Histone deacetylase 1 is required to repress Notch target gene expression during zebrafish neurogenesis and to maintain the production of motoneurones in response to hedgehog signalling

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Summary

Histone deacetylases (Hdacs) are widely implicated as key components of transcriptional silencing mechanisms. Here, I show that *hdac1* is specifically required in the zebrafish embryonic CNS to maintain neurogenesis. In *hdac1* mutant embryos, the Notch-responsive *E(spl)*-related neurogenic gene *her6* is ectopically expressed at distinct sites within the developing CNS and proneural gene expression is correspondingly reduced or eliminated. Using an *hdac1*-specific morpholino, I show that this requirement for *hdac1* is epistatic to the requirement for Notch signalling. Consequently, *hdac1*-deficient embryos exhibit several defects of neuronal specification and patterning, including a dramatic deficit of hedgehog-dependent branchiomotor neurones that is refractory to elevated levels of hedgehog signalling. Thus, in the zebrafish embryo, *hdac1* is an essential component of the transcriptional silencing machinery that supports the formation and subsequent differentiation of neuronal precursors.

Key words: Histone deacetylase, Chromatin, Neurogenesis, Zebrafish

Introduction

Neurogenesis is regulated by intercellular interactions involving the transmembrane-spanning proteins Notch and Delta (reviewed by Campos-Ortega, 1993; Artavanis-Tsakonas et al., 1999). In vertebrate and invertebrate embryos, neuronal precursor cells are born within small proneural clusters as a result of Notch- and Delta-dependent lateral inhibitory interactions (reviewed by Chan and Jan, 1999). Delta-bound Notch protein is cleaved intracellularly and the intracellular fragment promotes transcription of genes encoding transcriptional repressors of the hairy/Enhancer of split [E(spl)] family. Within a Notch-expressing cell, E(spl) proteins repress genes required for the adoption of a neuronal fate, including components of the *achaete-scute* complex and other bHLH genes such as *neurogenin* and *neuroD*. Homologues of *Drosophila* neurogenic genes such as *notch*, *delta* and *hairy/E(spl)* have been identified in many model vertebrates. In the zebrafish, three *delta* homologues have been described (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998), and seven different *Hairy/E(spl)* (*her*) genes have been characterised to date (www.ensembl.org/Danio_rerio).

The adoption of a neuronal fate in vertebrate embryos involves the sequential activation of bHLH proneural genes (reviewed by Brunet and Ghysen, 1999; Chitnis, 1999). In the mouse, achaete-scute orthologues such as *Mash1* (*Ascl1* – Mouse Genome Informatics) are required early on in neural cells to enable them to acquire a neuronal precursor identity (Cau et al., 1997). Expression of the murine bHLH genes neurogenin (*Ngn1*) and *Neurod1* (previously known as neuroD) requires *Mash1* activity (Cau et al., 1997). In the zebrafish, both *ash1a* and *ngn1* are required for specification of epiphysial neurones (Cau and Wilson, 2002). *ngn1* is also required for the formation of Rohon-Beard sensory neurones in the spinal cord (Cornell and Eisen, 2002), and later on in development for specification of dorsal root sensory ganglia (Andermann et al., 2002).

In the mouse embryo, the *E(spl)* protein Hes1 represses multiple proneural genes. Targeted inactivation of *Hes1* causes upregulation of *Mash1* and *Ngn1*, leading to accelerated neurogenesis and a decrease in the number of later born neurones (Ishibashi et al., 1995). Conversely, ectopic expression of *Hes1* prevents neuronal differentiation (Ishibashi et al., 1994). The zebrafish orthologue of *Hes1*, *her6*, is expressed in the developing CNS (Pasini et al., 2001), suggesting that it may perform a function related to that of *Hes1*. Murine Hes5, in contrast to Hes1, is not required for repression of *Mash1* during early neurogenesis but instead functions synergistically with Hes1 at a later stage to repress *Ngn1* (Cau et al., 2000). The zebrafish orthologue of *Hes5*, *her4*, similarly represses *ngn1* in the neural plate (Takke et al., 1999). Taken together, these studies indicate that different *E(spl)* homologues perform distinct roles during vertebrate neurogenesis. Increased understanding of the distinct functions of *E(spl)* genes, together with a better appreciation of how they are regulated, promise to yield important new insights into the molecular mechanisms controlling neurogenesis.

Covalent chromatin modifications play key roles in regulating eukaryotic gene expression (reviewed by Strahl and Allis, 2000). The acetylation of core histones on N-terminal

lysines is a major determinant of the transcriptionally active state of many genes and chromatin-associated histone acetyltransferases (HATs) are known to perform essential functions in embryonic development. By contrast, the recruitment of histone deacetylase (HDAC) enzymes to specific genes presages their transcriptional silencing. The results of these and other studies suggest that chromatin modifying enzymes may be involved in the establishment and maintenance of cell memory during embryonic development (reviewed by Turner, 2002). Indeed, targeted deletion of murine *hdac1* reduces embryonic growth, leading to morphological abnormalities in the head and allantois (Lagger et al., 2002). However, few other insights into the roles of vertebrate HDACs in embryonic development have emerged to date. Here, I have exploited a mutation in zebrafish *hdac1* to investigate the function of this gene in the developing nervous system. The results reveal for the first time a primary, in vivo requirement for *hdac1* to maintain vertebrate neurogenesis and evidence is presented that this occurs via repression of Notch targets, including *her6*, the zebrafish orthologue of murine *Hes1*. Moreover, I demonstrate that expression of proneural genes and neuronal specification are severely impaired in distinct CNS territories of mutant embryos within which strong ectopic expression of *her6* is observed. Although the *hdac1* mutant hindbrain is segmented, the patterning of post-mitotic neurones and glia within each rhombomere is disorganized. In addition, *hdac1* mutants fail to maintain the responsiveness of hindbrain neural precursor cells to hedgehog signalling, which results in the specification of very few branchiomotor neurones. Taken together, these results reveal a surprisingly specific requirement for *hdac1* to maintain neurogenesis and enable neuronal fates to be realised in the zebrafish CNS.

Materials and methods

Zebrafish stocks

hdac1^{hi1618} mutant fish were raised from embryos kindly supplied by Dr S. Farrington and Professor N. Hopkins at MIT, USA. *mindbomb* mutant fish were provided by Dr T. Whitfield; *smo*^{hi1640} mutant and *Isl1-GFP* transgenic fish were provided by Prof. P. W. Ingham, from stocks maintained at the University of Sheffield.

Homozygous *hdac1*^{hi1618} mutants were distinguished from siblings at 22-24 hours post-fertilization (hpf) by their reduced anterior hindbrain development. Homozygous *smo*^{hi1640} mutants were distinguished from siblings at 24 hpf by their U-shaped somites. Homozygous *mib* mutants were distinguished from siblings by their abnormal trunk morphology.

hdac1 cDNA cloning

Full-length *hdac1* cDNA clones were amplified by RT-PCR using the forward primer 5′-ggc agg cgc agg ctg taa tt-3′ and the reverse primer 5′-atg cat cca gga gga ctg gc-3′, based on the *hdac1* cDNA sequence deposited in the EMBL database (Accession Number, AF506201).

Microinjection of morpholino antisense oligonucleotides and synthetic mRNA

A morpholino (MO; Gene Tools, LLC) was designed to block translation initiation of *hdac1* mRNA, using DNA sequence obtained from the EMBL database (Accession Number, AF506201) and verified by independent cDNA cloning: *hdac1*-MO: 5′-ttg ttc ctt gag aac tca gcg cca t-3′.

A second MO identical in sequence to *hdac1*-MO apart from four mismatching nucleotides (highlighted in bold), was used to control for non-specific effects of MO injection: Control-MO: 5′-ttg **c**tc **g**tt gag aac tc**t** gc**a** cca t-3′

MOs were microinjected into zebrafish embryos at the one- to two-cell stage in a volume of ~2 nl, at a final concentration of 0.3 mM in water. Neither the control MO nor an irrelevant sequence MO known from previous studies to be biologically inert (5′-cct ctt acc tca gtt aca att tat a-3′) exhibited any observable effects on embryonic development after microinjection.

To modulate the expression level of *shh* in vivo, in vitro-synthesised capped mRNA encoding zebrafish Shh was microinjected into embryos at the one- to two-cell stage, at a dose of 100 pg/embryo along with the appropriate dose of MO.

Histology and immunohistochemistry

For histological analysis, embryos were fixed in 4% paraformaldehyde, embedded in paraffin wax, then 8 μm sections were taken and stained with Haematoxylin and Eosin. Immunohistochemistry was performed using standard procedures (Schulte-Merker, 2003), which incorporated a 5 minute permeabilisation step with ice-cold trypsin for embryos older than 28 hpf (omitted for Isl1 staining). Antibodies were used at the following dilutions: anti-Isl1 monoclonal (39.4D5; Developmental Studies Hybridoma Bank, Iowa, USA), 1:500; anti-Phospho-H3 polyclonal (Upstate), 1:500; anti-Hu (BD Biosciences), 1:500; anti-GFAP (a kind gift of Dr J. Clarke, UCL) (Nona et al., 1989), 1:160. Primary antibody binding was visualized with peroxidase- or FITC-conjugated secondary antibodies.

RNA in situ hybridisation

Digoxigenin-labelled probes were prepared as recommended by the manufacturer (Roche). Whole-mount in situ hybridisation was performed using standard procedures (Oxtoby and Jowett, 1993). Details of the probes used are available on request.

Results

hdac1 is expressed widely throughout the embryo and required for CNS growth and morphogenesis

A recessive mutation of zebrafish *hdac1* was recently reported to cause embryonic lethality (Golling et al., 2002). However, few details of the defects caused by loss of *hdac1* function were described in that report, and so a systematic analysis of the *hdac1* mutant phenotype was initiated in order to gain insights into the requirements for *hdac1* function during embryonic development. The expression pattern of *hdac1* during embryonic development was first documented using probes prepared from a full-length *hdac1* cDNA clone. Fig. 1 shows that *hdac1* is maternally expressed, with transcripts being detectable at all developmental stages analysed and in all regions of the embryo. Particularly strong zygotic *hdac1* expression is evident in the anterior CNS of embryos at the 18-somite stage and 24 hpf, suggesting that there might be a specific requirement for *hdac1* function in the developing brain. As development continues, *hdac1* transcripts selectively accumulate throughout the brain and spinal cord (Fig. 1).

