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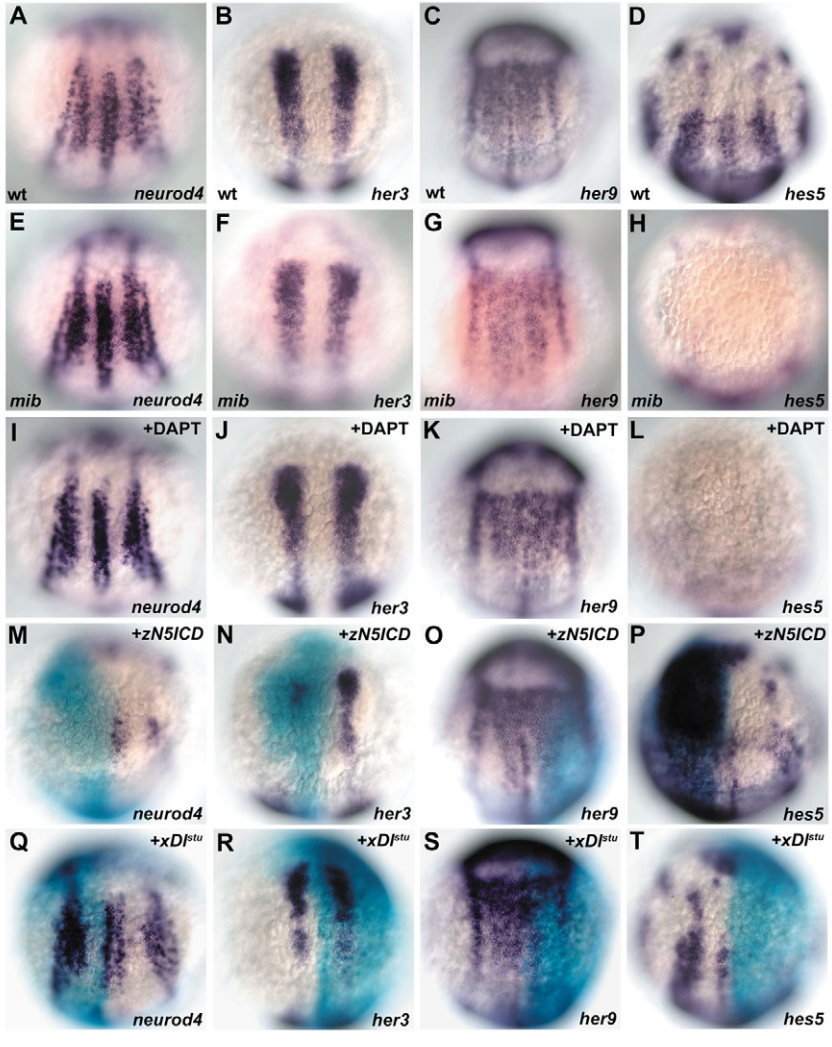
In Fig. 3 of this paper, panel C and panel K were inadvertently duplicated. Panel C has been replaced with the correct panel in the figure reprinted below. There are no changes to the figure legend, which is accurate. This error does not affect the conclusions of the study.

The authors apologise to readers for this mistake.



Patterning of proneuronal and inter-proneuronal domains by *hairy*- and *enhancer of split*-related genes in zebrafish neuroectoderm

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Summary

In teleosts and amphibians, the proneuronal domains, which give rise to primary-motor, primary-inter and Rohon-Beard (RB) neurons, are established at the beginning of neurogenesis as three longitudinal stripes along the anteroposterior axis in the dorsal ectoderm. The proneuronal domains are prefigured by the expression of basic helix-loop-helix (bHLH) proneural genes, and separated by domains (inter-proneuronal domains) that do not express the proneural genes. Little is known about how the formation of these domains is spatially regulated. We have found that the zebrafish *hairy*- and *enhancer of split*-related (Her) genes *her3* and *her9* are expressed in the inter-proneuronal domains, and are required for their formation. *her3* and *her9* expression was not regulated by Notch signaling, but rather controlled by positional cues, in which Bmp signaling is involved. Inhibition of Her3 or Her9 by antisense morpholino oligonucleotides led to ectopic expression of the proneural genes in part of the inter-proneuronal domains. Combined inhibition of Her3 and Her9 induced ubiquitous expression of proneural and neuronal genes in the neural plate, and abolished the formation of the inter-proneuronal domains. Furthermore, inhibition of Her3/Her9 and Notch signaling led to ubiquitous and homogeneous expression of proneural and neuronal genes in the neural plate, revealing that Her3/Her9 and Notch signaling have distinct roles in neurogenesis. These data indicate that *her3* and *her9* function as prepattern genes that link the positional dorsoventral polarity information in the posterior neuroectoderm to the spatial regulation of neurogenesis.

Key words: Neurogenesis, *hairy, enhancer-of-split*, Proneural gene, Proneuronal domain

Introduction

Spatial regulation of neurogenesis is a fundamental process that controls development of the central nervous system. Little is known about the mechanism by which neurogenesis is spatially regulated in vertebrates. Zebrafish embryos, like other anamniotes, have two distinct populations of neurons – primary and secondary neurons. Primary neurons are rapidly developing early neurons, which include primary-motor, primary-inter and Rohon-Beard (RB) neurons, and they are required for the coordinate movements of larvae soon after hatching (reviewed by Appel, 2000). Primary neurons are generated from proneuronal domains, also called neurogenic regions, that are established as three longitudinal stripes along the anteroposterior axis in the dorsal ectoderm during the late gastrulation and early segmentation periods. The proneuronal domains express basic helix-loop-helix (bHLH) proneural genes, *neurogenin1* (*neurog1*) (Blader et al., 1997; Kim et al., 1997), *olig2* (Park et al., 2002) and *neurod4* (previously called *zath3*) (Park et al., 2003; Wang et al., 2003), and a homeobox gene, *pnx* (Bae et al., 2003) in zebrafish. The proneuronal domains are separated by the inter-proneuronal domains, which do not express the proneural genes and do not exhibit neurogenesis when it occurs in the proneuronal domains.

As proposed in *Drosophila* (Campos-Ortega, 1993), proneural genes play an important role in neurogenesis. *neurog1* is required for the formation of RB neurons (Cornell and Eisen, 2002), and *olig2* is required for the formation of primary motoneurons (Park et al., 2002). Within the proneuronal domains, a subset of cells is selected to become neurons. This selection is controlled by the Notch-mediated lateral inhibition mechanism (Appel and Chitnis, 2002). In the context of lateral inhibition, cells that express proneural genes, such as *neurog1*, at high levels become neurons and simultaneously express the Notch ligands Delta or Serrate, which activate Notch signaling in neighboring cells (Appel and Eisen, 1998; Chitnis and Kintner, 1996; Haddon et al., 1998; Ma et al., 1996). These neighboring cells express Hairy and Enhancer of Split-related (Her) transcriptional repressors that inhibit neurogenesis (Takke et al., 1999). Although the mechanism that restricts the number of cells differentiating into neurons within the proneuronal domains is relatively clear, the mechanism that represses neurogenesis in the inter-proneuronal domains is largely unknown.

In *Drosophila*, Hairy functions differently from Enhancer of Split [E(spl)], which functions downstream of Notch signaling, in the context of sensory organ development (Orenic et al., 1993; Skeath and Carroll, 1991). *hairy* is expressed in four longitudinal stripes in the *Drosophila* pupal legs, located between stripes expressing the proneural gene *acheate*. In the absence of *hairy* function, *acheate* expression expands into the

inter-stripe regions that normally express *hairy*, fusing the *acheate* stripes and resulting in the disorganization of sensory organ bristles (Orenic et al., 1993), indicating that Hairy suppresses the neurogenesis in the inter-stripe region in the *Drosophila* leg. *hairy* expression is controlled by positional cues, and it is proposed to function as a prepattern gene that links positional information to the spatial regulation of neurogenesis (Kwon et al., 2004; Orenic et al., 1993; Skeath and Carroll, 1991) A similar role has been proposed for the Her/Hes-family genes in vertebrates. *Xenopus ESR6e*, which is expressed in the superficial layer of the ectoderm, is involved in protecting the ectodermal layer cells from becoming neurons (Chalmers et al., 2002), and mouse Hes1 represses neurogenesis in the olfactory placode independent of lateral inhibition (Cau et al., 2000). Her5 represses neurogenesis independently of Notch signaling in the midbrain-hindbrain boundary (MHB) in zebrafish (Geling et al., 2004). These reports suggest that Her/Hes-mediated prepattern mechanisms are involved in the spatial regulation of neurogenesis in vertebrates.

