in situ hybridization) before (A) and after (B) a 1-hour heat-shock. While no ectopic expression of *her5* is detected without heat-shock, ectopic *her5* expression is ubiquitously induced upon heat-shock (white arrows indicate endogenous *her5* expression at the MHB, black arrows indicate *hsp*-driven ubiquitous expression). (C) Stability of the induced *her5* mRNA upon heat-shock, determined by whole-mount in situ hybridization (in percentage of the estimated intensity of staining that immediately follows a 0.5-hour heat-shock pulse). Induced mRNAs become undetectable within 1.5 hours following the end of the heat-shock. (D-H,N,O) Ectopic expression of Her5 inhibits *ngn1* expression in the vcc and presumptive r2MN. Whole-mount in situ hybridization or immunocytochemistry with the markers indicated (bottom left, color coded) on transgenic embryos (tg) (E,F,H,O) and their wild-type siblings (wt) (D,G,N) at the 3-somite (D-F), 20-somite (G,H), and 36 hpf (N,O) stages, following a 1-hour heat-shock at late gastrulation (hs). D-F and N,O are dorsal views of the MH area in flat-mounted embryos, anterior to the top; G,H are sagittal views of the head, anterior to left; the insets show unperturbed *ngn1* expression in the spinal cord. The misexpression of *her5* during late gastrulation inhibits *ngn1* expression in the vcc and r2MN at the 3-somite stage (white asterisks in E). Non-heat-shocked transgenics display a *ngn1* profile indistinguishable from non-transgenic controls (not shown). This effect is maintained until at least the 20-somite stage (H), and is rescued upon injection of MO^{tg}, a morpholino oligonucleotide selective of the transgene (F). At 24 hpf, the number of nMLF neurons (brown arrows), which derive at least in part from the vcc, is also significantly reduced in *hsp-her5* transgenics (O) (red arrow to *her5* expression at the MH junction). (I) Genotyping results to identify transgenic embryos in H (PCR for the transgene). Lane 1: embryo H, lane 2: embryo G, lane 3: negative control, lane 4: positive control. An identical procedure was used to identify embryos in N,O. (J-M,P,Q) The inhibition of Her5 activity leads to the differentiation of ectopic neurons in place of the IZ. J,K: dorsal views of the MH area in flat-mounted embryos at the 3-somite stage, anterior to the top, probed for *ngn1* expression following injection of MO^{her5}, a morpholino selective of endogenous *her5* (K), compared to non-injected wild-type control embryos (J). Note that the vcc and r2MN clusters are bridged (double arrow), while other neuronal populations (e.g.r4MN, arrowhead) are unaffected. (L,M) TUNEL assay in wild-type (L) and MO^{her5}-injected (M) embryos shows that injections are not followed by increased apoptosis in the MH area (bar). (P,Q) At 36 hpf, an ectopic HNK1-positive neuronal cluster (brown arrows) lies across the MH junction (identified by *her5* expression, blue arrow) upon MO^{her5} injection. Note reduced *her5* levels at the MHB in Q (compared with P), a late event suggesting indirect positive autoregulation of *her5* expression. IZ, intervening zone; MH, midbrain-hindbrain domain; nMLF, nucleus of the medial longitudinal fascicle; r2MN, motorneurons of rhombomere 2; r4MN, motorneurons of rhombomere 4; vcc, ventrocaudal cluster.

of ectopic differentiated neurons at later stages: in most cases (67% of cases, *n*=12), bilateral clusters of HNK1-positive neurons formed across the MHB in MO^{her5}-injected embryos by 36 hpf, but not wild-type embryos (brown arrows in Fig. 3Q, compare with P and N). Together, our results demonstrate that Her5 is both necessary and sufficient for inhibition of neurogenesis in the medial MHB domain at the onset of neurogenesis, an activity that helps keep the MHB free of differentiated neurons during later development.

Her5 can act in a dose-dependent manner on newly selected neuroblasts to inhibit neurogenesis at least until 24 hpf

Because *her5* expression delineates the IZ until at least 24 hpf, we tested whether it might also be involved in inhibiting neurogenesis at these late stages. When *hsp-her5* transgenic embryos were heat-shocked for 2 hours at the 8- or 15-somite stages, *ngn1* expression was down-regulated across the entire neural plate (Fig. 4A-C,G,I), in a dose-dependent fashion (86% of cases, *n*=22) (data not shown, and compare Fig. 4B and C). Within and around the MH domain, this phenotype was stable over time (Fig. 4D,E), while in other territories, *ngn1* expression was restored within a few hours of development at normal temperature (data not shown, and blue arrows in Fig. 4E) (87% of cases, *n*=24). When ectopic Her5 activity was induced at 24 hpf, *ngn1* expression was decreased within the MH domain (77% of cases, *n*=18) (compare Fig. 4K and J), while other sites remained unaffected (blue arrows in Fig. 4K). Thus Her5 activity is capable of inhibiting neurogenesis throughout somitogenesis.

ngn1 transcripts identify neuronal precursors in which neuronal fate is still being determined and includes cells that might still be proliferating (Ma et al., 1996). To better define the targets of Her5 activity and test whether it could affect cells further engaged in the neuronal differentiation pathway, we monitored the effect of ectopic *her5* expression on the expression of HuC protein. In contrast to the early onset of *huC* RNA expression (Kim et al., 1996), HuC protein immediately labels post-mitotic precursors (Mueller and Wullimann, 2002). In striking contrast to *ngn1* expression, the HuC profile was only moderately affected, if at all, by ectopic Her5 activity (82% of cases, *n*=11) (Fig. 4F-I). Thus Her5 is sufficient to prevent *ngn1* expression and/or to revert newly selected neuroblasts (*ngn1*-positive but still HuC-negative) to a non-engaged state. However, it does not act on immediately committed precursors such as HuC-positive cells, an observation in line with the idea that the mitotic to post-mitotic transition represents an irreversible commitment.