Until 22-24 hpf, embryos homozygous for the *hdac1* mutant allele *hdac1*^{hi1618} are morphologically indistinguishable from unaffected siblings. However, at this time, specific abnormalities of hindbrain development first become apparent. Although the correct number of rhombomeres are formed, the anterior rhombomeres fail to undergo the characteristic mediolateral expansion typical of the hindbrain in sibling embryos. Subsequently, ventricular expansion in the midbrain also fails. By 48 hpf, homozygous mutant embryos adopt a

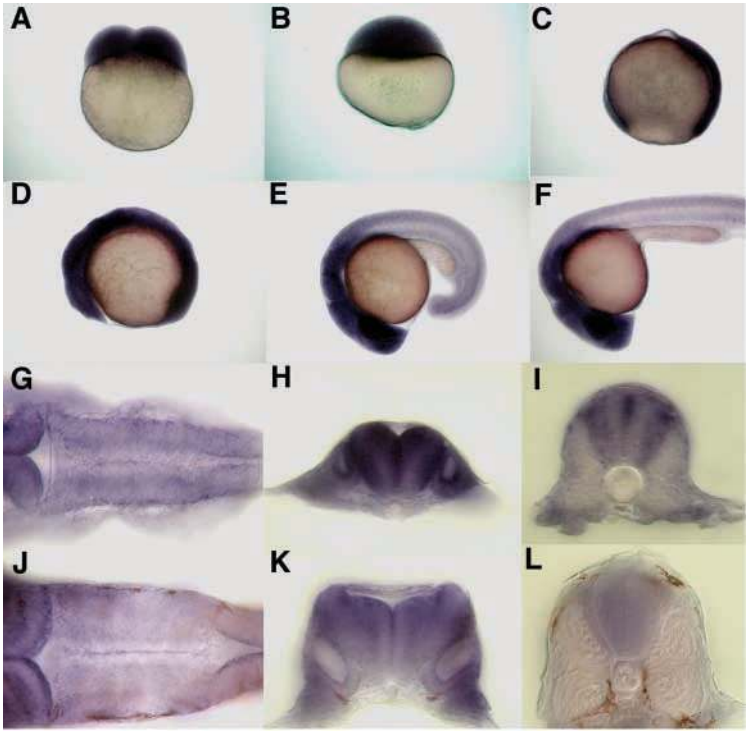


Fig. 1. Expression of *hdac1* is widespread in the embryo at all stages of development and most abundant in the anterior CNS. Whole-mount in situ hybridisation of an *hdac1* cDNA probe to zebrafish embryos at the (A) two-cell, (B) 1000-cell, (C) 80% epiboly, (D) 6-somite, (E) 18-somite and (F) 24 hpf stages. (G,J) Dorsal views of the hindbrain at 24 (G) and 48 (J) hpf. (H,K) Transverse sections through the hindbrain at the level of r3/r4, at 24 (H) and 48 (K) hpf. (I,L) Transverse sections through the anterior spinal cord at 24 (I) and 48 (L) hpf. Strongest expression of *hdac1* is observed in the brain and spinal cord; by contrast, notochord and somites express much lower levels of *hdac1* transcripts.

curled-down shape and their CNS is significantly smaller than that of unaffected siblings (Fig. 2A,B). Several additional morphological abnormalities are also apparent, including the absence of pectoral fins, the presence of a weakly beating heart lacking an atrio-ventricular valve, and the absence of epithelial projections forming the semi-circular canals in the developing otic vesicle (Fig. 2C-H). Histological analysis confirmed that the *hdac1* mutant brain is correctly subdivided into forebrain, midbrain and hindbrain, but all of these structures, as well as the spinal cord, are smaller than in unaffected sibling embryos (Fig. 2C,D). Transverse sections through the hindbrain and spinal cord also revealed abnormalities in their dorsoventral patterning (Fig. 2E-H). To determine whether the reduced dimensions of the hindbrain reflects reduced proliferation of neural cells, phospho-H3-positive mitotic cells were identified and counted in the hindbrain of *hdac1*^{hi1618} homozygous mutant and sibling embryos (Saka and Smith, 2001) over a period from 25 to 38 hpf (Fig. 3; Table 1). At 25 hpf, cell proliferation in the *hdac1*^{hi1618} mutant hindbrain is significantly lower than that in unaffected siblings. However, by 33 hpf, the mitotic indices of *hdac1*^{hi1618} mutants and siblings are similar and they remain so at 38 hpf (Fig. 3; Table 1). Thus, although there is a transient proliferation deficit in the *hdac1*^{hi1618} mutant hindbrain at 25 hpf, the normal proliferation rate of neural precursor cells is subsequently regained and maintained independently of *hdac1* function.

Mutation of *hdac1* does not affect regional specification of the neuroepithelium but neurogenesis is highly dependent on zygotic *hdac1* function

To explore the possibility that the morphological abnormalities in the CNS of *hdac1*^{hi1618} mutants are caused by primary patterning abnormalities within the neuroepithelium, the expression patterns of genes involved in neural patterning, such as *pax2a*, *epha4*, *hoxb1*, *hoxb4* and *fgf8,* were investigated. As exemplified by *pax2a* and *epha4* in Fig. 4, expression of all of these genes in the CNS is essentially unperturbed by loss of *hdac1* function, indicating that the mechanisms responsible for regional specification of the neuraxis do not require zygotic *hdac1* function (Fig. 4A-D).

To investigate the possibility that the *hdac1*^{hi1618} mutation affects neurogenesis, the expression patterns of the proneural genes *ash1b* (Allende and Weinberg, 1994) and *ngn1* (Blader et al., 1997) were compared in *hdac1*^{hi1618} mutants and siblings. At 25 hpf, expression of *ash1b* (*ashb* – Zebrafish Information Network) and *ngn1* is strikingly reduced throughout the CNS of *hdac1*^{hi1618} mutants (Fig. 4E-H), demonstrating that neurogenesis is significantly compromised by loss of *hdac1* function.

A Notch-activated repressor of proneural gene expression, *her6*, is derepressed in *hdac1* mutants

In view of the evidence that *hdac1* functions primarily in the transcriptional repression of target genes (reviewed by Ng and Bird, 2000), it seemed reasonable to postulate that the downregulation of proneural gene expression observed in *hdac1*^{hi1618} mutants could be an indirect consequence of de-repressing other genes that are themselves direct targets of hdac1-mediated repression. Proneural genes are well-characterised targets of Notch-dependent transcriptional repression by members of the hairy/E(spl) family of bHLH proteins (reviewed by Campos-Ortega, 1993; Chitnis, 1999). In the mammalian CNS, the Notch target gene *Hes1* is required during neurogenesis to co-ordinately repress transcription of both *Mash1* and *Ngn1* (Cau et al., 2000). This raised the possibility that its zebrafish orthologue, *her6* (Pasini et al., 2001), might be aberrantly expressed in *hdac1*^{hi1618} mutant embryos. At 26 hpf, *her6* is weakly expressed in the lateral hindbrain of wild-type embryos, but by 33 hpf, transcripts have accumulated in two distinct lateral stripes running caudally from the rhombic lip (Fig. 5A,C). In 26 hpf *hdac1*^{hi1618} mutant embryos, *her6* is expressed in the medial hindbrain, being particularly abundant in rhombomeres 5 and 6, and this aberrant pattern persists at 33 hpf (Fig. 5B,D). In wild-type embryos, the proneural genes *ash1b* and *ngn1* exhibit distinct, partially overlapping expression patterns in the hindbrain (Fig. 5G,I,M,O). However, in the *hdac1* mutant hindbrain, the abundance of *ash1b* and *ngn1* transcripts is dramatically reduced both at 26 hpf and 33 hpf (Fig. 5H,J,N,P). Transverse

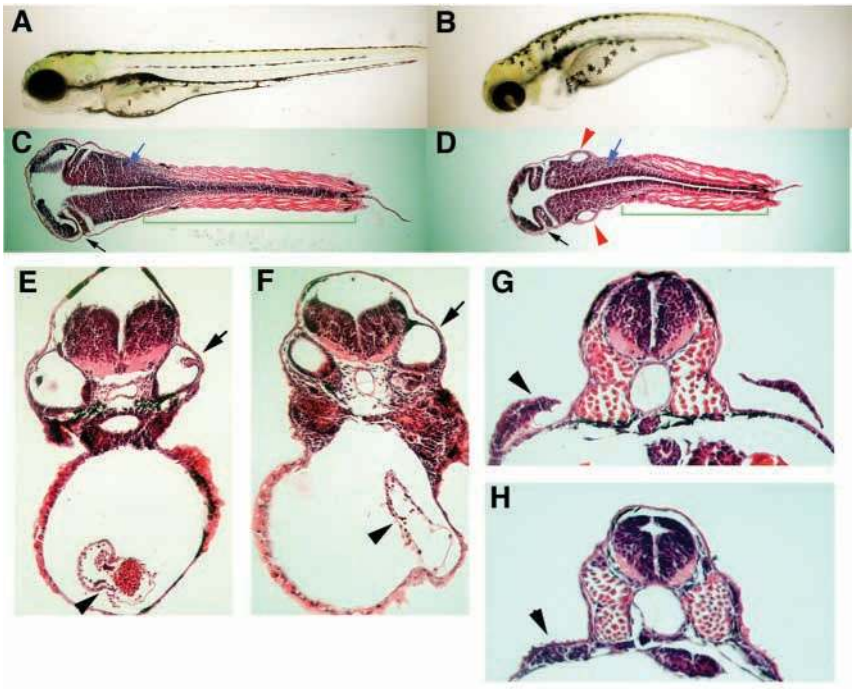


Fig. 2. Mutation of *hdac1* causes multiple developmental defects in the zebrafish embryo. (A,B) Bright-field images of live, 4-day-old *hdac1*^{hi1618} (A) sibling and (B) mutant embryos. Reduced brain size, absent jaw, curled down tail and pericardial oedema are clearly visible in the mutant. (C,D) Longitudinal sections of 3-day-old *hdac1*^{hi1618} (C) sibling and (D) mutant embryos taken at equivalent dorsoventral levels. Anterior is towards the left. Note the reduced size of the midbrain and anterior hindbrain and the dorsally open spinal cord in the mutant embryo. Position of midbrain-hindbrain boundary is marked with a black arrow. The hindbrain is indicated with a blue arrow and the row of somites (staining bright red) on the left side of each embryo is bracketed. Otic vesicles (lying below the plane of section in the sibling embryo) are marked with red arrowheads in D. (E,F) Transverse sections of 3-day-old *hdac1*^{hi1618} (E) sibling and (F) mutant embryos taken at equivalent anteroposterior levels passing through the otic vesicles and the heart. Note the absence of a heart valve (arrowhead) and semi-circular canal projections (arrow), and abnormal hindbrain shape in the mutant. (G,H) Transverse sections of 3-day-old *hdac1*^{hi1618} (G) sibling and (H) mutant embryos taken at equivalent anteroposterior levels passing through the pectoral fins. Note the absence of pectoral fin bud outgrowth (arrowhead) in the mutant.