Bmp activity has been shown to provide positional information for the posterior primary neurons (Barth et al., 1999; Nguyen et al., 2000). In embryos with mild defects in Bmp signaling, the RB neurons are absent, and the primary interneuron domains are expanded and located ventrally. In embryos with severe defects in Bmp signaling, both the RB neurons and primary interneurons are absent. These studies suggest that the position and width of the proneuronal and the inter-proneuronal domains are regulated directly or indirectly by Bmp signaling. It has been reported that the *Xenopus Zic*-related gene *Zic2* is expressed in the inter-proneuronal domains and is involved in patterning the proneuronal and inter-proneuronal domains (Brewster et al., 1998). However, none of the *zic*-related genes displays a similar expression pattern in zebrafish (Grinblat and Sive, 2001) or any other vertebrate species. There should be other genes that are controlled by positional information, in which Bmp signaling is involved, and that spatially control neurogenesis along the dorsoventral axis in the zebrafish posterior neuroectoderm.

We report that zebrafish *her3* and *her9*, which are expressed in the inter-proneuronal domains, repress the expression of proneural genes in the inter-proneuronal domains and thereby control the formation of the inter-proneuronal domains. *her3* and *her9* expression is not regulated by Notch signaling, but rather is controlled by positional information, in which Bmp signaling is involved. The data indicate that *her3* and *her9* function as prepattern genes, which spatially control neurogenesis through a mechanism similar to that involving *hairy* in *Drosophila*.

Materials and methods

Fish embryos

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of fish with the Oregon AB or AB/India genetic background. The following zebrafish mutants were used: *swr*^{jc300}, *snh*^{ty68a} and *mib*^{m178} (Mullins et al., 1996; Schier et al., 1996).

Plasmids and transcription detection

Expression plasmids for Myc-tagged Her9 (Her9MT), Her9VP16, Her9EnR and Her3MT were constructed in pCS2+ as previously published (Bae et al., 2003). Expression plasmids for the activated form of zebrafish Notch5 (zN5ICD), the antimorphic form of *Xenopus* Delta-1 (xDlu^{stu}) and β-galactosidase have also been published (Bae et al., 2003). The RNA for Her9MT, Her9VP16, Her9EnR, Her3MT, zN5ICD or xDlu^{stu} was co-injected with 50 pg of β-galactosidase RNA into one blastomere of two- to four-cell stage zebrafish embryos. The embryos were fixed and stained with X-gal and antisense riboprobes, as reported previously (Bae et al., 2003). To visualize the in situ hybridization, BM purple AP substrate was used as the substrate for alkaline phosphatase. The *neurod4* (Wang et al., 2003), *her2* (NM_131089), *her4* (Takke et al., 1999), *her12* (NM_205619), *her3* (Hans et al., 2004) and *hes5* (Raya et al., 2003) probes were amplified by PCR. *pnx* (Bae et al., 2003), *her9* (Leve et al., 2001), *olig2* (Park et al., 2002), *neurog1* (Kim et al., 1997), *islet1* (Bae et al., 2003), *deltaA* (Haddon et al., 1998) and *elavl3* (Kim et al., 1996) were detected as described previously.

DAPT treatment

Embryos were incubated with 100 μM DAPT from 60% epiboly until the one-somite stage.

Antisense morpholino oligonucleotides

Morpholino oligonucleotides (MOs) were generated by Gene Tools. *neurog1*-MO and *olig2*-MO have been previously published (Cornell and Eisen, 2002; Park et al., 2002). The other MOs used here were: *her9*ATG-MO, 5′-CTCCATATTATCGGCTGGCATGATC-3′; *her9*SD-MO, 5′-GTGATTTTTACCTTTCTATGCTCGC-3′; *her9*_5mis-MO (the lower-case letters indicate mispaired sequences in the control morpholino), 5′-CTCtATATgcTCGGCTGatATGATC-3′; *her3*-MO1, 5′-CTGTTGGATGCTGTAGCCATTGTCC-3′; and *her3*-MO2, 5′-TGCAGCCATTGTCCTTAAATGCTCA-3′ [the same sequence as published (Hans et al., 2004)]. We also used the standard MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′. We used two distinct MOs for *her3* (*her3*-MO1 and *her3*-MO2) and her9 (*her9*ATG-MO and *her9*SD-MO), and obtained essentially the same results from them. Neither the *her9*_5mis control MO nor the standard MO elicited significant abnormalities in neurogenesis at the doses used.

Results

her3 and *her9* are expressed in the inter-proneuronal domains

To reveal the mechanism by which proneuronal and inter-proneuronal domains are established, we sought genes that are specifically expressed in these domains. The bHLH proneural genes *neurog1* and *neurod4* and the homeobox gene *pnx* display spotty expression within the proneuronal domains (Bae et al., 2003; Blader et al., 1997; Kim et al., 1997; Park et al., 2003; Wang et al., 2003) (Fig. 1A,A′; Fig. 2A, data not shown), and *olig2* is also expressed in the proneuronal domains, which correspond to the primary motoneurons (Park et al., 2002) (Fig. 1G,G′). We found that the zebrafish Her genes displayed two distinct expression profiles in the neural plate (Fig. 1J). *her2*, *her4*, *her12* and *hes5* are expressed in the proneuronal domains (Takke et al., 1999) (Fig. 1D,D′, data not shown), similar to the expression of the proneural genes. By contrast, *her9* and *her3* were expressed in the inter-proneuronal domains (Fig. 1B,B′,E,E′,H,H′). It has previously been reported that *her9* is expressed as three longitudinal stripes (Leve et al., 2001), but it was not clear whether these expression domains corresponded to the proneuronal domains or the inter-proneuronal domains. Co-staining of early segmentation-stage embryos with *her9* and *pnx* or *neurog1* probes revealed that the expression of *her9* was

complementary to that of *neurog1* and *pnx* (Fig. 1C,C′, data not shown), and detected between the primary motoneurons and interneurons, and between the primary interneurons and RB neurons. Co-staining with *her3* and *hes5* or *olig2* probes showed that *her3* was expressed between the primary motoneurons and interneurons (Fig. 1F,F′,I,I′). The *her3* expression domains were more restricted than those of *her9* in the inter-proneuronal domain between the primary motoneurons and interneurons. These data suggest that *her3* and *her9* are expressed differently and have one or more different functions from other Her genes that are expressed in the proneuronal domains.

Expression of *her3* and *her9* is regulated by positional cues

The positions of the primary neurons are known to be controlled by Bmp in the zebrafish (Barth et al., 1999; Nguyen et al., 2000). It has previously been reported that the RB neurons are lost and primary interneuron domains expanded in *snailhouse* (*snh*)*/bmp7*(*snh*^{ty68}) and that both the RB neurons and interneurons are lost in *swirl* (*swr*)*/bmp2b* (*swr*^{tc300}) mutant embryos (Nguyen et al., 2000). In our experiments, the *snh* mutant embryos showed a similar phenotype to that described in the previous publication, and the lateral proneuronal domains that contain *neurod4*-expressing RB neurons and *hes5*-expressing cells were lost and the primary interneuron domains were expanded and located ventrally in these embryos (Fig. 2B,E). However, both the RB neurons and primary interneurons were lost in the *swr* mutant embryos (Fig. 2C,F). This phenotype was similar to that of *swr* mutant embryos into which *chordin* RNA was injected (Nguyen et al., 2000). The severity of the phenotypes of zebrafish mutants often depends on the genetic background, the age of the parent heterozygotes and the condition of the culture. The *swr*^{tc300} mutant embryos in our experiments displayed more severe Bmp signaling-defect phenotypes than did those reported previously (Nguyen et al., 2000). In these *snh* and *swr* mutant embryos, the inter-proneuronal spaces were expanded. In correlation with this, the *her3* and *her9* expression was expanded ventrally. The *her3* and *her9* expression did not reach the most ventral part of the *snh* mutant embryos (Fig. 2H,K), but in the *swr* mutant embryos, their expression was expanded and reached the most ventral part of the embryos (Fig. 2I,L). These data indicate that the position and width of the *her3* and *her9* expression is regulated by positional cues, in which Bmp signaling is involved directly or indirectly. The formation of the proneuronal and the inter-proneuronal domains is co-regulated by the positional information.