Her5 activity is not involved in patterning events within the MH domain

Because *her5* expression coincides with a number of markers that define MH identity or the MHB (Lun and Brand, 1998; Reifers et al., 1998; Belting et al., 2001; Reim and Brand, 2002), we asked whether Her5 activity is involved in controlling aspects of MH regionalization. Strikingly, ectopic *her5* expression from the onset of endogenous MH *her5* expression (70% epiboly) in *hsp-her5* transgenic embryos had no detectable effect on the expression of MH patterning markers (*iro1*, *iro7*, *pax2.1*, *eng2*, *eng3*) or IsO activity markers (*wnt1*, *fgf8*) at the 5- and 15-somite stages (*n*=35; Fig. 5A-C, also data not shown). Because a single short heat-shock pulse

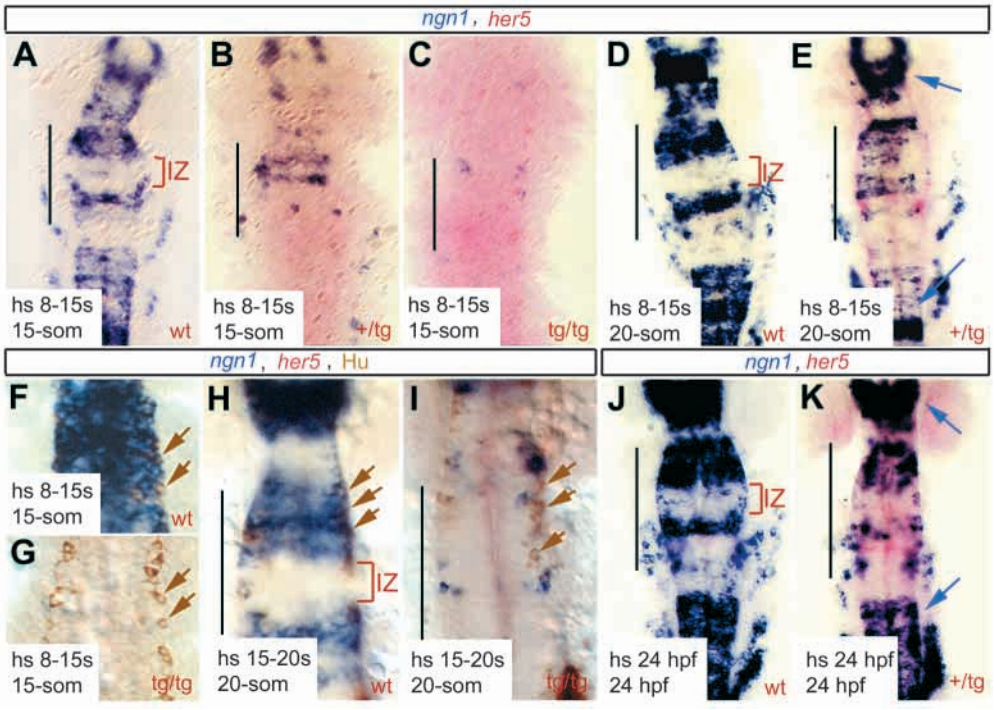

Fig. 4. Her5 activity can inhibit *ngn1* until 24 hpf, in a dose-dependent manner, but does not affect HuC expression. Whole-mount in situ hybridization for *ngn1* (blue) and *her5* (red), with immunocytochemistry for HuC protein expression (brown), at the stages indicated (bottom left) following a two-hour heat-shock (hs) between the 8- and 15-somite stages (A-G), the 15- and 20-somite stages (H,I) and at 24 hpf (J,K) in *hsp-her5* transgenic heterozygotes (+/tg), homozygotes (tg/tg) or their non-transgenic siblings (wt). Dorsal views of flat-mounted anterior neural tubes (A-E,H-K) or tails (F,G), anterior to the top; bracket indicates the IZ, vertical black bar indicates the MH domain. *ngn1* expression is downregulated in a dose-dependent manner immediately after heat-shock at any of these stages (B,C,I). This phenotype is stable in the MH domain (blue arrows to restored *ngn1* expression in the fore- and hindbrain in E). In contrast, neuronal precursors already expressing HuC are only moderately affected (brown arrows in F-I) [HuC is first detectable in vcc neurons at about the 20-somite stage (H,I), thus at the 15-somite stage we focused on HuC expression in the tail (F,G)[. At 24 hpf, only the MH domain is sensitive to ectopic Her5 activity (blue arrows to unaffected *ngn1* expression in the fore- and hindbrain in K). IZ, intervening zone; hs, heat-shock; som, somites.

might induce only a transient burst in Her5 activity, insufficient to trigger stable defects, we repeatedly heat-shocked *hsp-her5* embryos until 24 hpf. Again, even in these embryos that received a constant supply of ectopic *her5* mRNAs, no patterning defects were detected (*n*=25; Fig. 5D, also data not shown), although strong and ubiquitous ectopic expression of *her5* was achieved (Fig. 5D, red staining). These results indicate that ectopic expression of *her5* from late gastrulation onwards is not capable of altering neural patterning.