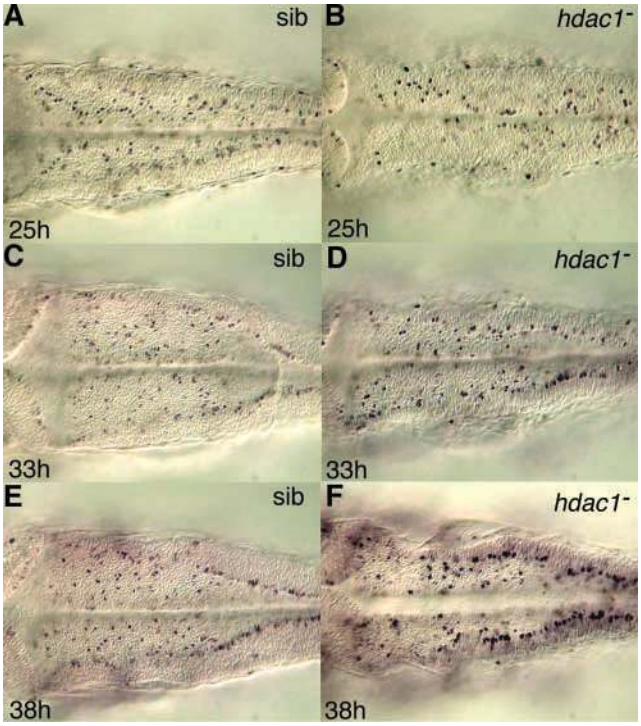


Fig. 3. Analysis of cell proliferation in the hindbrain of *hdac1*^{hi1618} (A,C,E) sibling and (B,D,F) mutant embryos between 25 and 38 hpf. Dorsal views of hindbrains from embryos at (A,B) 25, (C,D) 33 and (E,F) 38 hpf immunostained for the mitosis marker phospho-H3. Anterior is towards the left in all panels. At 25 hpf there are substantially fewer mitotic cells in the hindbrain of *hdac1*^{hi1618} mutants than in the hindbrain of sibling embryos. By 33 hpf, the numbers of mitotic cells in the hindbrain of mutant and sibling embryos are similar and they remain so at 38 hpf. For quantitation of hindbrain mitotic indices, see Table 1.

Table 1. Quantification of cell proliferation in the hindbrain of *hdac1*^{hi1618} mutant and unaffected sibling embryos at 25, 33 and 38 hpf

	Age		
	25 hpf	33 hpf	38 hpf
hdac1^{hi1618} siblings	285±11.5	306±2.9	300±17.8
hdac1^{hi1618} mutants	176±14.6	320±14.3	288±8.6

The total number of darkly stained phospho-H3-positive nuclei in the region of the hindbrain spanning rhombomeres 2-7 was counted under the compound microscope. Values given are mean cell counts (±s.e.m.) where *n*=4 individual embryos in each sample group (see Fig. 3 for examples).

sections through rhombomere 5 further reveal the complementary nature of the *her6* expression domain compared with those of *ash1b* and *ngn1*, in both *hdac1*^{hi1618} mutant and unaffected sibling embryos (Fig. 5). Thus, loss of *hdac1* function causes an increase in *her6* transcript levels and suppresses expression of *ash1b* and *ngn1* (Fig. 5E,F). In the dorsal diencephalon of wild-type embryos, the expression domain of *her6* is also complementary to those of *ash1b* and *ngn1* (Fig. 6), both at 26 hpf and at 33 hpf Moreover, the *her6* expression domain is expanded in the dorsal diencephalon of *hdac1*^{hi1618} mutants, whereas those of *ash1b* and *ngn1* are correspondingly reduced or eliminated, at 26 and 33 hpf (Fig. 6). Taken together, these results demonstrate that *hdac1* is required both to repress *her6* and to promote expression of *ash1b* and *ngn1* during the development of the hindbrain and dorsal diencephalon.

Mutation of *hdac1* causes defective production and patterning of hindbrain neurones and glia

In order to investigate the degree to which loss of *hdac1* function affected production of differentiated neurones in the

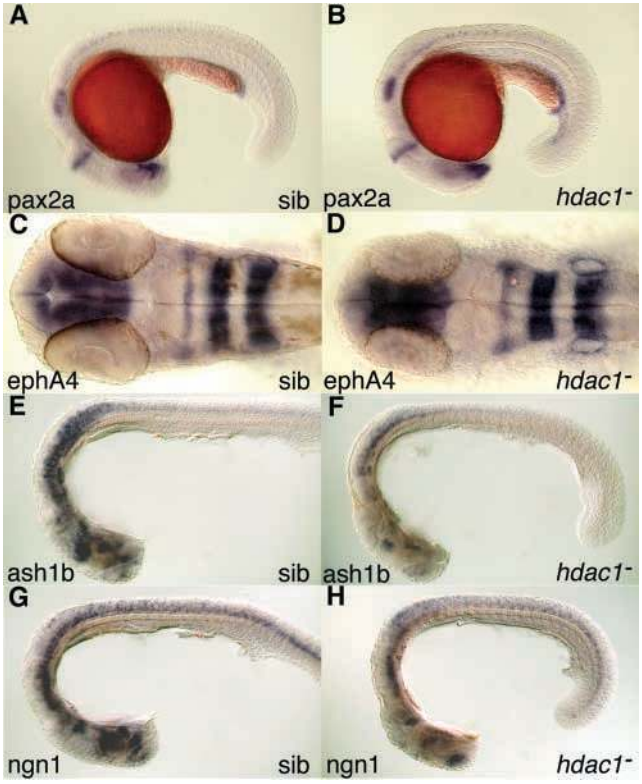


Fig. 4. Mutation of *hdac1* does not perturb primary neural patterning but neurogenesis is severely impaired. In situ hybridisation for expression of CNS patterning markers and proneural genes in *hdac1*^{hi1618} (A,C,E,G) sibling and (B,D,F,H) mutant embryos. (A,B) *pax2a* at 24 hpf marks the optic chiasm, midbrain-hindbrain boundary, otic vesicle and scattered spinal interneurones; (C,D) *epha4* at 32 hpf marks the forebrain and hindbrain rhombomeres 1, 3 and 5; (E,F) *ash1b* and (G,H) *ngn1* at 25 hpf mark neuronal precursors in brain and spinal cord. Expression of *pax2a* and *epha4* in the embryonic CNS is unperturbed by the *hdac1*^{hi1618} mutation, whereas expression of the proneural genes *ash1b* and *ngn1* is substantially reduced in *hdac1*^{hi1618} mutant embryos.

hindbrain, the distribution of cells expressing the pan-neuronal Hu proteins was compared in *hdac1*^{hi1618} mutant and sibling embryos, at 25, 34 and 38 hpf (Fig. 7). Within each rhombomere of the wild-type zebrafish hindbrain, Hu-positive post-mitotic neurones are arranged in a stereotyped segmental pattern that comprises a central cluster and an outer border separated by a layer of glial cells (Trevarrow et al., 1990) (Fig. 7). Strikingly, in the *hdac1*^{hi1618} mutant hindbrain, Hu-expressing cells fail to adopt the characteristic segmental arrangement in each rhombomere, and instead they assemble into an unsegmented column on each side of the hindbrain (Fig. 7A-F). Between 25 and 38 hpf, the number of Hu-positive cells within the *hdac1*^{hi1618} mutant hindbrain progressively increases, although in comparison to the hindbrain of sibling embryos, there are consistently fewer neurones. As the *hdac1*^{hi1618} mutant hindbrain develops, most of the newly born Hu-positive neurones accumulate in rhombomeres 2, 3 and 4, whereas relatively few are found in rhombomeres 5 and 6 (Fig. 7E,F), in stark contrast to the situation in wild-type embryos (Fig. 7E,F). Glia are also specified in the *hdac1* mutant hindbrain but they, like the Hu-expressing neurones, also fail to become organised into segmental arrays, and are distributed throughout the hindbrain in predominantly lateral locations, apparently intermingled with Hu-expressing neurones (Fig. 7G,H). Taken together, these results demonstrate that in the hindbrain *hdac1* is specifically required for the production and segmental patterning of both neurones and glia.

Multiple defects of neuronal specification in *hdac1*-deficient embryos

In order to characterise the extent to which the *hdac1*^{hi1618} mutation affected the specification of neuronal sub-types within the CNS, the development of Isl1-expressing epiphysial, branchiomotor and spinal cord neurones was compared in *hdac1*^{hi1618} mutant and unaffected sibling embryos (Korzh et al., 1993). Epiphysial neurones express the Isl1 transcription factor once they have been specified within the epithalamic region of the dorsal diencephalon (Masai et al., 1997). Branchiomotor and spinal motoneurones are induced by hedgehog signalling in the hindbrain and spinal cord, respectively, and they also express Isl1 protein in response to such signals (Chandrasekhar et al., 1997). Intriguingly, homozygosity for the *hdac1*^{hi1618} mutation eliminates Isl1 expression in the anterior half of the epiphysis (Fig. 8A,B), and prevents specification of almost all branchiomotor neuronal precursors except for two small clusters of trigeminal (nV) neurones in rhombomere 2 and two small groups of facial (nVII) precursors located in rhombomere 4 (Fig. 8C,D). To determine whether the Isl1-positive cells that were specified in the hindbrain of *hdac1*-deficient embryos were able to differentiate fully into motoneurones with axons, a morpholino targeted against the translation start site of *hdac1* mRNA was microinjected into embryos specifically expressing an Isl1-green fluorescent protein (GFP) transgene in branchiomotor neurones (Higashijima et al., 2000). At 30 hpf, *hdac1*-MO-injected embryos exhibited a pattern of four small clusters of Isl1-positive branchiomotor neurones identical to that observed in *hdac1*^{hi1618} mutants, two of which (nV neurones) were located in rhombomere 2, and two of which (nVII neurones) were located in rhombomere 4 (compare Fig. 8C,D with 8E-H; and see Table 2). Moreover, whereas the population of GFP-positive motoneurones in control-MO injected embryos was considerably increased in size between 30 hpf and 36 hpf (Fig. 8E,F), the rudimentary pattern of four small clusters of GFP-positive branchiomotor neurones remained unchanged in *hdac1*-MO-injected embryos (Fig. 8G,H). No GFP-positive cells were ever observed in the caudal part of the hindbrain that normally gives rise to nIX and nX motoneurones. Nevertheless, the GFP-positive motoneurones that were present in *hdac1*-MO-injected embryos did appear to differentiate normally and axon outgrowth could be clearly observed (Fig. 8G,H). These results demonstrate that relatively few branchiomotor neurones are specified in *hdac1*-deficient embryos, which remain spatially restricted to rhombomeres located anterior to rhombomere 5, and they do not undergo tangential migration posteriorly. Remarkably, however, these cells differentiate into motoneurones with axons that project anteriorly.