Expression of *her3* and *her9* is independent of Notch signaling

Many Her genes are known to be targets of Notch signaling in various zebrafish tissues (Henry et al., 2002; Itoh et al., 2003; Oates and Ho, 2002; Pasini et al., 2004; Raya et al., 2003; Takke and Campos-Ortega, 1999; Takke et al., 1999). We therefore examined the regulation of *her3* and *her9* by the Notch signal. *mind bomb* (*mib*) embryos have a mutation in the gene encoding a ubiquitin ligase for Delta and display a strong reduction in Notch signaling (Itoh et al., 2003). We also used a chemical compound, DAPT, which is a γ-secretase inhibitor that inhibits Notch signaling by preventing the generation of NotchICD (Geling et al., 2002), and RNAs for an activated form of Notch5 (zN5ICD) (Bae et al., 2003) or

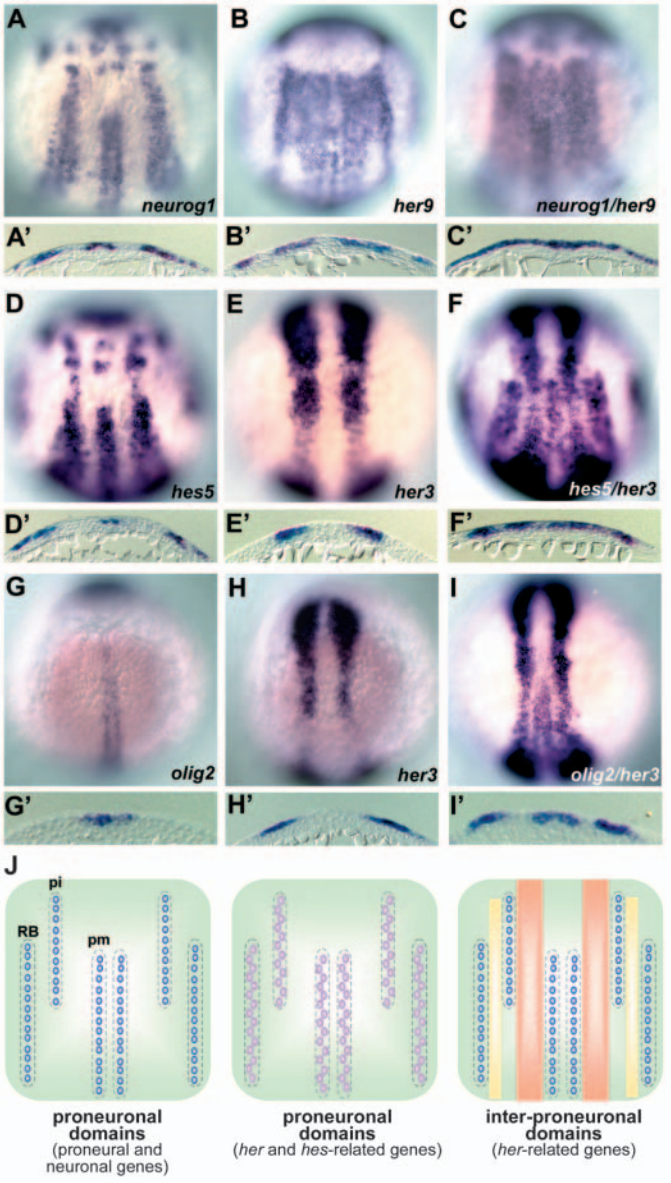

Fig. 1. Expression of Her genes in the zebrafish posterior neuroectoderm. Expression of *neurogenin1* (*neurog1*; A,A′), *her9* (B,B′), *hes5* (D,D′), and *her3* (E,E′) at the one-somite stage, and *olig2* (G,G′) and *her3* (H,H′) at the three-somite stage. Co-staining with probes for *neurog1* and *her9* (C,C′, one-somite stage), *hes5* and *her3* (F,F′, one-somite stage), and *olig2* and *her3* (I,I′, three-somite stage). Dorsal views of the hindbrain and spinal cord region. (J) Schematic representation of expression profiles of the genes expressed in the proneuronal and inter-proneuronal domains. pm, primary motoneuron; pi, primary interneuron; RB, Rohon Beard neurons. *neurog1* and *olig2* are expressed in the proneuronal domains (indicated by purple circles, left). *hes5* and *her4* are also expressed in the proneuronal domains, but not in the cells that express proneural genes (indicated by pink circles, middle). *her3* is expressed between the primary motoneurons and interneurons (orange stripes), and *her9* is expressed in all the inter-proneuronal domains (orange and yellow stripes, right).

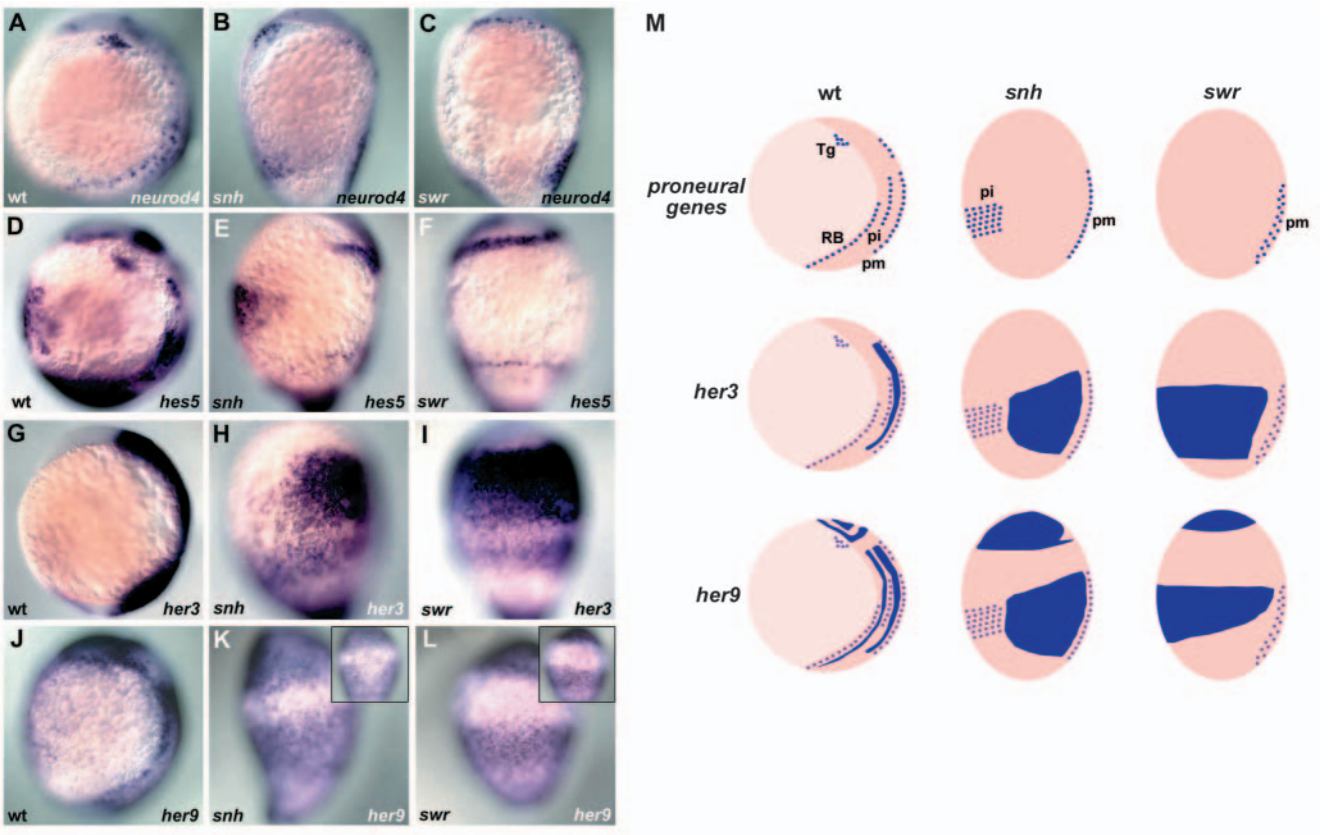

Fig. 2. Regulation of *her3* and *her9* expression by Bmp signaling. Expression of *neurod4* (A-C), *hes5* (D-F), *her3* (G-I) and *her9* (J-L) in wild-type (A,D,G,J), *snail house/bmp7* (*snh*; B,E,H,K) and *swirl/bmp2b* (*swr*; C,F,I,L) mutant embryos at the one-somite stage. Lateral views with dorsal towards the right and ventral views (insets in K,L). (M) Schematic representation of the expression profiles of the proneuronal gene *neurod4*, and of *her3* and *her9* in the wild-type, *snh* and *swr* mutant embryos. Tg, trigeminal neurons. In the snh^{ty68a} mutant embryos, the RB neurons are lost and the primary interneurons are shifted ventrally (primary neurons are marked by *neurod4* expression). In the swr^{tc300} mutant embryos, both the RB and primary interneurons are absent. In correlation with the expansion of the inter-proneuronal domains in these mutant embryos, the expression domains of *her3* and *her9* were strongly expanded ventrally. The expression reached the most ventral side in the swr^{tc300} mutant embryos.