Similarly, when wild-type embryos were injected with MO^{her5}, no defects were observed in the induction or maintenance of MH patterning (*n*=30; Fig. 5E-G, also data not shown). Thus, in contrast to its prominent effect on neurogenesis, Her5 activity is not required for the establishment and early maintenance of MH identities. To ascertain whether Her5 activity could dissociate neurogenesis from MH patterning in a single embryo, we colabeled embryos injected with MO^{her5} for MH patterning markers (e.g. *pax2.1*) and neurogenesis markers (e.g. *ngn1*). Both marker types appeared co-expressed across the MH junction (Fig. 5H), a combination never normally observed in vivo (see Fig. 1A). Thus, while *her5* expression in the MH is determined by early patterning cues, its function does not control regional patterning within this domain. Thus Her5 is an essential factor that translates early axial patterning information into a distinct pattern of neurogenesis in the MH domain.

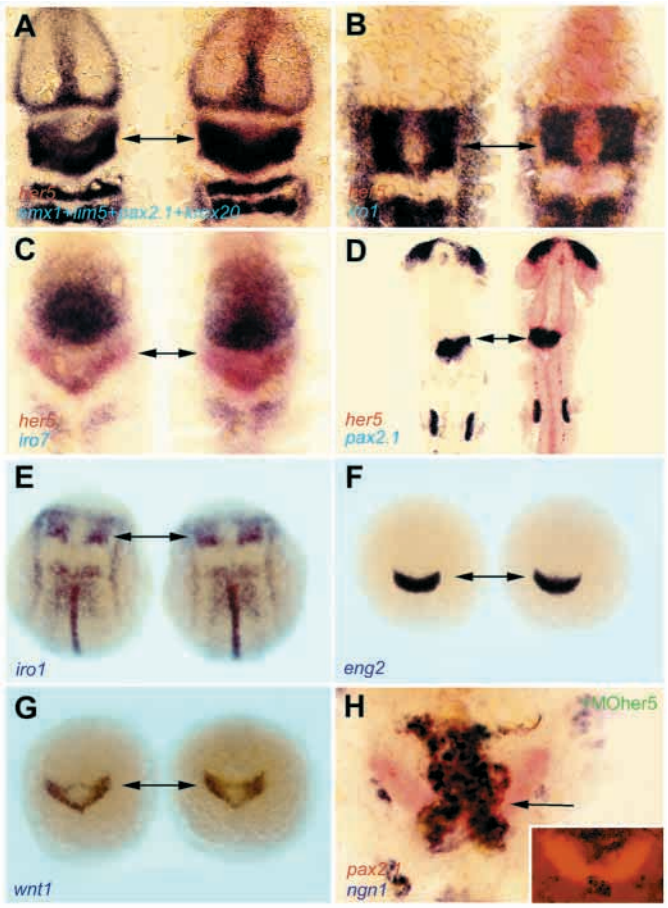

Fig. 5. Her5 activity does not control MH regional patterning. Whole-mount in situ hybridization at the 3-somite (E-H), 5-somite (A-C), and 24 hpf (D) stages for the expression of patterning and neurogenesis markers, as indicated (bottom left, color coded), following up- or down-regulation of Her5 activity. All panels are dorsal views of whole-mount (E-G) or flat-mounted (A-D,H) embryos, anterior to the top; arrows point to the MH junction. (A-D**)** MH patterning is not altered in transgenic *hsp-her5* embryos (right in each panel) compared to non-transgenic siblings (left) by heat-shock during late gastrulation (A-C) or by repetitive heat-shocks (D). (E-G) MH patterning also remains unaltered in MO^{her5}-injected embryos (right in each panel) compared to controls (left). (H) Co-expression of *ngn1* and *pax2.1* (see also fluorescent view, inset) across the MHB in a single embryo upon MO^{her5} injection demonstrates that neurogenesis and patterning can be uncoupled by Her5 activity.

Her5 activity regulates cell proliferation and the expression of the zebrafish cyclin-dependent kinase inhitor-encoding gene *p27^{Xic1}-a*

We next examined the cellular mode of Her5 action. Neuronal differentiation generally correlates with cell cycle exit (Ross, 1996; Ohnuma et al., 2001) suggesting that Her5 activity might be associated with the maintenance of a proliferating state. To test this hypothesis, we counted the number of dividing cells per cell row (phosphohistone H3-immunoreactive, indicating M phase) across the neural plate in wild-type and Her5-manipulated contexts at the onset of neurogenesis (Fig. 6A-F).

Counts of dividing cells in wild-type embryos revealed differences within the IZ. The medial domain (Fig. 6A, A domain) has more cells in M phase than the dorsolateral domain (Fig. 6A, B domains) (*n*=5) (Fig. 6F, right panel; Fig. 6B,D, brown arrows). Thus intrinsic mediolateral differences in the proliferation status of the IZ in vivo parallel its medially heightened response to ectopic neurogenesis-promoting factors (Fig. 1E) and to lack of Her5 activity (Fig. 3K).