In the developing spinal cord of *hdac1*^{hi1618} mutant embryos, the number of Isl1-positive motoneurones is considerably reduced in comparison with that observed in unaffected siblings (Fig. 8I,J). Similarly, *hdac1*-MO-injected

embryos exhibited a substantially reduced population of spinal motoneurones in comparison with control MO-injected embryos (Fig. 8K,L). To a lesser degree, the population of Rohon-Beard sensory neurones is also reduced in the dorsal spinal cord of *hdac1* mutants and morphants (Fig. 8I,L). Thus, loss of *hdac1* function profoundly impairs neuronal specification in the epiphysis, hindbrain and spinal cord, which is consistent with the observed widespread reduction of proneural gene expression and ectopic expression of the Notch target gene *her6* in the CNS of *hdac1* mutants.

her6 is derepressed, proneural gene expression is extinguished, and neuronal specification is impaired in *hdac1*-deficient embryos, independently of Notch signalling

Notch signalling is essential for proper transcriptional activation of many *E(spl)* genes during neurogenesis (for a review, see Chitnis, 1999). In vitro studies indicate that Notch-mediated transcriptional activation of *E(spl)* genes is antagonised by a protein complex that contains Hdac1 (Kao et al., 1998). However, the in vivo requirements for *hdac1* functions in repression of *E(spl)* genes, particularly in relation to the effects of Notch signalling, have not been defined. The *mind bomb* (*mib*) mutation profoundly impairs Notch signalling such that extensive, premature neuronal differentiation occurs throughout the embryonic CNS (Itoh et al., 2003). To test the hypothesis that *hdac1* function is required for repression of *her6* in the developing CNS, and that Notch signalling is required to relieve this repression, *her6* expression was analysed in the *mib* mutant under conditions where levels of hdac1 activity were either unperturbed or reduced by *hdac1*-MO microinjection. Homozygosity for the *mib* mutation significantly reduces the abundance of *her6* transcripts both in the dorsal diencephalon and the hindbrain (Fig. 9), confirming that notch signalling is essential for proper expression of *her6*. In stark contrast, microinjection of the *hdac1*-MO into either *mib* mutant or sibling embryos caused a dramatic derepression of *her6* both in the dorsal diencephalon and in hindbrain rhombomeres 5 and 6 (Fig. 9, arrows; Table 3). These results demonstrate that hdac1 does indeed act as a repressor of *her6* in the hindbrain and dorsal diencephalon, and also that the repressive effect of hdac1 on *her6* is normally alleviated by Notch signalling. In further confirmation of these findings, expression of the proneural gene *ngn1* in the CNS was strictly dependent on wild-type levels of *hdac1* activity, irrespective of whether *hdac1*-deficient embryos were homozygous for the *mib* mutation or not (Fig. 9I,L). Finally, immunostaining for Isl1 protein revealed that loss of *hdac1* function severely impaired neuronal specification in both the epiphysis and the hindbrain in a *mib*-independent manner (Fig. 9M-T; Table 3). Reduced levels of hdac1 eliminated Isl1 expression in the anterior epiphysis, both in *mib* siblings and mutants (Fig. 9M-P). The hindbrain of *hdac1* morphants developed with two pairs of Isl1-positive cell clusters in r2 and

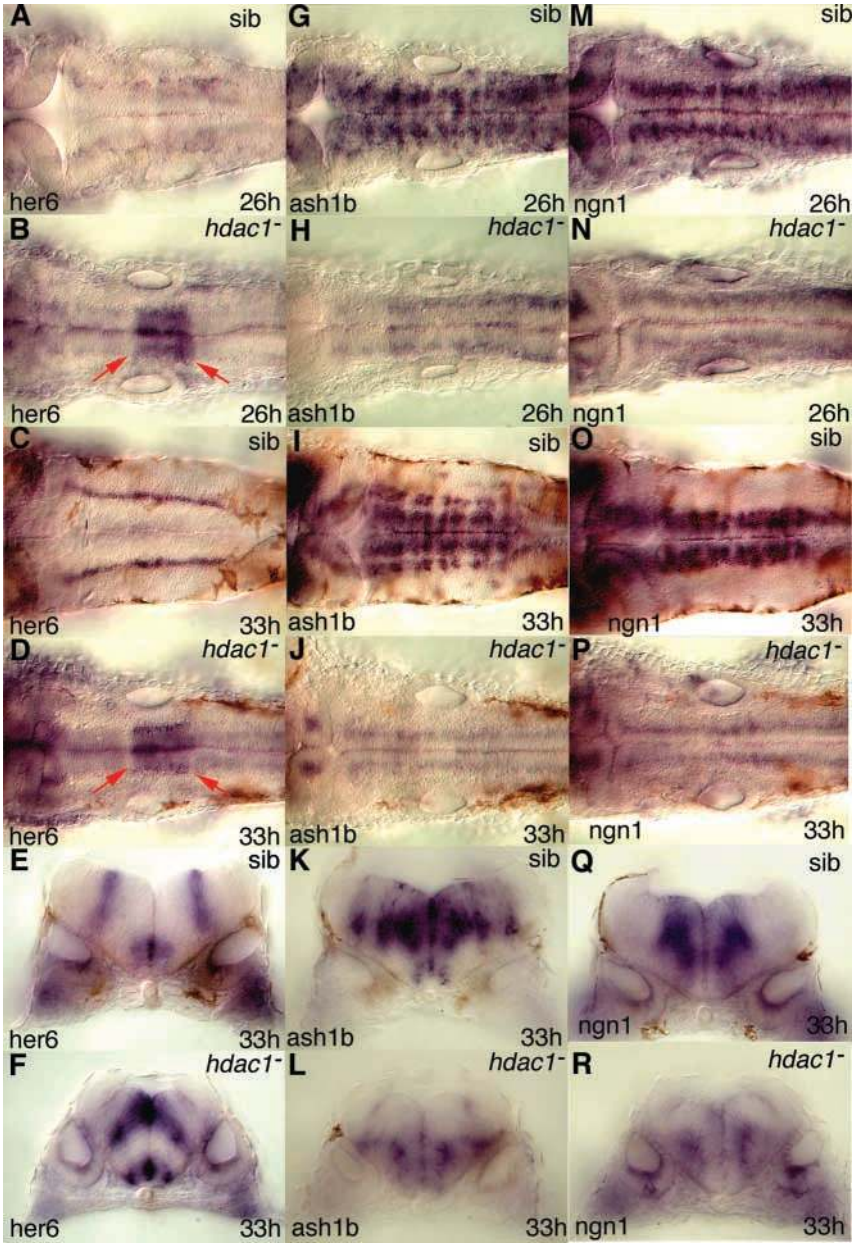


Fig. 5. In situ hybridisation analysis comparing the expression domains of *her6*, *ash1b* and *ngn1* in the hindbrain of *hdac1*^{hi1618} sibling and mutant embryos. *her6* is aberrantly expressed in the medial hindbrain of *hdac1*^{hi1618} mutants at 26 and 33 hpf, and most strongly in rhombomeres 5 and 6 (arrows). By contrast, expression of *ash1b* and *ngn1* in the *hdac1*^{hi1618} mutant hindbrain is almost completely extinguished. (A-F) *her6*; (G-L) *ash1b*; (M-R) *ngn1* expression patterns. (A,B,G,H,M,N) Dorsal views of hindbrain, 26 hpf; anterior is towards the left. (C,D,I,J,O,P) Dorsal views of hindbrain, 33 hpf; anterior is towards the left. (E,F,K,L,Q,R) Transverse sections through rhombomere 5 of hindbrain, 33 hpf; dorsal is uppermost.

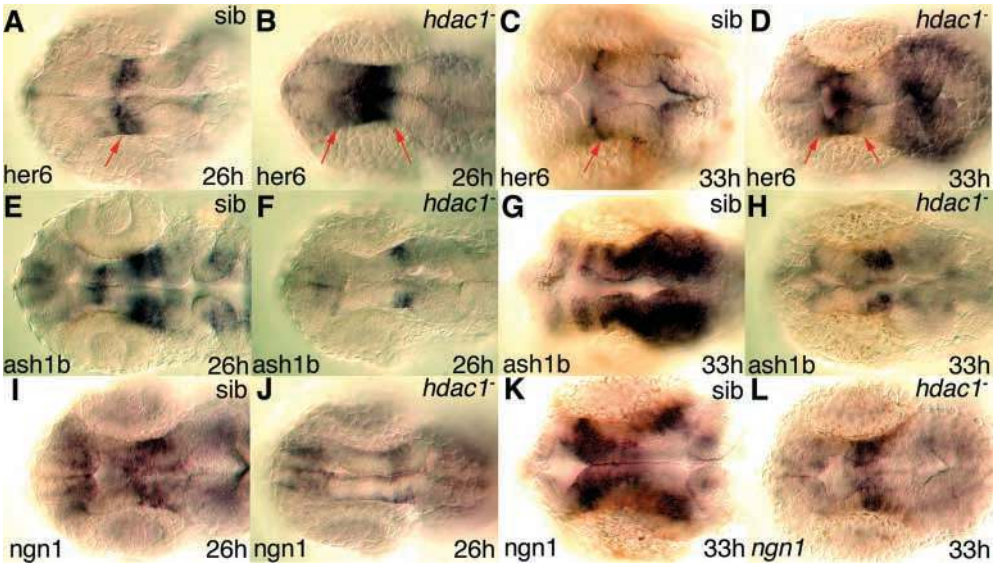
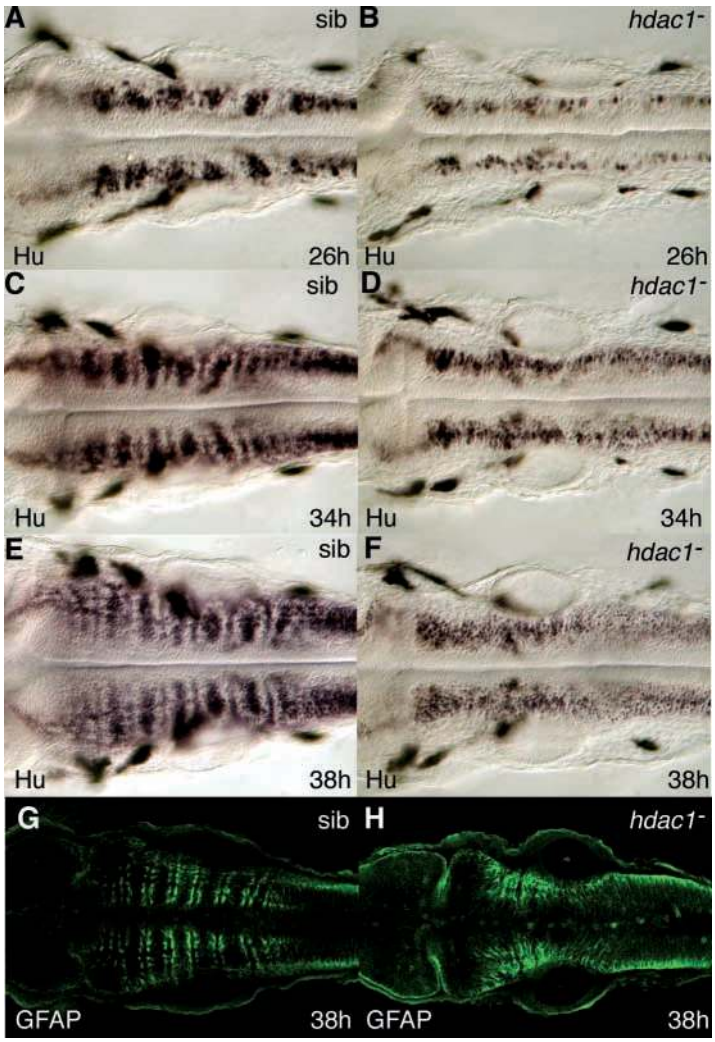