an antimorphic form of *Xenopus* Delta (xDl^{stu}), which inhibits Notch signaling (Chitnis et al., 1995). In the *mib* mutant embryos and embryos treated with DAPT, *hes5* expression was strongly reduced or abolished, and the *neurod4* expression became homogenous within the proneuronal domains (Fig. 3E,H,I,L). This is a typical expression pattern seen in Notch-defective embryos (Appel et al., 2001; Itoh et al., 2003) and is consistent with *hes5* being a target of Notch signaling (Raya et al., 2003). By contrast, the expression of *her3* and *her9* was not significantly affected at the early segmentation stage in these Notch-defective embryos (Fig. 3F,G,J,K). Expression of zN5ICD strongly induced the ectopic expression of *hes5* and abolished the *neurod4* expression (Fig. 3M,P). By contrast, expression of xDl^{stu} suppressed the *hes5* expression and induced a homogenous expression of *neurod4* within the proneuronal domains (Fig. 3Q,T). However, neither zN5ICD nor xDl^{stu} affected the *her9* expression (Fig. 3O,S). The *her3* expression was suppressed by zN5ICD, as reported (Hans et al., 2004), but was not affected by xDl^{stu} (Fig. 3N,R). These data indicate that neither the *her3* nor the *her9* expression requires Notch signaling at the early segmentation stage. The expression of *her3* and *her9* was partly dependent on Notch signaling at later stages (data not shown).

Expression of *her3* and *her9* is independent of the formation of the proneuronal domains

The *her3* and *her9* expression is complementary to that of the proneural genes, and the expression of *her3, her9* and the proneural genes is co-regulated in embryos that are defective in Bmp signaling. These data raise the possibility that *her3* and *her9* expression is negatively controlled by the proneural genes or established upon the formation of the proneuronal domains. Positional information-dependent *her3* and *her9* expression might be mediated through the proneural genes or their downstream genes. To address this issue, we examined the expression of *her3* and *her9* in embryos that misexpressed or had defects in the function of proneural genes. Misexpression of *neurog1* induced the ectopic expression of *neurod4* and the postmitotic neuronal marker *elavl3* (Kim et al., 1997) (data not shown), suggesting that the misexpression of *neurog1* could induce ectopic proneuronal domains. Nevertheless, the misexpression of *neurog1* did not affect the expression of *her9* (Fig. 4B). The *neurog1* misexpression reduced the expression

of *her3* (Fig. 4A), probably through the upregulation of *delta* and the activation of Notch signaling (Takke et al., 1999). Consistent with this, the ectopic expression of *hes5* was induced by the misexpression of *neurog1* (Fig. 4C).

The injection of antisense morpholino oligonucleotides (MOs) for *neurog1* and *olig2* inhibits the formation of RB neurons and primary motoneurons, respectively (Cornell and Eisen, 2002; Park et al., 2002). Consistent with this, the co-injection of *neurog1*-MO and *olig2*-MO inhibited the expression of *islet1* and *elavl3* in RB neurons and primary interneurons (Fig. 4F,I; data not shown), but did not affect the expression of *her3* and *her9* (Fig. 4D,E,G,H), indicating that the regulation of *her3* and *her9* expression is independent of the proneural genes and is not established upon the formation of the proneuronal domains.

Her9 functions as a transcriptional repressor to inhibit neurogenesis

To reveal the functions of *her3* and *her9*, we injected the *her3*-MO and/or *her9*-MO into wild-type or *mib* mutant embryos. We used two different MOs for each of Her genes and obtained essentially the same results (see Materials and methods). We show the data from the *her9*ATG-MO and *her3*-MO1 in Figs 5 and 6. In the *her9*-MO-injected embryos, *neurod4* was detected in the inter-proneuronal domains between the RB neurons and primary interneurons, whereas *neurod4* was not expressed in this region in the control embryos at the early segmentation stage (Fig. 5A,B,E,F). In the *her9*-MO-injected wild or *mib* mutant embryos, the *neurod4*-expressing intermediate and lateral domains were fused and became homogenous (Fig. 5C,D,G,H) (94%, n=129 for Fig. 5C,G; and 96%, n=45 for Fig. 5D,H), indicating that *her9* is required for the suppression of *neurod4* expression in the inter-proneuronal domains between the RB neurons and the primary interneurons. *her9* is not required for restricting the number of *neurod4*-expressing cells within the proneuronal domains. In the *her9*-MO-injected embryos, the fourth ventricle in the hindbrain became smaller and the domain containing the *atoh1*-expressing neural precursors in the subventricular area was reduced at the pharyngula stage (88%, n=67 at 30 hours post fertilization, hpf), compared with the control embryos. The data suggest that Her9 is involved in the generation or maintenance of neural progenitor cells in the hindbrain, which are located in the ventricular zone of the fourth ventricle. As reported previously, injection of the *her3*-MO induced ectopic *neurog1* expression only in rhombomeres 2 and 4 (Hans et al., 2004) (86%, n=52 for Fig. 5M,N). These data suggest redundant roles for Her3 and Her9 in the inter-proneuronal domains that are located between the primary motoneurons