In *hsp-her5* transgenic embryos that were heat-shocked during late gastrulation, the number of cells in M phase was significantly increased throughout the presumptive midbrain and hindbrain regions (Fig. 6A, A-D domains). This included the domain endogenously expressing *her5* (Fig. 6A, A and B domains) as well as the 16 cell rows immediately anterior and posterior to it (Fig. 6A, C and D domains), from which the vcc and r2MN originate (Fig. 6B, C and Fig. 6F, left panel) (*n*=5). In the presumptive forebrain (Fig. 6A, E domain) the number of dividing cells was not altered, although this area also prominently expressed *her5* (Fig. 6C). Conversely, in MO^{her5}-injected embryos, the number of dividing cells was significantly and selectively reduced in domain A (Fig. 6D, E; Fig. 6F, left panel) (*n*=5). Proliferation in this domain was not abolished but rather brought to a level equivalent to other neural plate territories (Fig. 6F). Together, these results suggest that Her5 activity can influence cell proliferation within and around the MH domain, and that it specifically accounts for the increased number of dividing cells within the medial IZ. In addition, Her5 loss-of-function results point to a strict correlation between domains with a decrease in cell proliferation and an increase in neuronal differentiation.

Cell proliferation involves the tight spatiotemporal control of expression and activity of a number of cellular factors including the cyclin-dependent kinases (Cdk) inhibitors p27 and p57 (O'Farrell, 2001; Ohnuma et al., 2001). Among these, $p27^{Xic1}$ (Bourguignon et al., 1998; Ohnuma et al., 1999), its mammalian relative $p27^{Kip1}$ (Lyden et al., 1999; Levine et al.,

2000; Dyer and Cepko, 2001; Li et al., 2002) and p57^{Kip2} (Dyer and Cepko, 2000) play prominent roles in the control of developmental neurogenesis downstream of neurogenic cascades in *Xenopus* and mouse. To identify potential downstream effectors of Her5 proliferative activity, we conducted database searches for zebrafish Cdk inhibitors-encoding genes. Three clones or ESTs encoding probable zebrafish homologs of p27^{Kip1} and two closely related forms of p27^{Xic1} (-a and -b) were recovered (see Materials and Methods), and the corresponding genes were PCR-amplified from tail bud-stage cDNA. In situ hybridization analyses revealed that only *p27^{Xic1}-a* was expressed in wild-type embryos at the onset of neurogenesis (Fig. 6G,I, also data not shown). Most interestingly, *p27^{Xic1}-a* expression strongly resembles that of *ngn1*, identifying the first primary neurons of the neural plate and avoiding the IZ (Fig. 6G,I). This expression profile is compatible with a role in linking cell cycle arrest with the differentiation of primary neurons. We thus addressed whether *p27^{Xic1}-a* expression was modulated by Her5 activity. Upon a brief heat-shock at late gastrulation, *p27^{Xic1}-a* expression was severely down-regulated in *hsp-her5* transgenic embryos, while it was unaffected in heat-shocked wild-type siblings (80% of cases, *n*=20; Fig. 6G,H). Conversely, in embryos where Her5 activity was abolished, *p27^{Xic1}-a* expression expanded ectopically across the IZ, overlapping the unaffected expression of *pax2.1* (82% of cases, *n*=22; Fig. 6I,J). Thus, modulating Her5 activity triggers opposite effects on cell proliferation and *p27^{Xic1}-a* expression. This suggests that down-regulation of *p27^{Xic1}-a* expression by Her5 might be involved in mediating the Her5-effected higher cell proliferation of the medial IZ domain in wild-type embryos.