Fig. 6. In situ hybridisation analysis comparing the expression domains of *her6*, *ash1b* and *ngn1* in the dorsal diencephalon of *hdac1*^{hi1618} sibling and mutant embryos at 26 and 33 hpf. Loss of *hdac1* function causes a stable expansion of the *her6* expression domain in the dorsal diencephalon (arrows), as well as reductions in the diencephalic expression domains of *ash1b* and *ngn1*. (A-D) *her6*; (E-H) *ash1b*; (I-L) *ngn1* expression patterns. In all panels, views are dorsal and anterior is towards the left.

r4 and none posterior to r4, as was observed in *hdac1*^{hi1618} mutants (Fig. 9S). By contrast, supernumerary Isl1-positive cells were found throughout the hindbrain of *mib* mutants (Fig. 9R). However, microinjection of the *hdac1*-MO into *mib* mutant embryos suppressed this widespread, ectopic neurogenesis and instead restricted the specification of Isl1-positive cells to the two pairs of cell clusters in rhombomeres 2 and 4 that are characteristic of *hdac1*^{hi1618} mutants (Fig. 9T).



hdac1 maintains the response of motoneurone precursors to hedgehog signalling

The results described above demonstrate that *hdac1* is required to repress Notch targets in order to promote proneural gene expression and to specify several different neuronal cell types, including motoneurones. However, formation of motoneurones in the zebrafish also requires ventral midline-derived hedgehog signals (Chen et al., 2001; Varga et al., 2001; Lewis and Eisen, 2001). It was therefore of interest to investigate the relationship between hedgehog signalling and the requirement for *hdac1* function in motoneurone specification. In situ hybridisation analysis for expression of *shh* and the shh target gene *ptc1* revealed that *hdac1* does not play a major role in determining the expression patterns of these genes in motoneurone-forming regions because *shh* is expressed in ventral midline cells in both *hdac1*^{hi1618} mutant and sibling embryos, and expression of the shh target *ptc1* is relatively unperturbed both in the CNS and paraxial mesoderm (Fig. 10A-D). A localised reduction in expression of both *shh* and *ptc1* is found more anteriorly, however, in the zona limitans intrathalamica of the forebrain (zli, Fig. 10A-D). In wild-

Fig. 7. Defective production and patterning of post-mitotic neurones and glia in the hindbrain of *hdac1*^{hi1618} mutant embryos. Immunohistochemistry for Hu-expressing post-mitotic neurones (A-F) and GFAP-positive glia (G,H) in the hindbrain of *hdac1*^{hi1618} sibling and mutant embryos at (A,B) 26, (C,D) 34 and (E-H) 38 hpf. Although the efficiency of neurone formation is reduced there is a progressive increase in the number of Hu-positive neurones in the hindbrain of *hdac1*^{hi1618} mutants between 26 and 38 hpf. However, these neurones are arranged in continuous longitudinal tracts extending through the hindbrain and a segmented arrangement is not adopted. Glia also fail to adopt their characteristic arrangement in each rhombomere and instead accumulate aberrantly in the anterior hindbrain.

type embryos, the level of *shh* expression is strictly limiting for the number of branchiomotor neurones that are specified because over-expression of *shh* induces supernumerary branchiomotor neurones (Chandrasekhar et al., 1998). Although specification of the majority of branchiomotor neurones requires *hdac1*, small clusters of *hdac1*-independent motoneurones do form in rhombomeres 2 and 4 of *hdac1*-deficient embryos. Therefore, to determine whether *shh* overexpression could expand the population of branchiomotor neurones formed in *hdac1*-deficient embryos, *shh* mRNA was microinjected into Isl1-GFP transgenic embryos with either a *hdac1*-MO or a control MO. Microinjection of *shh* mRNA along with the control MO induced many supernumerary branchiomotor neurones in the hindbrain of Isl1-GFP embryos. However, no supernumerary branchiomotor neurones were formed when the *hdac1*-MO was co-injected with *shh* mRNA and instead only the rudimentary pattern of branchiomotor neurones that is characteristic of *hdac1*^{hi1618} mutants was observed (Fig. 10; Table 4). Thus, although the hedgehog pathway is active in *hdac1*-deficient embryos (Fig. 10A-D) and there are many mitotically active cells in the hindbrain (Fig. 3; Table 1), very few Isl1-expressing motoneurones are produced in response to hedgehog signals (Fig. 10E-L). To confirm that the branchiomotor neurones that did form in *hdac1*-deficient embryos were nevertheless dependent on hedgehog signalling, the effect of knocking down *hdac1* function in *smoothened* mutants (Chen et al., 2001; Varga et al., 2001) was analysed. At 26 hpf, the first cells to detectably express Isl1 protein in the hindbrain of control-MO-injected, unaffected sibling embryos were a small group of rostrally located trigeminal (nV) motoneurones in rhombomere 2 (Fig. 11A). Although loss of *smoothened* function completely abolishes the specification of these first-detected branchiomotor neurones (Fig. 11C), loss of *hdac1* function had no observable effect on their formation (Fig. 11B). By 32 hpf, control-MO-injected, unaffected siblings had developed a properly expanded and appropriately positioned set of branchiomotor neurones (Fig. 11E), whereas once more, no Isl1-positive cells were detectable in the hindbrain of *smoothened* mutants (Fig. 11G). As observed previously, at 32 hpf *hdac1*-deficient embryos developed only the two further small clusters of facial (nVII) motoneurones in r4 in addition to the trigeminal (nV) population first detected in r2 at 24

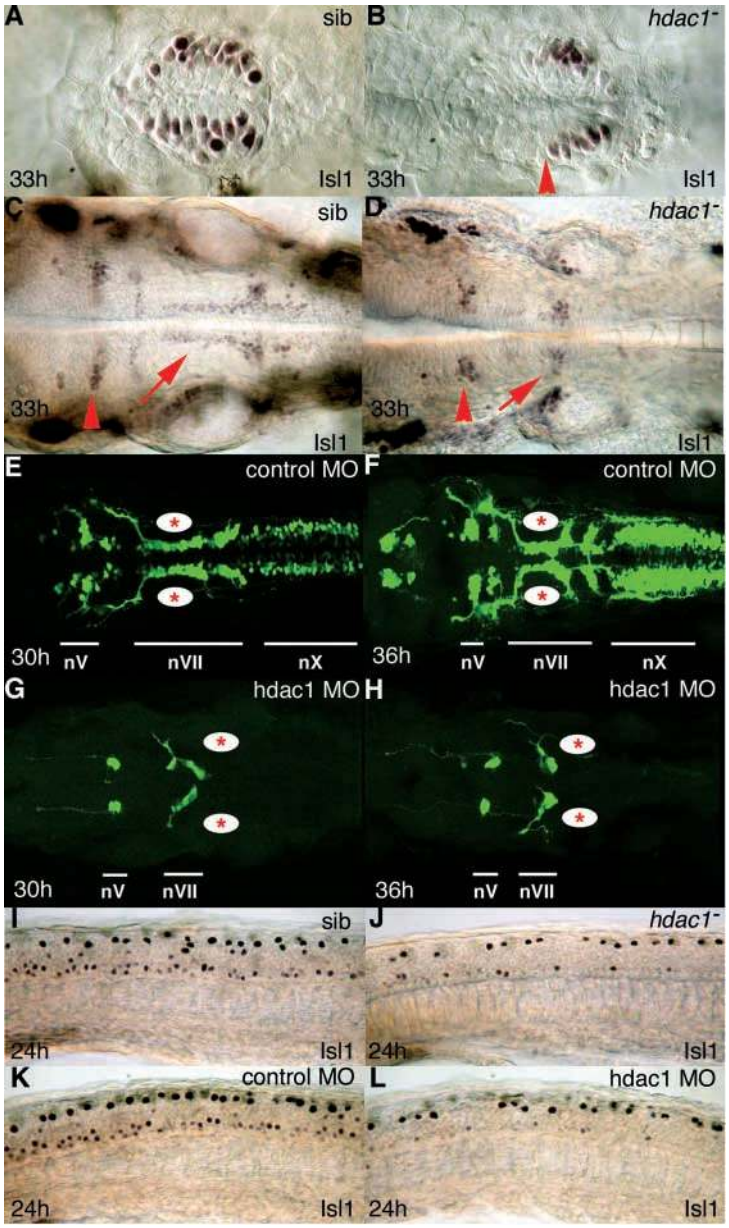


Fig. 8. Loss of *hdac1* function disrupts specification and patterning of Isl1-expressing neurones. (A,B) Immunohistochemistry for Isl1-expressing neurones in the epiphysis of *hdac1*^{hi1618} (A) sibling and (B) mutant embryos. Note the absence of Isl1-positive cells in the anterior half of the mutant epiphysis (left of arrowhead). (C,D) Immunohistochemistry for Isl1-expressing branchiomotor neurones in the hindbrain of *hdac1*^{hi1618} (C) sibling and (D) mutant embryos. Note that the characteristic curved arrangements of nVII branchiomotor neurones spanning rhombomeres 4, 5 and 6 in the sibling hindbrain are absent in the mutant hindbrain and replaced by two small clusters of Isl1-positive neurones in rhombomere 4 (arrows). Trigeminal (nV) motoneurones in rhombomere 2 are marked (arrowheads). (E-H) Expression of an Isl1-GFP transgene reveals the position and morphology of differentiated branchiomotor neurones in embryos microinjected with (E,F) control MO or (G,H) *hdac1*-MO, at (E,G) 30 hpf and (F,H) 36 hpf. Positions of otic vesicles are marked with a white oval and red asterisk. In A-H, views are dorsal, anterior is towards the left. At both 30 and 36 hpf, *hdac1*-MO-injected embryos exhibited the same four, small clusters of differentiated branchiomotor (trigeminal and facial) neurones with axons that projected anteriorly, and there were no Isl-GFP-positive neurones located posterior to rhombomere 4. By stark contrast, control MO-injected embryos developed a normal population of Isl1-GFP-expressing branchiomotor neurones which increased in size and morphological complexity between 30 and 36 hpf (I,J) Immunohistochemistry for Isl1-expressing neurones in the spinal cord of *hdac1*^{hi1618} (I) sibling and (J) mutant embryos, and wild-type WIK embryos microinjected with (K) a control MO or (L) an *hdac1*-MO, at 24 hpf. Lateral views, anterior is towards the left. Isl1-positive motoneurones lie in the ventral spinal cord; Rohon-Beard cells are located in the dorsal spinal cord and stain relatively strongly for Isl1 protein. Homozygosity for the *hdac1*^{hi1618} mutation or microinjection of an *hdac1*-MO reduces the number of Isl1-expressing neurones formed in the spinal cord.