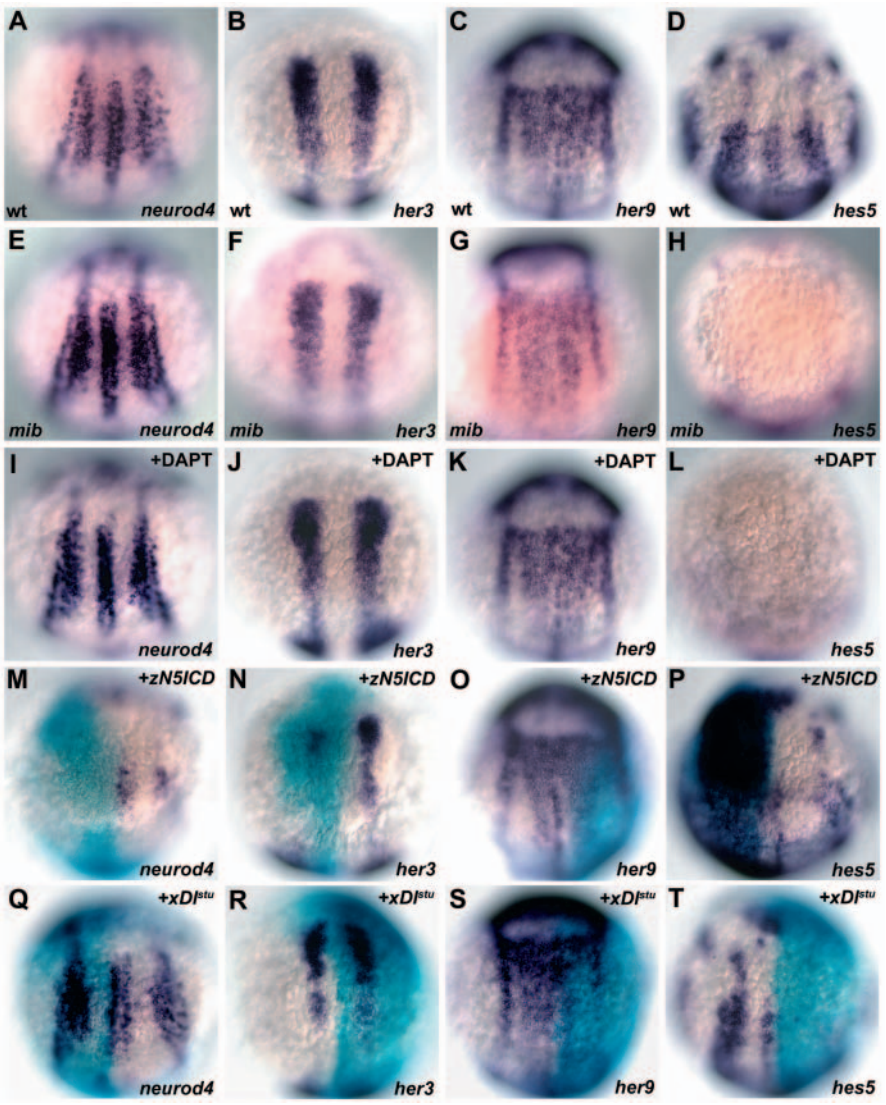

Fig. 3. *her3* and *her9* expression is independent of Notch signaling. (A-L) Expression of *neurod4* (A,E), *her3* (B,F), *her9* (C,G) and *hes5* (D,G) in wild-type (A-D), *mind bomb*^{m178} mutant (*mib*; E-H) and DAPT (γ-secretase inhibitor)-treated embryos (+DAPT; I-L) at the one-somite stage. Dorsal views with anterior towards the top. The expression of *neurod4* became homogeneous within the proneuronal domains, and the expression of *hes5* was abolished in the *mib*^{m178} mutant and the DAPT-treated embryos. The expression of *her3* and *her9* was not affected in the *mib*^{m178} mutant or the DAPT-treated embryos. (M-T) Expression of *neurod4*, *her3*, *her9* and *hes5* in embryos that had received an injection of β-galactosidase RNA (50 pg) and RNA for a constitutively active zebrafish *notch5* (50 pg, *+zN5ICD*; M-P) or an antimorphic *Xenopus Delta-1* (100 pg, *+xDl*^{stu}; Q-T) at the one-somite stage. Co-staining with the riboprobes and X-gal. Dorsal views with anterior towards the top. Misexpression of zN5ICD abolished the *neurod4* expression and reduced the *her3* expression at variable levels, and strongly induced the ectopic expression of *hes5*. By contrast, the expression of xDl^{stu} increased the *neurod4* expression within the proneuronal domain and abolished the *hes5* expression, but did not affect the *her3* expression. The expression of *her9* was not affected by the expression of either zN5ICD or xDl^{stu}.

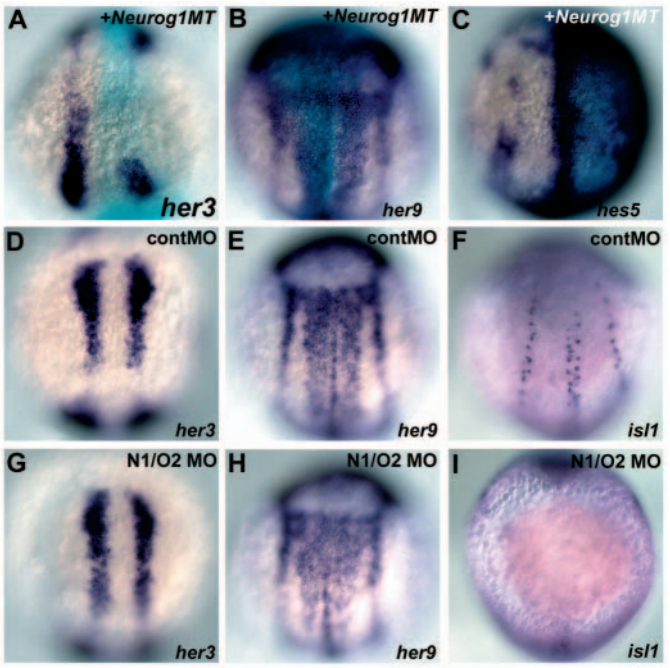

Fig. 4. Expression of *her3* and *her9* is independent of proneural genes. (A-C) Expression of *her3*, *her9* and *hes5* in embryos that had received an injection of RNA for Myc-tagged Neurog1 (Neurog1MT, 50 pg) and β-galactosidase (50 pg) (the injection was into one blastomere of two- to four-cell stage embryos) at the one-somite stage. Co-staining with the riboprobes and X-gal. (D-L) Expression of *her3*, *her9* and *islet1* in embryos that received injections of control MO (2 ng, D-F) or 1 ng *neurog1*-MO and 2 ng *olig2*-MO (N1/O2 MO, G-I) at the one-somite stage. Dorsal views with anterior towards the top. Misexpression of Neurog1MT reduced the *her3* expression and strongly induced the ectopic expression of *hes5*, but did not affect the *her9* expression. *neurog1/olig2* morphant embryos showed the loss of RB and primary motoneurons, which express *islet1*, but displayed a normal expression of *her3* and *her9*.

and interneurons, and in the position posterior to rhombomere 4.

We also examined whether Her9 functions as a transcriptional repressor for the formation of the inter-proneuronal domains. To address this issue, we co-injected RNAs for Myc-tagged Her9 (Her9MT), a fusion protein of Her9 and the transcriptional activator VP16 (Her9VP16), or a fusion protein of Her9 and the transcriptional repressor domain of *Drosophila* Engrailed (Her9EnR), with β-galactosidase RNA into one blastomere of two- to four-cell stage embryos. In the majority of the *her9MT* RNA-injected embryos, the cells that received the *her9MT* RNA were localized in the dorsal part of the embryos. These cells did not localize to the proneuronal domains, but rather to the inter-proneuronal domains in the neural plate (Fig. 5O, Table 1). However, in those cells that localized to the proneuronal domains, *neurod4* expression was reduced or absent (Fig. 5P, Table 1). Similar results were obtained in *her9EnR* RNA-injected embryos (Table 1). The cells that received the *her9VP16* RNA were evenly distributed. The expression of Her9VP16 disrupted the proneuronal domain-specific *neurod4* expression and induced an ectopic and spotty pattern of expression (Fig. 5Q). We also found that the misexpression of Her3 repressed the expression of *neurod4* in the proneuronal domains (Fig. 5R, Table 1). These data indicate that Her9, like Her3 (Hans et al., 2004), functions as a transcriptional repressor to inhibit neurogenesis in the inter-proneuronal domains.

Role of Her3 and Her9 in the establishment of inter-proneuronal domains

As the expression of *her3* and *her9* overlaps in the inter-proneuronal domain between the primary motoneuron and interneuron, it is likely that *her3* and *her9* function redundantly in the inter-proneuronal domain. We co-injected *her3*-MO and *her9*-MO into wild-type or *mib* mutant embryos, and examined the expression of *neurog1*, *neurod4*, *deltaA* and *elavl3*. Co-injection of *her3*-MO and *her9*-MO induced the ubiquitous expression of *neurog1*, *deltaA*, *neurod4* and *elavl3* within the neural plate, but not in the midline region that includes the floor plate [Fig. 6C (96%, *n*=78), Fig. 6G (94%, *n*=59), Fig. 6K (97%, *n*=66), Fig. 6O (99%, *n*=71)], indicating that Her3 and Her9 function redundantly in the inter-proneuronal domains. These data also show that Her3 and Her9 are required to repress the proneural genes and their downstream events in the inter-proneuronal domains.

As the ectopic expression of *neurog1*, *deltaA*, *neurod4* and *elavl3* was detected in the position of the inter-proneuronal domains of the *her3/her9*-MO-injected embryos, it is possible that some population of differentiated cells was increased or ectopically generated in the position of the inter-proneuronal domains. We examined the expression of *islet1* and *tlx3a*, which are expressed in the primary motoneurons and RB neurons, and in the RB neurons, respectively (Andermann and Weinberg, 2001; Inoue et al., 1994; Langenau et al., 2002), in the *her3/her9*-MO-injected embryos. The *islet1* and *tlx3a* expression was not affected in the *her3/her9* morphant embryos [Fig. 6R (100%, *n*=55), Fig. 6T (100%, *n*=63)]. Furthermore, there was no prominent change in the expression of the interneuron markers, *lim1* (Nguyen et al., 2000), *hlx1/2* (Fjose et al., 1994; Seo et al., 1999), *eng1b* (Higashijima et al., 2004), *evx1* (Thaeron et al., 2000), *vsx1/2* (Passini et al., 1997), *sax2* (Bae et al., 2004) and *pax2.1* (Mikkola et al., 1992) at the early and late segmentation stages, or at the pharyngula stage (data not shown). These data suggest that, although ectopic neurogenesis took place in cells located in the inter-proneuronal domains of the *her3/her9* morphant embryos, they did not undergo differentiation to specific neuronal cell-types at the early segmentation stage or they differentiated into neurons that are not recognized by the genetic markers.