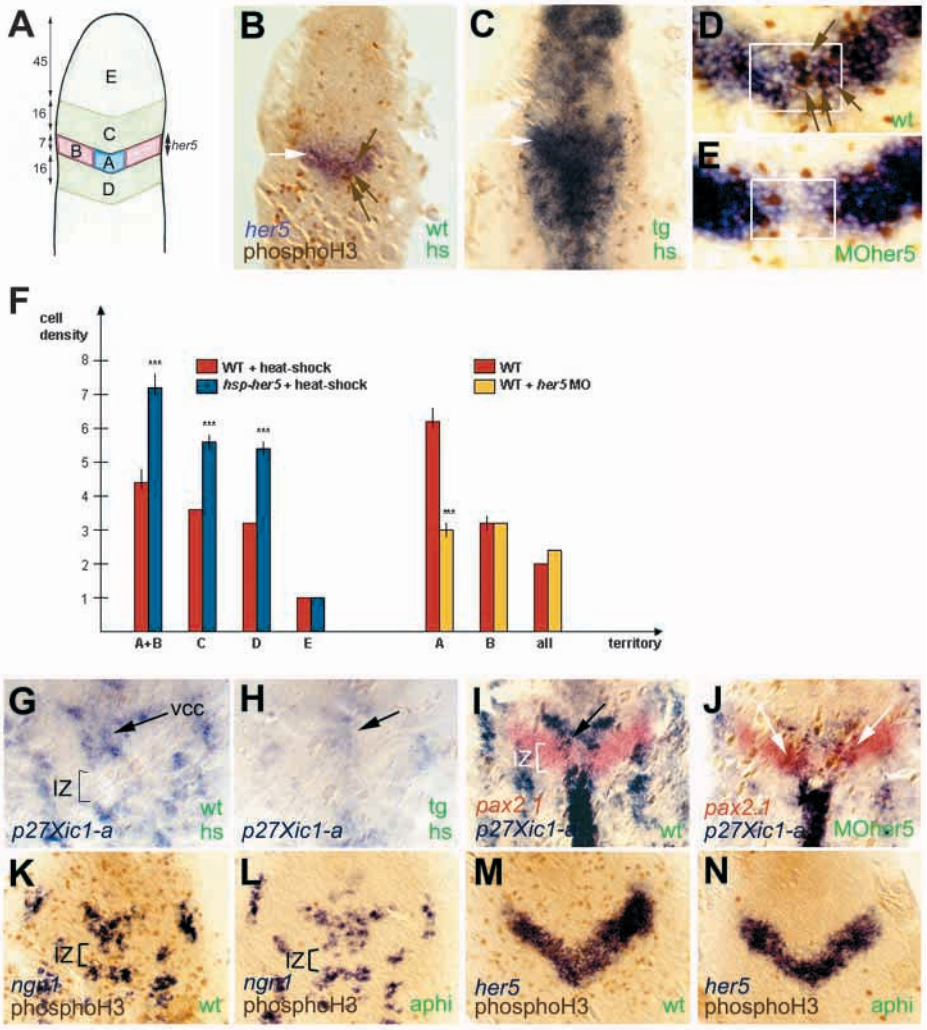

Fig. 6. Her5 activates cell proliferation within the MH domain, but this process is in itself insufficient to account for the regulation of *ngn1* expression. (A,F) *her5* expression (in situ hybridization, blue staining) and density of cells in M phase (brown anti-phosphoH3 immunostaining) (in number of positive cells per cell row) in the anterior neural plate at the 3-somite stage (in territories schematized in A) in *hsp-her5* transgenics (C), their wild-type siblings (B) (both heat-shocked), wild-type (D) and MO^{her5}-injected embryos (E). A is a schematic representation of the neural plate in B,C; B-E are flat-mounted views of the anterior neural plate (B,C) or the endogenous *her5* domain (D,E), anterior to the top. White arrow in B,C, the endogenous domain of *her5* expression (territories A + B); box in D,E indicates territory A. Proliferation is enhanced in territory A compared to other neural plate domains in wild-type embryos (brown arrows in B,D). Her5 is sufficient to increase proliferation within the MH domain upon ectopic expression (territories A-D) (F, left panel), and is necessary for the increased level of proliferation of territory A (F, right panel). (G-J) Expression of the cyclin-dependent kinase inhibitor-encoding gene *p27^{Xic1}-a* is downregulated by Her5 within the IZ. Expression of *p27^{Xic1}-a* and *pax2.1*, as indicated (bottom left, color-coded), in *hsp-her5* transgenic embryos after heat-shock (H) and MO^{her5}-injected embryos (J) compared to their wild-type siblings (G,I) at the 3-somite stage. In the vcc and IZ, *p27^{Xic1}-a* expression is strikingly similar to that of *ngn1* (e.g. Fig. 3D). *p27^{Xic1}-a* expression is down-regulated within the neural plate following ectopic *her5* expression (arrow in H), and is activated across the IZ when Her5 activity is blocked (white arrows in J). Concomitantly in the latter case, *p27^{Xic1}-a* expression is partially reduced in the vcc area, a phenomenon at present unexplained but independent of cell migration (A.G. and L.B.-C., unpublished data). (K-N) The direct inhibition of cell proliferation does not affect IZ formation and *her5* expression. Expression of *ngn1* or *her5* (blue in situ hybridization staining) and anti-phosphoH3 immunostaining (brown nuclei) at the 3-somite stage in embryos treated with the cell proliferation inhibitor aphidicolin at the onset of neurogenesis (L,N) compared to mock-treated siblings (K,M). Although phosphoH3 staining is virtually abolished upon aphidicolin treatment, both IZ size (bracket in K,L) and *her5* expression appear normal. Aphi, aphidicholin-treated embryo; hs, heat-shocked embryo; IZ, intervening zone; tg, transgenic; vcc, ventrocaudal cluster.

To determine whether Her5-induced effects on neurogenesis and proliferation are causally linked, we assessed neurogenesis in embryos where cell proliferation was blocked. To block cell proliferation, we incubated wild-type embryos in aphidicolin from the onset of *her5* expression until early neurogenesis. Although this treatment virtually abolished cell division (Fig. 6K-N, phosphoH3 staining), it had no effect on *ngn1* (*n*=20; Fig. 6L) or *her5* (*n*=20; Fig. 6N) expression. Thus the activation of cell proliferation by Her5 is not an intermediate step in its inhibition of *ngn1* expression across the IZ. Conversely, our results demonstrate that enhanced Her5 activity in *hsp-her5* transgenics can further upregulate cell proliferation within the endogenous *her5*-positive territory (Fig. 6F, left panel, A+B domain), which is devoid of *ngn1* expression and neurogenesis at all stages. Thus, at least within the IZ, the inhibition of *ngn1* expression by Her5 is unlikely to be an intermediate step in its activation of cell proliferation. Together, these results suggest that the regulation of neurogenesis and cell proliferation across the medial IZ in vivo reflect two parallel but distinct activities of endogenous Her5.