Table 2. Specific effect of an *hdac1*-MO on development of branchiomotor neurones in Isl1-GFP transgenic zebrafish embryos (see Fig. 8 for examples)

Treatment	Wild-type pattern of branchiomotor neurones	Defective pattern of branchiomotor neurones	Non-specific defects
Uninjected	125	0	3
Control-MO	218	0	4
hdac1-MO	9	193	1

hpf (Fig. 11F), all of which were strictly *smoothened*-dependent (Fig. 11H). Taking the results described in Figs 10 and 11 together, it can be concluded that the hedgehog signalling pathway is intact in *hdac1* mutants, but sustained production of hedgehog-dependent motoneurones, although initially normal, is not efficiently maintained even when the level of hedgehog expression is experimentally increased. Therefore, consistent with its function as an antagonist of Notch signalling, these results reveal that *hdac1* is required in

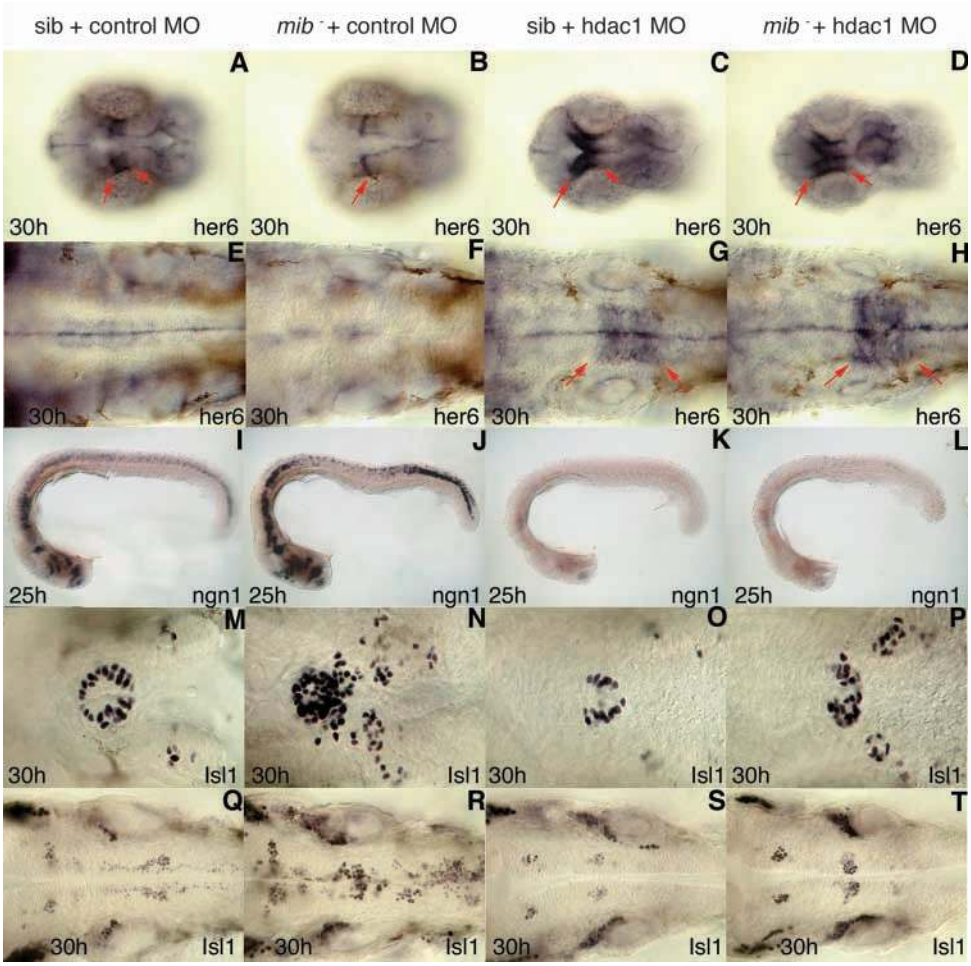


Fig. 9. The *hdac1* morphant CNS phenotype is epistatic to that of the *mind bomb* mutant. (A,E,I,M,Q) Unaffected *mib* siblings injected with control MO. (B,F,J,N,R) *mib* homozygous mutant embryos injected with control MO. (C,G,K,O,S) *mib* siblings injected with *hdac1*-MO. (D,H,L,P,T) *mib* homozygous mutant embryos injected with *hdac1*-MO. (A-H) In situ hybridisation analysis of *her6* expression in (A-D) dorsal diencephalon (30 hpf) or (E-H) hindbrain (30 hpf). Loss of *mib* function reduces the abundance of *her6* transcripts in the dorsal diencephalon and hindbrain, whereas loss of *hdac1* function derepresses *her6* in the dorsal diencephalon and hindbrain both in *mib*-siblings and mutants. (I-L) In situ hybridisation analysis of *ngn1* expression in 25 hpf embryos. Loss of *mib* function increases *ngn1* expression throughout the CNS, whereas loss of *hdac1* function abolishes *ngn1* expression in the CNS both in *mib*-siblings and mutants. (M-T) Immunohistochemistry for Isl1-expressing (M-P) epiphysial and (Q-T) branchiomotor neurones in the hindbrain of 30 hpf embryos; dorsal views, anterior is leftwards. Loss of *mib* function produces supernumerary Isl1-positive cells in the epiphysis and throughout the hindbrain, whereas loss of *hdac1* function severely impairs the production of Isl1-positive cells both in *mib*-siblings and mutants.

Table 3. Specific effects of an *hdac1*-MO on *her6* expression and specification of Isl1-expressing cells in the CNS of *mib* mutants and siblings (see Fig. 9 for examples).

	Wild-type *her6* expression	Ectopic *her6* expression	Reduced *her6* expression	Wild-type Isl1 pattern	Deficiency of Isl1+ve cells	Excess Isl1+ve cells
Uninjected						
mib siblings	72	0	0			
mib mutants	0	0	18			
Control-MO						
mib siblings	39	0	0	31	0	0
mib mutants	0	0	11	0	0	11
hdac1-MO						
mib siblings	1	40	0	0	28	0
mib mutants	0	16	1	0	7	0

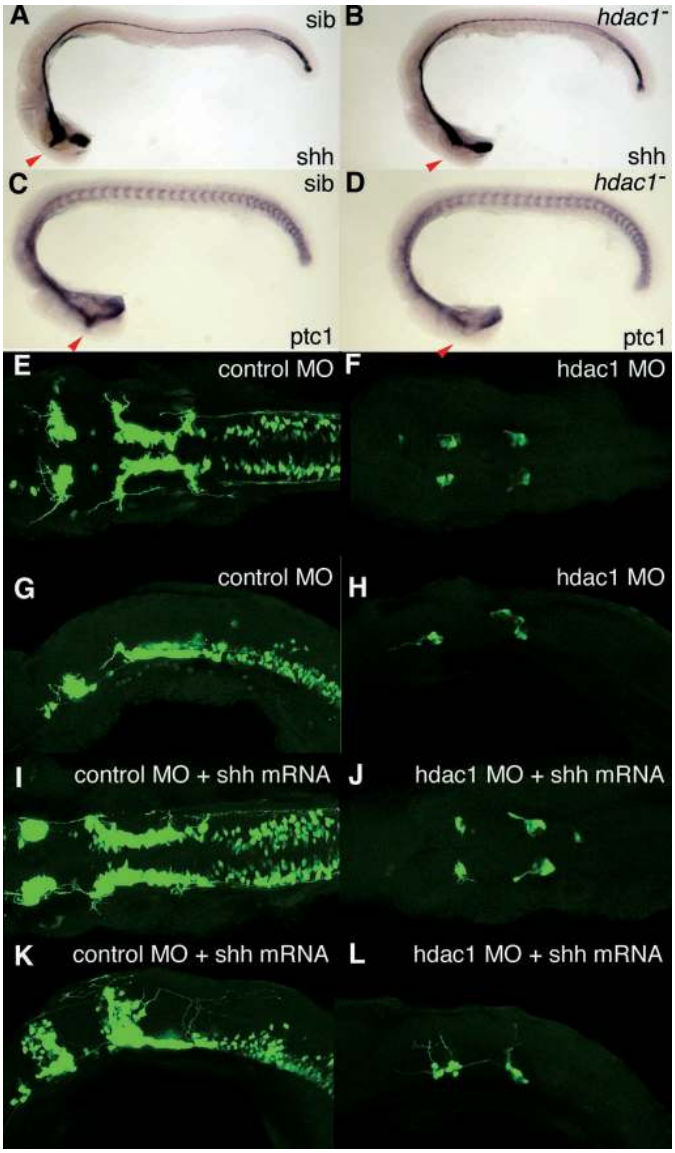


Fig. 10. The hedgehog signalling pathway is intact in *hdac1* mutant embryos but motoneurone precursors require wild-type levels of *hdac1* activity to respond to elevated levels of *hedgehog* signalling. (A-D) In situ hybridisation analysis of (A,B) *shh* and (C,D) *ptc1* expression in *hdac1*^{hi1618} (A,C) sibling and (B,D) homozygous mutant embryos at 24 hpf. Positions of zli are indicated (arrowheads). Loss of *hdac1* function does not significantly affect *shh* expression in ventral midline cells of hindbrain and spinal cord, and expression of the *shh* target gene *ptc1* is relatively unpertubed in CNS and paraxial mesoderm. (E-L) Expression of an Isl1-GFP transgene reveals the position and morphology of branchiomotor neurones in 30 hpf embryos microinjected with (E,G) control MO; (F,H) *hdac1*-MO; (I,K) control MO plus 100 pg *shh* mRNA; (J,L) *hdac1*-MO plus 100 pg *shh* mRNA. (E,F,I,J) Dorsal views of hindbrain, anterior is towards the left; (G,H,K,L) lateral views of hindbrain, anterior is towards the left. Overexpression of *shh* dramatically increased the number of Isl1-GFP-positive branchiomotor neurones. No supernumerary branchiomotor neurones were formed when an *hdac1*-MO was co-injected with *shh* mRNA and only the rudimentary pattern of Isl1-expressing neurones characteristic of *hdac1*-deficient embryos was observed.