In the *her3/her9*-MO-injected embryos, *neurog1*, *deltaA*, *neuroD4* and *elavl3* still displayed spotty expression patterns, suggesting that the cells expressing these genes in the inter-proneuronal domains were still subjected to lateral inhibition. Consistent with this, in the *her3/her9*-MO-injected *mib* embryos, all of these genes were expressed ubiquitously and homogenously within the neural plate, except in the midline region [Fig. 6D (100%, *n*=31), Fig. 6H (96%, *n*=22), Fig. 6L (100%, *n*=26), Fig. 6P (100%, *n*=27)], further indicating that Her3/Her9 and Notch signaling play different roles in neurogenesis. Notch signaling functions in lateral inhibition to restrict the numbers of neuronal cells in the proneuronal domains. Her3 and Her9 function as prepattern genes that spatially repress neurogenesis and thereby generate the inter-proneuronal domains.

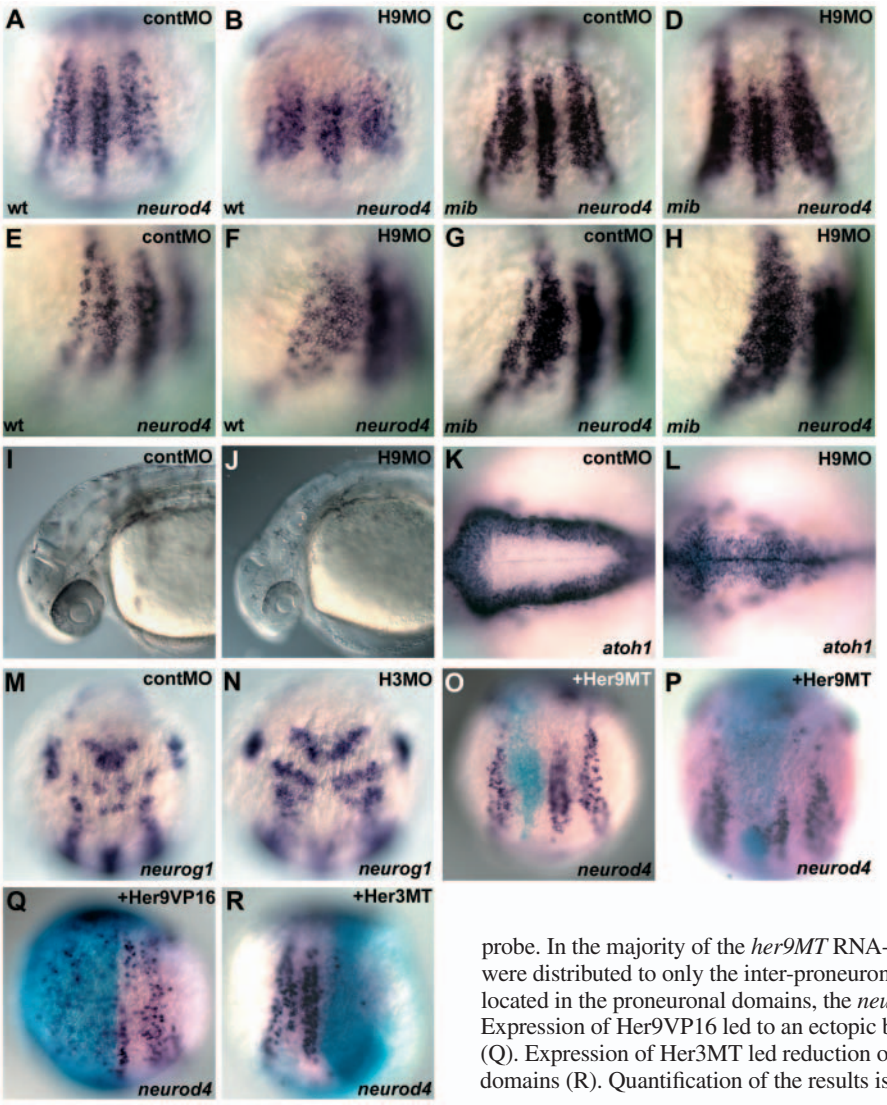

Fig. 5. Her3 and Her9 repress the expression of proneural genes. (A-H) Expression of *neurod4* in the wild-type control embryos (A,E), embryos that received an injection of 2 ng *her9*ATG-MO (H9MO; B,F), *mib* mutant embryos (C,G) and *mib* mutant embryos that received an injection of *her9*ATG-MO (D,H) at the one-somite stage. Dorsal views (A-D) and dorsolateral views (E-H). *neurod4* was expressed in the inter-proneuronal domain between the RB neurons and primary interneurons in the *her9*ATG-MO-injected wild-type and *mib* mutant embryos, where *neurod4* was not expressed in the control embryos. (I-L) The *her9*-MO-injected embryos had a reduced and narrow fourth ventricle at 30 hours post fertilization (hpf) (J,L), compared with the control embryos (I,K). *atoh1* marks neuronal precursor cells located in the subventricular zone of the fourth ventricle. Lateral views (I,J) and dorsal views of the hindbrain region (K,L). (M-N) Expression of *neurog1* in 1 ng of control MO (M) and *her3*-MO1-injected embryos (H3MO; N) at the one-somite stage. Dorsal views for the anterior hindbrain. Ectopic expression of *neurog1* was detected only in rhombomeres 2 and 4 of the *her3* morphant embryos. (O-R) Her9 functions as a transcriptional repressor. RNAs for Myc-tagged Her9 (Her9MT, 5 pg; O,P), Her9-VP16 fusion protein (Her9VP16, 5 pg; Q) or Myc-tagged Her3 (Her3MT, 20 pg; R) together with 50 pg β-galactosidase RNA, were injected into one blastomere of two- to four-cell stage embryos. The embryos were fixed at the one-cell stage, and stained with X-gal and the *neurod4* probe. In the majority of the *her9MT* RNA-injected embryos, cells that received *her9MT* RNA were distributed to only the inter-proneuronal domains (O). However, when the cells were located in the proneuronal domains, the *neurod4* expression was reduced or abrogated (P). Expression of Her9VP16 led to an ectopic but spotty expression of *neurod4* in the neural plate (Q). Expression of Her3MT led reduction of the *neurod4* expression in the proneuronal domains (R). Quantification of the results is shown in Table 1.

Table 1. Effects of the expression of Her3, Her9, Her9VP16 and Her9EnR on the *neurod4* expression

RNA	Dose (pg)	Normal (%)	Inter-proneuronal domain-specific contribution* (%)	Reduced expression† (%)	Ectopic and spotty expression‡ (%)	*n*
β-gal only	50	100	0	0	0	38
Her9MT	5	18	67	15	0	139
	20	13	79	8	0	78
Her9EnR	2	10	78	12	0	46
	10	9	83	8	0	33
Her9VP16	5	24	0	2	74	116
	20	17	0	3	80	39
Her3MT	20	61	6	33	0	94
	100	44	8	46	0	69

RNAs for Her9MT, Her9VP16, Her9EnR or Her3MT with β-galactosidase (β-gal) RNA were co-injected into one blastomere of two- to four-cell stage embryos, and the embryos were fixed at the three-somite stage and stained with X-gal and the *neurod4* riboprobe. The embryos were classified into four categories by the expression of *neurod4* and the contribution of the blue cells, which received the exogenous RNA.

*Embryos had the blue cells only in the inter-proneuronal domains (Fig. 5O).

†Embryos showed reduced or abolished expression of *neurod4* in the proneuronal domains (Fig. 5P,R).

‡Embryos showed ectopic and spotty expression of *neurod4* in the neural plate (Fig. 5Q). The embryos that did not show any changes in the *neurod4* expression or inter-proneuronal domain-specific contribution of the blue cells were classified as 'normal'. The embryos with blue cells in the ventral epidermis or the mesoderm were also classified as 'normal'.