DISCUSSION

In this study, we addressed the mechanisms establishing the pattern of neurogenesis of the vertebrate MH domain. We demonstrated that neuronal differentiation is actively repressed at the MHB, and that this process is necessary for the maintenance of MHB integrity. We provided evidence that the non-differentiation zone (IZ) that splits midbrain from hindbrain neuronal clusters at the MHB consists of a medial and lateral domain with intrinsically different patterns of cell proliferation and potential for neurogenesis. We demonstrated that knock-down of Her5 function uncovers a cryptic proneuronal domain in the medial IZ that is continuous with vcc neurons rostrally and r2 motor neurons caudally. We also demonstrated that Her5 is essential for maintaining relatively high levels of proliferation in this medial domain by a mechanism that may be independent of effects on neurogenesis and involve the Cdk inhibitor $p27^{Xic1}$-a. Finally, we showed that Her5 activity does not influence MH patterning. Together, our results establish that a local process actively inhibiting neurogenesis at the MHB shapes the MH neuronal differentiation pattern and is essential to MHB maintenance. We identify Her5 as one crucial molecular component of this partially redundant pathway, and demonstrate that Her5 is a key regulator linking early axial patterning information to a distinct pattern of neurogenesis and cell proliferation in the MH domain. These findings more generally shed light on the mechanisms underlying the combinatorial control of patterning, neurogenesis and proliferation events within the vertebrate neural plate.

Differential competence of the MH junction towards neurogenesis

A first conclusion of our findings is that the IZ is not a homogeneous territory but is composed of two subdomains that differ strikingly both in their proliferation properties and in their competence to undergo neurogenesis. The medial IZ exhibits a single block in the differentiation pathway, encoded by Her5 activity, while the dorsolateral IZ likely bears multiple blocks, one operating upstream of *ngn1* expression, and at least one operating downstream or in parallel to this step. These intrinsic differences are unlikely to reflect general lateral versus medial properties of the entire neural plate, since neurons develop elsewhere in lateral domains at the same time as in basal territories (for instance the sensory neurons of r2). They might be due to other local inhibitors redundant to Her5 function in the laterodorsal territory. The transcriptional inhibitors Eng2 and 3 (Ekker et al., 1992), also expressed within the IZ from the onset of neurogenesis (Lun and Brand, 1998), do not appear to be sufficient cofactors. Indeed their ectopic expression is capable of inhibiting *ngn1* expression within the MH, however blocking the activities of Her5, Eng2 and Eng3 together by co-injecting the relevant morpholinos does not extend the neurogenic phenotype triggered by the lack of Her5 activity alone (M.I. and A.C., unpublished). Other candidates might be found within antagonists to neurogenic bHLH proteins, such as Hairy/E(spl) or non-basic HLH factors (A.T. and L.B.-C., unpublished), or among factors related to known neurogenesis inhibitors such as Zic2 (Brewster et al., 1998). The combined use of multiple inhibitors to locally prevent neurogenesis has been postulated to explain the non-differentiation of the superficial ectoderm layer in *Xenopus* (Chalmers et al., 2002). Our results thus provide a new example of this strategy to delimit neuronal differentiation domains during neural plate development.

An intriguing aspect of the phenotype triggered by Her5 gain-of-function is its prominence around and within the MH domain (Fig. 3E,H and Fig. 4), suggesting the presence of local cofactors. These might act on the regulatory elements of *ngn1* or of genes encoding redundant proneural factors to potentiate Her5 activity, or might behave as partners of Her5 to reinforce its activity and/or the stability of the Her5 protein. In favor of these ideas, the *ngn1* enhancer contains an element driving expression preferentially within the MH domain (Blader et al., 2003). In addition, we found that a mutant form of Her5, deleted of its C-terminal Groucho-binding WRPW domain, was inactive in regulating *ngn1* expression (A.G. and L.B.-C., unpublished), suggesting that Groucho-like cofactors are necessary to Her5 function. Along this line, *groucho4* is selectively expressed within the MH domain in the mouse and chick (Sugiyama et al., 2000; Ye et al., 2001).

Her5 activity shapes the midbrain-hindbrain neurogenesis pattern

Her5 acts in vivo as a local inhibitor of neurogenesis at the MHB. Our findings suggest that in the basal MH area, neurogenesis is primarily shaped into a pattern of separate neuronal clusters by a process of local inhibition that likely splits a continuous MH proneural field. Recent studies in *Xenopus* brought to attention the role of neurogenesis inhibitors in organizing zones of differentiation within the neural plate (Bourguignon et al., 1998; Brewster et al., 1998; Chalmers et al., 2002). Our analysis of IZ formation illustrates how neurogenesis inhibitors, superimposed on differentiation-competent territories, are crucial elements in shaping the embryonic neurogenesis pattern in vertebrates.