Table 4. Suppressive effect of an *hdac1*-MO on induction of supernumerary branchiomotor neurones by co-injected *shh* mRNA in Isl1-GFP-transgenic embryos (see Fig. 10 for examples).

Treatment	Wild-type pattern of branchiomotor neurones	Deficiency of branchiomotor neurones	Supernumerary branchiomotor neurones
Uninjected	21	0	0
Control-MO	13	0	0
Control-MO + *shh* mRNA	1	0	44
hdac1-MO	0	13	0
hdac1-MO + *shh* mRNA	0	44	0

the hindbrain to maintain the production of branchiomotor neurones in response to hedgehog signalling.

Discussion

Previous studies have demonstrated that Hdac1 enzymes are deployed in a variety of developmental contexts to mediate transcriptional silencing. In the mouse, targeted mutation of *Hdac1* derepressed CDK inhibitor genes leading to reduced cell proliferation and post-gastrulation embryonic lethality (Lagger et al., 2002). In *Drosophila*, a loss-of-function mutation in the *hdac1* orthologue *Rpd3* causes a pair-rule phenotype (Mannervik and Levine, 1999), whereas in *C. elegans,* mutation of *hda-1* causes post-embryonic defects in gonadogenesis and vulval development (Dufourcq et al., 2002). In zebrafish, mutation of *hdac1* (Golling et al., 2002) causes a phenotype in which, as shown here, distinct aspects of the development of several organs and tissues are affected.

Hdac1 as a repressor of Notch targets in neural precursor cells

Histological analysis of the CNS in *hdac1* mutants initially indicated that neurogenesis may be sensitive to loss of *hdac1* function. The ensuing analysis demonstrated unequivocally that *hdac1* is indeed required to promote neurogenesis and evidence is presented that this is accomplished by transcriptional repression of Notch-activated target genes such as *her6. Her6* is the orthologue of mammalian *Hes1*, which is required for Notch-driven repression of *Mash1* and *Ngn1* (Cau et al., 2000). Consistent with these observations, transcripts of the proneural genes *ash1b* and *ngn1* were almost completely absent in *hdac1*-mutant embryos, suggesting that, as for Hes1 in mammals, Her6 is a specific repressor of multiple proneural genes. Derepression of *her6* was greatest in the dorsal diencephalon and hindbrain rhombomeres 5 and 6, two regions of the CNS where neuronal specification, as revealed by Isl1 immunostaining, was particularly severely affected (Fig. 8). Intriguingly, *her6* derepression caused by *hdac1* deficiency was epistatic to the *mind bomb* neurogenic phenotype, both in the dorsal diencephalon and in rhombomeres 5 and 6 of the hindbrain. These observations unequivocally show that a function of Notch signalling is to relieve *hdac1*-mediated transcriptional repression of Notch targets in the CNS, which is consistent with studies in other species (Kao et al., 1998; Barolo and Posakony, 2002). Interestingly, loss of *hdac1* function did not cause widespread derepression of *her6* throughout the embryo. Instead, *her6* derepression was

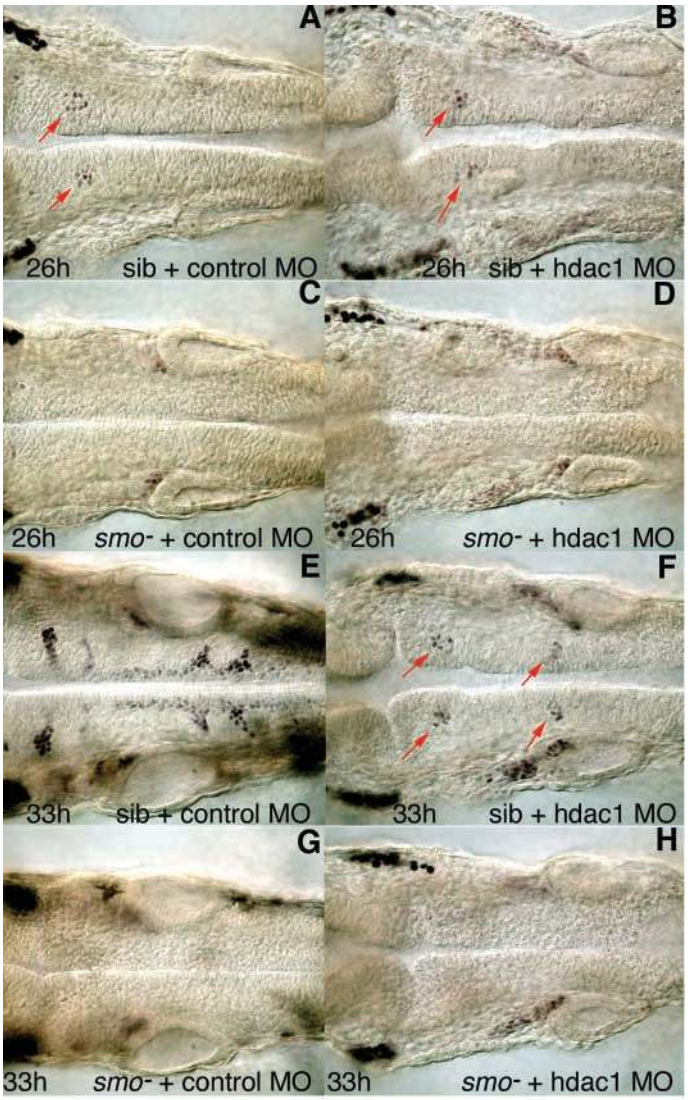


Fig. 11. Sustained production of smoothened-dependent branchiomotor neurones requires wild-type levels of *hdac1* activity. Immunohistochemistry for Isl1-expressing branchiomotor neurones in the hindbrain of *smo*^{hi1640} (A,B,E,F) sibling and (C,D,G,H) homozygous mutant embryos, at (A-D) 26 hpf and (E-H) 32 hpf. Embryos were microinjected with either (A,C,E,G) control MO or (B,D,F,H) *hdac1*-MO. Dorsal views, anterior is towards the left. Specification of all Isl1-expressing branchiomotor neurones requires *smoothened* function. Initial specification of Isl1-positive trigeminal motoneurones at 26 hpf was *hdac1* independent, but further production of *smoothened*-dependent branchiomotor neurones was impaired in *hdac1*-deficient embryos and only two further small clusters of Isl1-positive facial neurones had formed by 33 hpf, in contrast to the situation in control-MO-injected embryos.

strongest in CNS territories undergoing neuronal specification, implying that hdac1-mediated transcription silencing is selective for neural patterning processes, perhaps through interactions with neural-specific repressors. The results described here also imply that the products of Notch target genes such as the her6 transcriptional repressor can function in an *hdac1*-independent manner, possibly through redundant interactions with other class I HDACs.

In *Drosophila*, Notch target genes are actively repressed by DNA-bound Suppressor of Hairless [Su(H)], which recruits co-repressor molecules such as groucho and CtBP to the target locus via interactions with the Hairless protein (Barolo et al., 2002). Other studies in cultured mammalian cells previously found that the Su(H) orthologue CBF1 bound the HDAC1-containing SMRT complex (Kao et al., 1998). Moreover, in *Xenopus* animal cap explants, the broad specificity HDAC inhibitor trichostatin A caused a two-fold increase in expression of the Notch target gene *ESR1* (an orthologue of zebrafish *her4*) in response to overexpression of *Xdelta1* mRNA (Kao et al., 1998), suggesting a role for histone deacetylase in repression of Notch targets. The experiments reported here extend these observations significantly by demonstrating that in the zebrafish embryonic CNS, *hdac1* is required for repression of Notch targets in a manner that still renders these genes inducible by Notch signalling. No vertebrate orthologue of the *hairless* gene has yet been described, and the SMRT protein can interact directly with the orthologue of Su(H) (Kao et al., 1998). However, proteins such as SHARP and SKIP may also modulate connectivity between DNA-bound Su(H) and Hdac1 in vertebrate embryos (Oswald et al., 2002). It will now be of great interest to determine whether interactions between Hdac1, SHARP, SKIP and Su(H) orthologues are essential for repressing Notch targets during neurogenesis in the zebrafish embryo.

A widespread requirement for hdac1 in neuronal specification and patterning

Immunostaining for expression of Hu proteins and GFAP revealed that formation of neurones and glia was highly abnormal in the hindbrain of *hdac1* mutant embryos (Fig. 7). There are fewer post-mitotic neurones in *hdac1* mutant embryos than in siblings at each of the three stages analysed, and the characteristic segmental organisation of these neurones with their associated glia is lost. However, loss of *hdac1* function does not cause a general arrest of CNS growth and development, because although cell proliferation in the *hdac1* mutant hindbrain is reduced at 25 hpf in comparison to the situation in sibling embryos, it regains its normal proliferative capacity by 33 hpf and a similarly high level of mitotic activity persists at 38 hpf. Moreover, throughout the period from 25 hpf to 38 hpf, the size of the Hu-positive neuronal population in the hindbrain progressively increases. It is possible that the transient reduction in cell proliferation within the *hdac1* mutant hindbrain specifically affects a particular step of neurogenesis, because the defects in neurogenesis are irreversible and become more profound with time, whereas cell proliferation within the hindbrain recovers. It is also possible that *hdac1* mutants selectively accumulate proliferating neural precursors as a direct consequence of derepressing Notch target genes (Solecki et al., 2001). Future experiments will investigate these possibilities. However, taken together with the finding that patterning markers such as *epha4* are properly segmentally expressed in the hindbrain (Fig. 4), the observed abnormalities clearly illustrate that *hdac1* is required to efficiently couple neurogenesis to the mechanisms determining segmental patterning of the hindbrain.