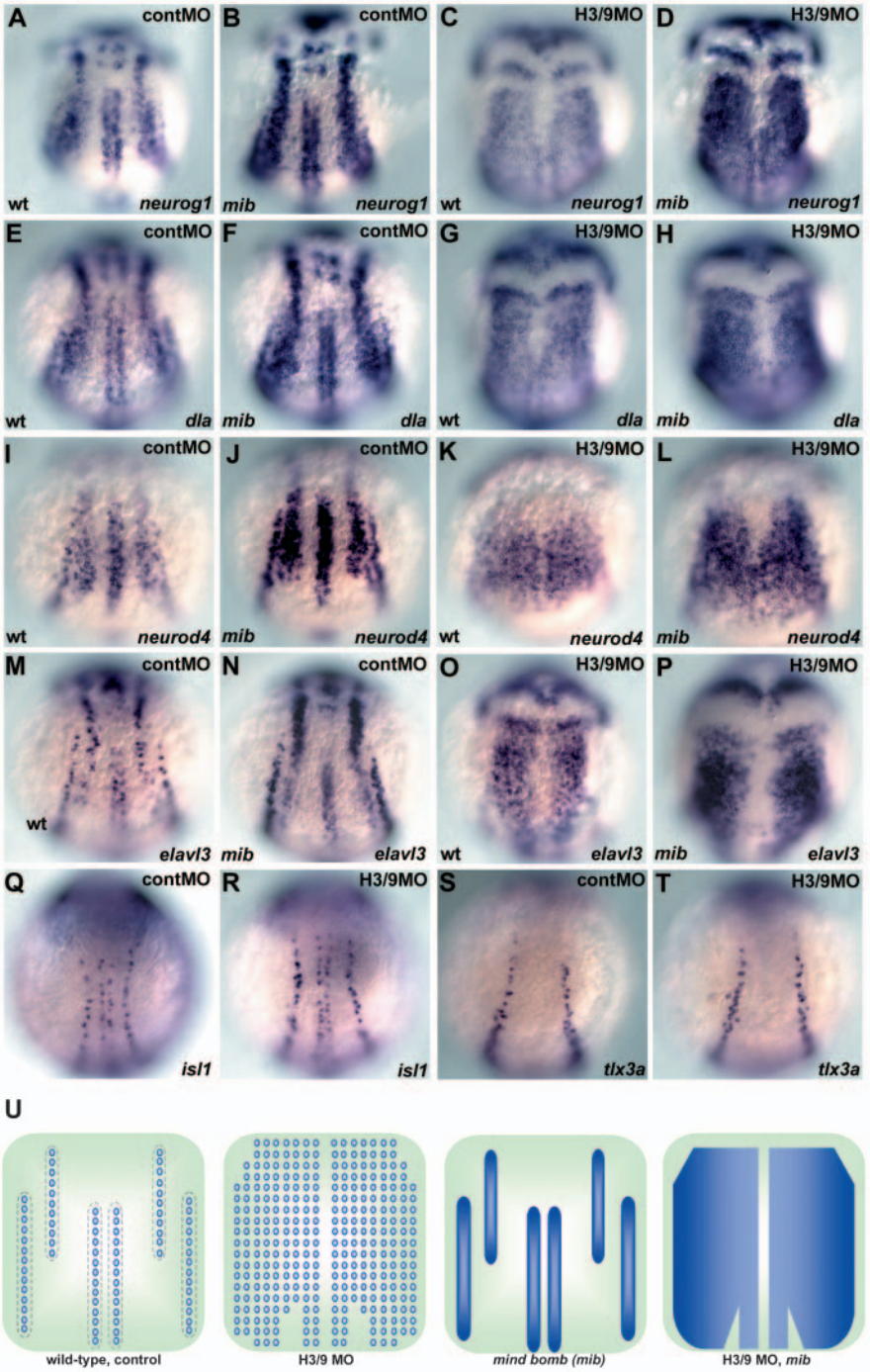

Fig. 6. Her3 and Her9 are required for formation of the inter-proneuronal domains. (A-P) Expression of *neurog1* (A-D), *deltaA* (*dla*; E-H), *neurod4* (I-L) and *elavl3* (M-P) in wild-type embryos that received 2 ng control MO (A,E,I,M) or 1 ng *her3*-MO1 and 2 ng *her9*ATG-MO (C,G,K,O), or in *mib* mutant embryos that received control MO (B,F,J,N) or *her3/her9*-MO (D,H,L,P) at the one-somite stage. Dorsal views. (Q-T) Expression of *islet1* (*isl1*, a marker for primary motoneurons and RB neurons) and *tlx3a* (a marker for RB neurons) in the control (Q,S) and the *her3/her9*-MO-injected embryos (R,T) at the three-somite stage. Expression of *islet1* and *tlx3a* was not affected in the *her3/her9* morphant embryos. (U) Schematic representation of the expression profiles of *neurog1*, *dla*, *neurod4* and *elavl3* in wild-type or *mib* embryos that received control or *her3/her9*-MO injections. The expression of these genes became ubiquitous in the neural plate, except for the midline region including the floor plate in the *her3/her9* morphant embryos, and became homogenous within the proneuronal domains in the *mib* mutant embryos. The *her3/her9* morphant *mib* mutant embryos showed ubiquitous and homogeneous expression of these genes in the neural plate, except for the midline region.

Discussion

her3 and *her9* expression are controlled by positional information

We demonstrated that there are two types of Her genes that are regulated by different mechanisms and exhibit distinct roles in neurogenesis in the zebrafish posterior neuroectoderm. *her2*, *her4*, *her12* and *hes5* are regulated by Notch signaling, whereas *her3* and *her9* are regulated by positional information (Figs 2, 3). There are several signaling molecules that are involved in the formation of the dorsoventral body axis and regulate the formation of neuroectoderm, including Bmps, Bmp antagonists and Fgfs. However, only the reduction of Bmp signaling affects the position and width of the proneuronal and inter-proneuronal domains (Barth et al., 1999; Nguyen et al., 2000). It is not clear how Bmp signaling regulates the periodic stripe expression of the proneural genes *her3* and *her9*. Turing's 'reaction diffusion' mechanism is proposed to be a good model to explain the formation of stripes, such as skin stripes (Kondo, 2002; Meinhardt, 2003). In this model, a certain chemical activator (such as a growth factor) induces a feedback inhibitor that can diffuse over a long distance, and the summation of the activator and the inhibitor generates a stripe of high activity of the activator. In the case of Bmp signaling, there are several feedback regulators reported, but none of them has been shown to form stripes of activity or to be involved in the stripe formation of the proneuronal domains, so far. Furthermore, periodic (striped) Bmp signaling activity (e.g. the phosphorylation of Smad1) has not been reported. Bmp signaling might regulate the position of the proneuronal and inter-proneuronal activity through the regulation of other signaling cascades. Alternatively, each stripe of *her3* and *her9* (and proneural gene) expression may be regulated by different mechanisms. A combination of dorsal and ventral signals might act on the distinct regulatory elements of the *her3* and *her9* genes, and generate the striped expression. Promoter analysis of *her3* and *her9* in transgenic zebrafish will be required to clarify the mechanism for their striped expression. *Drosophila hairy* also shows a periodic expression pattern in

the pupal leg (Orenic et al., 1993). It has been reported that *hairy* expression is regulated in part by Dpp, a *Drosophila* Bmp (Kwon et al., 2004). In this sense, there is some conservation between the regulation of *Drosophila hairy* and zebrafish *her3/her9* expression.

It has previously been reported that *her3* is negatively regulated by Notch signaling (Hans et al., 2004). We also observed the repression of *her3* expression by the expression of NotchICD or the misexpression of Neurog1. As the misexpression of *neurog1* upregulates the *hes5* expression (Fig. 4C), the suppression of *her3* is likely to be mediated through Delta-Notch signaling. However, the expression of *her3* was not affected in *mib* mutant, DAPT-treated or antimorphic Delta-expressing embryos, indicating that Notch signaling is not required for the endogenous expression of *her3*. The situation is similar to that of the expression of *her5,* which is proposed to function as a prepattern gene (described below) in the zebrafish mid-hindbrain boundary. *her5* expression is repressed by NotchICD expression but is not affected in Notch-defective embryos (Geling et al., 2004). It is possible that NotchICD activates a gene cascade(s) that is not activated under physiological conditions, and renders the neuroectoderm incompetent to express *her3*. In any case, the striped expression of *her3* and *her9* does not require Notch signaling.

her2, *her4*, *her12* and *hes5* are expressed in the proneuronal domains, where the proneuronal genes and *pnx* are also expressed (Fig. 1). The expression of these Her genes is absent or strongly reduced in Notch-defective embryos (Fig. 3, data not shown), indicating that they are strictly regulated by Notch signaling. This is in contrast to the homogenous expression of *neurog1*, *neurod4* and *deltaA* in the Notch-defective embryos (Fig. 6). As the proneural gene *neurogenin* activates the *delta* expression and subsequently activates Notch signaling in the neighboring cells (Takke et al., 1999), the cells expressing these Her genes are different from those expressing the proneural genes within the proneuronal domains, although their expression may overlap transiently during the lateral inhibition. All of these data indicate that the regulation of *her3* and *her9*, and that of the other Her genes, is different: the latter are controlled by Notch signaling and the former by positional information.