MO^{her5}-injected embryos display ectopic neurogenesis across the medial IZ from the very onset of *ngn1* expression (Fig. 3K), demonstrating that Her5 activity is essential to the establishment of this neuron-free zone. Whether Her5 is also

involved in medial IZ maintenance at later stages cannot be directly concluded from our loss-of-function data. Such a role, however, would be in line with the observation that *her5* expression continues to define the neuron-free area untill the 24 hpf stage and that ectopic *her5* expression can prevent neurogenesis within the MH domain at least until 24 hpf (Fig. 4). In the mouse, IZ maintenance relies on the combined action of two other Hairy/E(spl) bHLH factors, Hes1 and Hes3 (Hirata et al., 2001), which separately inhibit neurogenesis in a number of instances in vivo (Ishibashi et al., 1994; Ishibashi et al., 1995; Ohtsuka et al., 1999): *Hes1*$^{-/-}$*;Hes3*$^{-/-}$ double-mutant embryos display premature neuronal differentiation across the MH junction from late somitogenesis (E10.5) (Hirata et al., 2001). No earlier neurogenic phenotype was detected in these embryos, however, suggesting that Hes1 and Hes3, unlike zebrafish Her5, are not involved in IZ generation. These observations are in keeping with the relatively late onset of *Hes1* and *Hes3* expression within the MH domain (Lobe et al., 1997; Allen and Lobe, 1999; Hirata et al., 2001), and with the observation that Hes1 and Hes3 are more related in sequence to zebrafish Her6 and Her3 than to Her5. Whether Her5 function is, all or in part, relied on by other Her factors at late stages to maintain medial IZ development in the zebrafish will require further study.

Her5 effectors in the control of MH neurogenesis

Her5 belongs to the Hairy/E(spl) class of bHLH transcription factors, generally functioning as transcriptional repressors (see Kageyama et al., 1997; Fischer and Caudy, 1998; Davis and Turner, 2001). Indeed, we demonstrated previously that Her5 functions as an inhibitor of transcription during a first developmental cell fate choice event required for endoderm patterning (Bally-Cuif et al., 2000). The direct targets of Hairy/E(spl) factors remain largely unknown outside of *achate-scute*-related genes (Chen et al., 1997) and some instances of autoregulation (Takebayashi et al., 1994). Our results demonstrate that a rapid response to manipulating Her5 activity is the regulation of *ngn1* expression. Thus the most parsimonious interpretation of Her5 function is that it directly inhibits the transcription of *ngn1*. Alternatively, Her5 might primarily inhibit expression (or activity) of upstream proneural factors such as those belonging to the Ash or Ath bHLH familes. Several such factors have been isolated in the zebrafish (Allende and Weinberg, 1994; Masai et al., 2000; Itoh and Chitnis, 2001), but their expression in the early neural plate was not reported. Addressing this point will be an important issue.

Her5 might also act at other steps of the neurogenic cascade, but our results indicate that the time-window of Her5 action is limited. Upstream of *ngn1* expression are the specification of the MH proneural field (possibly by Iro1 and 7) (Lecaudey et al., 2001; Itoh et al., 2002), the definition of proneural clusters and the singling-out of individual precursors by the Notch-dependent lateral inhibition process (Haddon et al., 1998; Lewis, 1998; Chitnis, 1999; Takke et al., 1999). An action of Her5 at any of these upstream steps is unlikely. First, knocking-down Her5 activity has no effect on the expression of markers of the MH proneural fields or clusters (Fig. 5D and data not shown). In contrast, perturbing Iro function affects *her5* expression (M.I. and A.C., unpublished), placing *her5* downstream of these factors. Second, manipulating the lateral inhibition machinery, and in particular suppressing Notch signaling, did not affect IZ formation (A.G. and L.B.-C., unpublished), arguing against a role for Her5 upstream of Notch signaling. Further, Her5 does not act far downstream of *ngn1* expression in the neurogenic cascade, as expression of the post-mitotic marker HuC protein (Mueller and Wullimann, 2002) was never reversed upon ectopic Her5 activation (Fig. 4). In the same individuals, *ngn1* expression was virtually abolished. Thus our results support a role for Her5 in regulating the expression (or activity) of proneural factors at a level equivalent to Ngn1 in the neuronal differentiation process.

An important question is, to what extent the mechanism regulating neurogenesis at the MHB differs from those operating elsewhere in the neural plate. All studied bHLH neurogenesis inhibitors in the vertebrate central nervous system act as downtream effectors of Notch activity, with the exception of *Xenopus* HES6 and mouse Hes3. Her5 joins these exceptions as both its expression and activity within the neural plate are independent of Notch signaling in vivo (A.G. and L.B.-C., unpublished). Within the neural plate, Her5 expression and function appear more reminiscent of those of *Drosophila* Hairy than of other vertebrate Hairy/E(Spl) factors known to date. Indeed Hairy operates independently of Notch signaling and is involved in pre-patterning broad non-differentiation zones within the *Drosophila* notum, prior to the onset of neurogenesis (Fischer and Caudy, 1998; Davis and Turner, 2001). Similarly, mouse Hes1 was proposed to negatively delimit neurogenesis domains within the olfactory epithelium (Cau et al., 2000). Her5 appears as the first vertebrate Hairy/E(spl) factor with similar function within the neural plate, and it will be interesting to determine whether our findings can be extended to other family members.