Hdac1-deficient embryos exhibited a range of defects in specification of neuronal subtypes, as revealed by immunostaining for Isl1 and by confocal microscopy of an

Isl1-GFP transgenic line. In the dorsal diencephalon of *hdac1* mutants, there was a striking deficit of anterior epiphysial neurones in a position corresponding to the diencephalic territory within which strong expression of *her6* and extinction of proneural gene expression was observed (Figs 6, 8). In the hindbrain, nascent clusters of Isl1-positive trigeminal (nV) and facial (nVII) motoneurones were produced in rhombomeres 2 and 4 of *hdac1*-deficient embryos, but these clusters failed to expand properly and no additional branchiomotor neurones were formed. Nevertheless, those nV (r2) and nVII (r4) motoneurones that were specified persisted in their original positions within the hindbrain and they produced correctly oriented axons that are characteristic of properly differentiated neurones. Interestingly, there was no evidence of tangential migration caudally (Chandrasekhar et al., 1997), by branchiomotor neurones born in rhombomere 4, into rhombomeres 5 and 6 of the *hdac1* mutant hindbrain, where *her6* was strongly expressed. It remains unclear whether this abnormality solely reflects a tangential migration defect in nVII neuronal precursors of *hdac1* mutants that were born in rhombomere 4, or whether the observed defect is also the consequence of a failure to specify nVII motoneurones from separate precursors originating in rhombomeres 5 and 6.

Within the trunk, loss of *hdac1* function caused a substantial reduction in the size of the spinal motoneurone population throughout the spinal cord, as revealed by Isl1 immunostaining, and a milder effect on the number of Rohon-Beard cells was also evident (Fig. 8), indicating that neuronal specification can be initiated throughout the length of the spinal cord but in the absence of *hdac1* function it is not efficiently maintained.

hdac1 facilitates the response of neuronal precursors to hedgehog signalling and the acquisition of motoneurone identity

All branchiomotor neurones require hedgehog signalling for specification and at the onset of this developmental process, *hdac1*-deficient and wild-type embryos were indistinguishable (Fig. 11). However, later phases of branchiomotor specification were defective in *hdac1*-deficient embryos, and this could not be averted by overexpression of *shh* (Figs 10, 11). Thus, *hdac1* is required to maintain the response of neural precursors in the hindbrain to hedgehog signals. Impairment of this response could be a direct consequence of increased Notch target gene expression in *hdac1*-deficient embryos. Previous work demonstrated that expression of the proneural gene *ngn1* in the developing CNS is positively regulated by hedgehog signalling (Blader et al., 1997), raising the possibility that loss of *hdac1* function directly inhibits the response to hedgehog signalling by preventing proneural gene expression. Recent studies in *Drosophila* have demonstrated that Notch signalling also prevents the hedgehog-mediated activation of *collier* in the wing margin (Glise et al., 2002). As vertebrate homologues of *collier* have previously been implicated in control of neurogenesis (Bally-Cuif et al., 1998; Dubois et al., 1998), it is conceivable that derepression of Notch targets such as *her6* in the *hdac1* mutant hindbrain could directly inhibit hedgehog-mediated expression of *collier* homologues. This possibility will now be investigated. Hdac1 protein has been found in physical association with numerous transcriptional repressors, and may be required for the deacetylation of histones associated with many different target genes in neural cells. Nevertheless, the results presented here unveil *her6* as a likely direct target of *hdac1*-mediated transcriptional repression and imply that the *her6* locus is hyperacetylated in *hdac1* mutant embryos. Deacetylated core histones are substrates for lysine-methylation by a large family of SET-domain-containing histone methyltransferases (for a review, see Turner, 2002), and so the *her6* locus of wild-type embryos may also exhibit histone methylation patterns that are under-represented in *hdac1* mutant embryos. These possibilities will now be investigated.

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References

Allende, M. L. and Weinberg, E. S. (1994). The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant. *Dev. Biol.* **166**, 509-530.

Andermann, P., Ungos, J. and Raible, D. W. (2002). Neurogenin 1 defines zebrafish cranial sensory ganglia precursors. *Dev. Biol.* **251**, 45-58.

Appel, B. and Eisen, J. S. (1998). Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* **125**, 371-380.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Bally-Cuif, L., Dubois, L. and Vincent, A. (1998). Molecular cloning of Zcoe2, the zebrafish homologue of *Xenopus* Xcoe2 and mouse EBF-2, and its expression during primary neurogenesis. *Mech. Dev.* **77**, 85-90.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev.* **16**, 1167-1181.

Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev.* **16**, 1964-1976.

Blader, P., Fischer, N., Gradwohl, G., Guillemot, F. and Strahle, U. (1997). The activity of neurogenin 1 is controlled by local cues in the zebrafish embryo. *Development* **124**, 4557-4569.

Brunet, J. F. and Ghysen, A. (1999). Deconstructing cell determination: proneural genes and neuronal identity. *BioEssays* **21**, 313-318.

Campos-Ortega, J. (1993). Mechanisms of early neurogenesis in *Drosophila melanogaster*. *J. Neurobiol.* **10**, 1305-1327.

Cau, E., Gradwohl, G., Fode, C. and Guillemot, F. (1997). *Mash1* activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611-1621.

Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R. and Guillemot, F. (2000). *Hes* genes regulate sequential stages of neurogenesis in the olfactory epithelium. *Development* **127**, 2323-2332.

Cau, E. and Wilson, S. W. (2002). Ash1a and neurogenin function downstream of Floating head to regulate epiphysial neurogenesis. *Development* **130**, 2455-2466.

Chan, Y. M. and Jan, Y. N. (1999). Conservation of neurogenic genes and mechanisms. *Curr. Opin. Neurobiol.* **9**, 582-588.

Chandrasekhar, A., Moens, C. B., Warren, J. T., Jr, Kimmel, C. B. and Kuwada, J. Y. (1997). Development of branchiomotor neurons in zebrafish. *Development* **124**, 2633-2644.

Chandrasekhar, A., Warren, J. T., Jr, Takahashi, K., Schauerte, H. E., van Eeden, F. J., Haffter, P. and Kuwada, J. Y. (1998). Role of sonic hedgehog in branchiomotor neuron induction in zebrafish. *Mech. Dev.* **76**, 101-115.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish *smoothened* mutant reveals conserved and divergent functions of Hedgehog activity. *Development* **128**, 2385-2396.

Chitnis, A. (1999). Control of neurogenesis – lessons from frogs, fish and flies. *Curr. Opin. Neurobiol.* **9**, 18-25.

Cornell, R. A. and Eisen, J. S. (2002). Delta/Notch signaling promotes formation of zebrafish neural crest by repressing Neurogenin 1 function. *Development* **129**, 2639-2648.

Dornseifer, P., Takke, C. and Campos-Ortega, J. (1997). Overexpression of a zebrafish homologue of the *Drosophila*neurogenic gene *Delta* perturbs differentiation of primary neurons and somite development. *Mech. Dev.* **63**, 159-171.

Dubois, L., Bally-Cuif, L., Crozatier, M., Moreau, J., Paquereau, L. and Vincent, A. (1998). XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in *Xenopus*. *Curr. Biol.* **8**, 199-209.

Dufourcq, P., Victor, M., Gay, F., Calvo, D., Hodgkin, J. and Shi, Y. (2002). Functional requirement for histone deacetylase 1 in *Caenorhabditis elegans* gonadogenesis. *Mol. Cell. Biol.* **22**, 3024-3034.

Glise, B., Jones, D. L. and Ingham, P. W. (2002). Notch and wingless modulate the response of cells to hedgehog signalling in the *Drosophila* wing. *Dev. Biol.* **248**, 93-106.

Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., Burgess, S., Haldi, M., Artzt, K., Farrington, S., Lin, S.-Y., Nissen, R. M. and Hopkins, N. (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat. Genet.* **31**, 135-140.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998). Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-370.

Higashijima, S., Hotta, Y. and Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* **20**, 206-218.

Ishibashi, M., Moriyoshi, K, Sasai, Y., Shiota, K., Nakanishi, S. and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *EMBO J.* **13**, 1799-1805.

Ishibashi, M., Ang, S.-L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian *hairy* and *Enhancer of split* homolog-1 (*HES-1*) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis and severe neural tube defects. *Genes Dev.* **9**, 3136-3148.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L., Weissmann, A. M., Lewis, J., Chandrasekharappa, S. C. and Chitnis, A. B. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Kao, H.-Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M. and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the notch signal transduction pathway. *Genes Dev.* **12**, 2269-2277.

Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118**, 417-425.

Lagger, G., O'Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuettengruber, B., Hauer, C., Brunmeir, R., Jenuwein, T. and Seiser, C. (2002). Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor expression. *EMBO J.* **21**, 2672-2681.

Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signalling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.

Mannervik., M. and Levine, M. (1999). The Rpd3 histone deacetylase is required for segmentation of the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **96**, 6797-6801.

Masai, I., Heisenberg, C. P., Barth, K. A., Macdonald, R., Adamek, S. and Wilson, S. W. (1997). floating head and masterblind regulate neuronal patterning in the roof of the forebrain. *Neuron* **18**, 43-57.

Ng, H. H. and Bird, A. (2000). Histone deacetylases: silencers for hire. *Trends Biochem. Sci.* **25**, 121-126.

Nona, S. N., Shehab, S. A., Stafford, C. A. and Cronly-Dillon, J. R. (1989). Glial fibrillary acidic protein (GFAP) from goldfish: its localisation in visual pathway. *Glia* **2**, 189-200.

Oswald, F., Kostezka, U., Astrahantseff, K., Bourteele, S., Dillinger, K., Zechner, U., Ludwig, L., Wilda, M., Hameister, H., Knochel, W., Liptay, S. and Schmid, R. M. (2002). SHARP is a novel component of the Notch/RBP-JK signalling pathway. *EMBO J.* **21**, 5417-5426.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

Pasini, A., Henrique, D. and Wilkinson, D. G. (2001). The zebrafish Hairy/Enhancer-of-split-related gene *her6* is segmentally expressed during early development of hindbrain and somites. *Mech. Dev.* **100**, 317-321.

Saka, Y. and Smith, J. C. (2001). Spatial and temporal patterns of cell division during early *Xenopus* embryogenesis. *Dev. Biol.* **229**, 307-318.

Schulte-Merker, S. (2003). Looking at zebrafish embryos. In *Zebrafish: A Practical Approach* (ed. C. Nusslein-Volhard and R. Dahm), pp. 39-58. Oxford: Oxford University Press.

Solecki, D. J., Liu, X. L., Tomoda, T., Fang, T. and Hatten, M. E. (2001). Activated Notch2 signaling inhibits differentiation of cerebellar granule neuron precursors by maintaining proliferation. *Neuron* **31**, 557-568.

Strahl, B. D. and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* **403**, 41-45.

Takke, C., Dornseifer, P., von Weiszacker, E. and Campos-Ortega, J. (1999). *Her4*, a zebrafish homologue of the *Drosophila* neurogenic gene *E(spl)*, is a target of notch signalling. *Development* **126**, 1811-1821.

Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**, 669-679.

Turner, B. M. (2002). Cellular memory and the histone code. *Cell* **111**, 285-291.

Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y.-L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish *smoothened* functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.