her3 and *her9* function as prepattern genes

In *Drosophila*, *hairy* and *enhancer of split* have different roles in neurogenesis. *E(spl)* functions downstream of Notch signaling and is involved in the lateral inhibition mechanism, whereas *hairy* does not function downstream of Notch signaling, but rather as a prepattern gene that acts in the interface between positional information and neurogenesis (Davis and Turner, 2001; Fisher and Caudy, 1998). *hairy* shows a periodic longitudinal expression pattern in the *Drosophila* leg and determines the position of sensory organs by repressing the proneural gene *acheate* (Orenic et al., 1993; Skeath and Carroll, 1991). Although the structure of the zebrafish neural plate is very different from that of the *Drosophila* leg, the role of Her3 and Her9 in the zebrafish neural plate is similar to that of Hairy in the *Drosophila* leg. We propose that Her3 and Her9 function as prepattern genes and control the position of the proneuronal and inter-proneuronal domains through a conserved mechanism, by which Hairy also controls the position of sensory organs in the *Drosophila* leg. Among the Her genes in vertebrates, only a few are reported to function in a similar way. Mouse *Hes1* is expressed in the olfactory placodal domains independent of Mash1 activity, thus *Hes1* expression is suggested to be Notch signal independent (Cau et al., 2000). Combined disruption of *Hes1* and the *Hes5* gene, whose expression is dependent on Mash1 and thus possibly controlled by Notch signaling, leads to a strong upregulation of *neurog1* expression in the olfactory epithelium (Cau et al., 2000). The situation is similar to the effect of the combined inhibition of Her3/Her9 and Notch signaling in the zebrafish posterior neuroectoderm (Fig. 6). Zebrafish *her5* and *him*, which are expressed in the MHB, function to repress neurogenesis in the MHB (Geling et al., 2004; Ninkovic et al., 2005). Although the mechanism that induces the expression of *her5* and *him* is not clear, the maintenance of *her5* and *him1* in the MHB involves Pax2.1, Eng2/3 and Fgf8, which provide positional information in the MHB (Geling et al., 2003; Geling et al., 2004; Ninkovic et al., 2005). In this sense, the function of *her3* and *her9* is similar to that of *her5* and *him*. Mouse *Hes1* and zebrafish *her5* function downstream of positional information related to the anteroposterior (AP) axis and control neurogenesis in the specific position of the AP axis, whereas *her3* and *her9* function as prepattern genes that control neurogenesis in the context of the dorsoventral (DV) axis in the neuroectoderm. Intriguingly, the inhibition of Her5 and Him function leads to ectopic *neurog1* expression in the MHB, while leaving a *neurog1*-negative domain in the lateral region of MHB (Ninkovic et al., 2005). *her3* is expressed in the lateral region of the MHB (Fig. 1) (Hans et al., 2004). It is possible that *her3* contributes to the repression of proneural genes in the lateral region of the MHB. *her3, her5* and *him* might redundantly function in this region, as the inhibition of Her3 function did not lead to ectopic *neurog1* expression in the MHB (Fig. 5). All of these data support the role of a subset of Her genes in the prepatterning that functions downstream of the positional information linked to the DV and AP axes.

We found a difference in the activity of Her3 and Her9 by misexpression studies (Fig. 5). Cells expressing exogenous *her3* contributed to the proneuronal domains and repressed the expression of *neurod4* in the proneuronal domains. When cells that expressed exogenous *her9* were located in the proneuronal domains, *neurod4* expression was repressed. However, the majority of exogenous *her9*-expressing cells was located in the inter-proneuronal domains and did not contribute to the proneuronal domains. These data suggest that Her9 may play an additional role other than the repression of neurogenesis (which it shares with Her3). Her9 may confer on the cell its ability to be localized to the inter-proneuronal domains. Her9 may be involved in the regulation of genes that encode cell-adhesion molecules, or repulsive or attractant molecules. This Her9-mediated activity to localize cells to the inter-proneuronal domains is attributable to the clear separation of the proneuronal and the inter-proneuronal domains.

In amniotes, there is no discernible primary neuron. However, some Hes/Her genes show a spatially restricted expression pattern in the spinal cord during a specific developmental period (Hatakeyama et al., 2004; Wu et al., 2003), and these Hes/Her genes may function in the spatial regulation of neurogenesis similar to *her3* and *her9*. As observed for mouse *Hes1* in the olfactory placode (Cau et al., 2000), a specific Her/Hes gene may be regulated by the

positional information early and controlled by Notch signaling later. Consistent with this idea, the expression of *her3* and *her9* also becomes dependent on Notch signaling at the later stages (data not shown). Notch-mediated Her9 function may be involved in later processes of neurogenesis, such as the reduction of *atoh1*-expressing neural precursor cells in the hindbrain region (Fig. 5L).

Role of the inter-proneuronal domains in neurogenesis

Although *neurog1*, *deltaA*, *neurod4* and *elavl3* were ectopically expressed in the inter-proneuronal domains in the *her3/her9* morphant embryos, we did not detect ectopic/aberrant expression of the genes that are expressed in primary motoneurons (*islet1* and *islet2*), primary interneurons (*lim1*), RB neurons (*islet1*, *islet2*, *tlx3a*) or of other interneuronal markers (*hlx1/2*, *eng1b*, *evx1*, *vsx1/2*, *sax2* and *pax2.1*) (Fig. 5, data not shown). There are two possible explanations for this result. First, the cells ectopically expressing *neurog1*, *deltaA*, *neurod4* and *elavl3* may not undergo differentiation to specific types of neurons at the early segmentation stage. Alternatively, these cells may become neurons that are not detected by the markers we used, or neurons that do not normally exist. The motor-, inter- and sensory-neuronal markers used in this study correspond to the chick and mouse genetic markers that cover the most of the neuronal types along the DV axis, in particular, mid-ventral interneurons, in the spinal cord. Therefore, it is more likely that these cells do not differentiate into specific types of neurons until the physiological timing of the differentiation. These data suggest that Her3 and Her9 are not involved in the specification of neurons. We also noticed that glial populations, which were marked by Gfap, Blbp (brain lipid-binding protein) and MBP, were not prominently affected in the *her3/her9* morphant embryos, whereas they were strongly reduced in the *mib* mutant embryos (data not shown). These data support the idea that Her3 and Her9 do not function in the specification of glia versus neurons. Considering these observations together, Her3 and Her9 are involved in the delay of the neuronal differentiation, and the inter-proneuronal domains, which require the function of Her3 and Her9, provide the field that maintains undifferentiated neural cells.

It is not yet clear what types of neurons and glia are generated from the inter-proneuronal domains. In Notch-defective embryos, the numbers of primary neurons are increased and the secondary neurons are strongly reduced (Appel et al., 2001). This is consistent with the finding that Notch-defective embryos show increased *elavl3*-expressing neuronal cells and a strong reduction of *elavl3*-negative cells within the proneuronal domains (Itoh et al., 2003) (Fig. 6). Thus, it is conceivable that both the primary and the secondary neurons are generated from the proneuronal domains, and the inter-proneuronal domains give rise to only the non-neuronal populations – glia. However, inhibition of the inter-proneuronal domains by *her3/her9*-MO did not strongly affect the glial population in the spinal cord (data not shown). Therefore, it is more likely that the inter-proneuronal domains generate the secondary neurons after the expression of *her3* and/or *her9* is reduced or terminated at later periods. Notch signaling could function in the inter-proneuronal domains in the absence of Her3 and Her9 function (Fig. 6). Therefore, Notch signaling may function in the specification of glia versus neurons in the inter-proneuronal domains at the later stages. Precise fate mapping of cells in the inter-proneuronal domains will be required to clarify the fates of the cells in the inter-proneuronal domains.

In summary, *her3* and *her9* function as prepattern genes, the expression of which is controlled by positional information linked to the dorsoventral polarity of the posterior neuroectoderm, and their expression spatially controls neuronal differentiation at the beginning of neurogenesis. The Her-mediated prepattern mechanism contributes to the establishment of the central nervous system through the spatially coordinated regulation of neurogenesis.

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