Proliferation and neurogenesis at the MH junction

Two classes of G1 CyclinD:Cdk inhibitors play a prominent role in a developmental context: p16, and the Cip/Kip family members p21, p27 and p57 proteins (O'Farrell, 2001; Ohnuma et al., 2001; Ho and Dowdy, 2002). Zebrafish *p27*^{Xic1}*-a* expression is negatively regulated by Her5 activity, adding strong support to the idea that Cdk inhibitors control spatiotemporally regulated cell cycle events during embryogenesis and are, at least in part, controlled themselves at the transcriptional level (see Dyer and Cepko, 2000; Dyer and Cepko, 2001; Hardcastle and Papalopulu, 2000; Levine et al., 2000; Ohnuma et al., 1999; Ohnuma et al., 2001). Our findings strongly suggest that the transcriptional inhibition of *p27*^{Xic1}*-a* is a downstream event of Her5 activity in its activation of cell proliferation within the medial IZ. Her5 thus appears reminiscent of mammalian Hes1 and 3, which inhibit the expression of Cip/Kip family members in vitro (Kabos et al., 2002), and it is possible, like for other Hes factors (Sasai et al., 1992; Kageyama et al., 1997; Hirata et al., 2000; Pagliuca et al., 2000), that *p27*^{Xic1}*-a* is a direct transcriptional target of Her5. However, our data also suggest that additional cell cycle regulators are responsive to Her5 activity in this domain, since an increased dose of Her5 at the MHB further enhances cell proliferation in *hsp-her5* transgenics compared to wild-type embryos while this domain does not express *p27*^{Xic1}*-a*.

In its regulation of cell proliferation, Her5 does not appear as an all-or-none switch, but rather as a modulator. Indeed a

basal level of proliferation is maintained in the absence of Her5 activity within the medial IZ. In addition, the laterodorsal IZ, which also expresses *her5*, does not proliferate at a higher rate than other neural plate domains. Several hypotheses might account for these observations. Her5 might act as a permissive factor that enhances the competence of its expressing cells towards extrinsic or intrinsic proliferation triggers. Alternatively, Her5 might alter the length of cell cycle phases to shorten those where cells are responsive to differentiation signals. Finally, Her5 might not act on the cell cycle *per se* but rather orient cell divisions towards a symmetrical mode at the expense of an asymmetrical one.

Finally, our results suggest that the effects of Her5 on cell proliferation and neurogenesis are distinct. In *hsp-her5* transgenics, increased Her5 activity upregulates proliferation even at the MHB, a neurogenesis-free territory. Conversely, blocking cell proliferation does not induce *ngn1* expression at the MHB. Thus, the activation of proliferation by Her5 is not simply a consequence, and is also unlikely to be an upstream step, of its inhibition of neurogenesis. Rather, our results support a model where these two processes are, at least in part, independently regulated by Her5 activity in vivo. Her5 would thus appear as a coordinator of cell division and neuronal differentiation within the MH domain, in a manner reminiscent of the key regulator XBF-1 within the *Xenopus* anterior neural plate (Hardcastle and Paplopulu, 2000). A striking and relevant example is also provided by the bifunctional *Xenopus* protein $p27^{Xic1}$, which uses separate molecular domains to regulate both cell cycle and cell differentiation in the retina (Ohnuma et al., 1999).

Linking patterning, neurogenesis and proliferation at the MHB

Patterning of the MH domain relies on two series of components, IsO-derived signals (e.g. Wnts and Fgfs) and general MH identity factors (e.g. Pax2/5/8 and Eng proteins) (Martinez et al., 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The expression of markers of both types was reproducibly unaltered at any stage in response to gain- and loss-of-function of Her5, under conditions that influenced MH neurogenesis and proliferation. Her5 activity thus strikingly differs from that of its probable *Xenopus* homolog XHR1 (Shinga et al., 2001) and from mouse Hes1/Hes3 (Hirata et al., 2001), all of which were interpreted as primarily acting on MH patterning. Ectopically expressed *XHR1* markedly enhances *En2* expression, and its dominant-negative forms down-regulated *XPax2* and *En2* in *Xenopus* (Shinga et al., 2001). Similarly, in double $Hes1^{-/-};Hes3^{-/-}$ mouse mutant embryos, the loss of organizer-specific gene expression such as *Pax2*, *Wnt1* and *Fgf8* precedes neuronal differentiation defects (Hirata et al., 2001). *her5* expression is established by early axial patterning cues, and later responds to isthmic organizer activity (Lun and Brand, 1998; Reifers et al., 1998; Belting et al., 2001; Reim and Brand, 2002). Our results show that Her5 selectively controls neurogenesis and proliferation without retroacting on MH patterning. Thus Her5 is part of a key coupling pathway activated at the MHB to translate early axial patterning and later isthmic organizer information into a local control of neurogenesis and proliferation.

At early somitogenesis stages, the isthmic organizer controls MH patterning and growth (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Later, the MHB remains a prominent source of proliferating cells (Wullimann and Knipp, 2000), proposed to permit the massive and sustained growth of MH structures relative to other neural territories in all vertebrates. Our findings demonstrate that maintenance of an MHB neuron-free zone results from an active mechanism, and further attests the biological significance of this process for MH development, by demonstrating that neurogenesis must be prevented at the MHB to maintain MHB integrity. The inhibitory process involving Her5 might perhaps speculatively be viewed as a self-protective mechanism permitting the maintenance of MHB activity over time, in a manner possibly reminiscent of other signaling boundaries, such as, for instance the *Drosophila* wing margin.

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