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Opposing actions of histone deacetylase 1 and Notch signalling restrict expression of *erm* and *fgf20a* to hindbrain rhombomere centres during zebrafish neurogenesis

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ABSTRACT The rate and pattern of neurogenesis in the developing vertebrate nervous system are controlled by a complex interplay of intercellular signalling pathways and transcriptional control mechanisms. In the zebrafish hindbrain, Fgf20a promotes transcription of the gene encoding the ETS-domain transcription factor Erm in the non-neurogenic centres of rhombomeres. Here, we demonstrate that the epigenetic regulator, Histone Deacetylase 1 (Hdac1) and the Notch signalling pathway have opposing functions in regulating expression of both *erm* and *fgf20a* in the zebrafish hindbrain. Our results show that Hdac1 is required for expression of *erm* and *fgf20a* in rhombomeres, and that the Hdac1-dependent expression of these two genes is attenuated in rhombomere boundary regions by Notch signalling activity, thereby restricting *erm* and *fgf20a* transcripts to narrow stripes of cells at rhombomere centres.

KEY WORDS: *neurogenesis, transcription, Hdac1, Notch, zebrafish*

Introduction

Throughout the developing vertebrate central nervous system (CNS), proliferating multipotent neural progenitors give rise to a remarkable variety of neuronal and glial cell types. Some of the crucial steps underlying lineage restriction and the commitment of neural progenitors to specific differentiated neuronal or myelinating glial fates include progenitor withdrawal from the cell cycle, transcriptional silencing of neural progenitor determinants and transcriptional activation of genes encoding bHLH transcription factors such as *ascl1b*, *neurog1* (Bertrand *et al*., 2002) and *olig2* (Rowitch, 2004). However, the processes driving commitment of proliferating progenitors to neuronal and glial fates in the CNS are countered by the regulated expression of neural progenitor maintenance factors such as the SoxB1 proteins (Pevny and Placzek, 2005) as well as the regulated activity of the Wnt, Notch and FGF signalling pathways (Ille and Sommer, 2005; Louvi and Artavanis-Tsakonas, 2006; Gonzalez-Quevedo *et al.,* 2010), which provide negative feedback that limits the rate and pattern of neurogenesis.

Histone deactylases (HDACs) are components of the epigenetic machinery that regulates gene transcription during embryonic development (Cunliffe, 2008). In zebrafish and the mouse, the class I HDAC, Hdac1, plays important roles in the transformation of neural progenitors into neurones and myelinating glia during development of the vertebrate CNS, by promoting cell cycle exit and transcription of neurogenic genes (Cunliffe, 2004; Yamaguchi *et al*., 2005; Stadler *et al*., 2005; Cunliffe and Casaccia-Bonnefil, 2006; Harrison *et al*., 2011; Montgomery *et al*., 2009; Ye *et al*., 2009). In the hindbrain, *hdac1* is required for the specification of branchiomotor neurones and oligodendrocytes (Cunliffe, 2004; Cunliffe and Casaccia-Bonnefil, 2006). Our previous studies demonstrated that Hdac1 functions in the hindbrain by a mechanism that involves promoting expression of transcription factors such as those encoded by proneural genes, attenuating Notch target gene expression, and enabling neural fate-determining responses to Hedgehog pathway activity (Cunliffe, 2004; Cunliffe and Casaccia-Bonnefil, 2006; Harrison *et al*., 2011).

Accumulating evidence suggests that FGF-regulated transcription factors, such as the ETS-domain proteins Erm, Pea3 and Etv5, play important roles in neural specification and/or patterning within the developing CNS (Gonzalez-Quevedo *et al.,* 2010; Raible and Brand, 2001; Roussigné and Blader, 2006). In the hindbrain, transcripts encoding Erm and its positive regulator Fgf20a are restricted

Abbreviations used in this paper: ETS, E26 Transformation-Specific; Erm, ETS-related molecule; fgf, fibroblast growth factor; hdac1, histone deacetylase 1.

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to stripes of tissue within rhombomeres (Roussigné and Blader, 2006; Gonzalez-Quevedo *et al.*, 2010), and recent work suggests that Erm could function in the establishment of Fgf20a-dependent, non-neurogenic zones at rhombomere centres (Gonzalez-Quevedo *et al.,* 2010). Here, we demonstrate opposing roles for Hdac1 and Notch pathway signalling activity in regulating expression of both *erm* and *fgf20a* in the zebrafish hindbrain, which further suggests crucial functions for this transcription factor and its upstream signal in regulation of neurogenesis.

Results

Hdac1 promotes expression of the ETS transcription factor gene erm *in the developing embryonic CNS*

Previous studies in this laboratory revealed that in the zebrafish hindbrain, loss of *hdac1* function severely impairs neuronal and glial differentiation without affecting the primary patterning of the neuroepithelium, including its subdivision into rhombomeres (Cunliffe, 2004). We wondered whether Hdac1 might therefore regulate expression of genes involved in neuronal patterning within rhombomeres, such as the transcription factor gene *erm, fgf20a*-dependent transcription of which is restricted to a series of robust stripes that are located at rhombomere centres (Roussigné and Blader, 2006; Gonzalez-Quevedo *et al.*, 2010). We addressed this question by first analysing expression of *erm* in wild-type and *hdac1* mutant embryos at 21, 24 and 31 hours post-fertilisation (hpf), which correspond to developmental time points shortly before and after the morphological phenotype of *hdac1* mutant embryos first becomes apparent (Cunliffe, 2004). At 21 hpf, *erm* is expressed in the hindbrain of wild-type sibling embryos in a series of clearly demarcated stripes at rhombomere centres, with the strongest expression corresponding to stripes of tissue that are located within rhombomeres 4, 5 and 6. *erm* expression is also abundant within other regions of the 21 hpf embryo, including the midbrain-hindbrain boundary, posterior spinal cord, somites, tailbud, midbrain, optic vesicles and forebrain (Fig. 1). In 21 hpf *hdac1* mutant embryos, *erm* expression in the hindbrain is limited to a weaker, more diffuse expression domain encompassing rhombomeres 4, 5 and 6. Reduced expression of *erm* was also observed in the midbrain and midbrain-hindbrain boundary of the *hdac1* mutant CNS, whereas *erm* transcript levels in the trunk and tailbud are relatively unaffected. By 24hpf, a strong, centrally-located stripe of *erm* expression is visible in each of rhombomeres 2-7 (Fig. 1).

By contrast, in the hindbrain of 24 hpf *hdac1* mutant embryos, expression of *erm* is extinguished in all rhombomeres apart from rhombomere 4 (Fig. 1), where expression persists, albeit relatively weakly. Transverse sections of the wild-type hindbrain in 24 hpf embryos at the level of rhombomere 5 reveal a broad domain of *erm* expression encompassing both the ventricular zone and

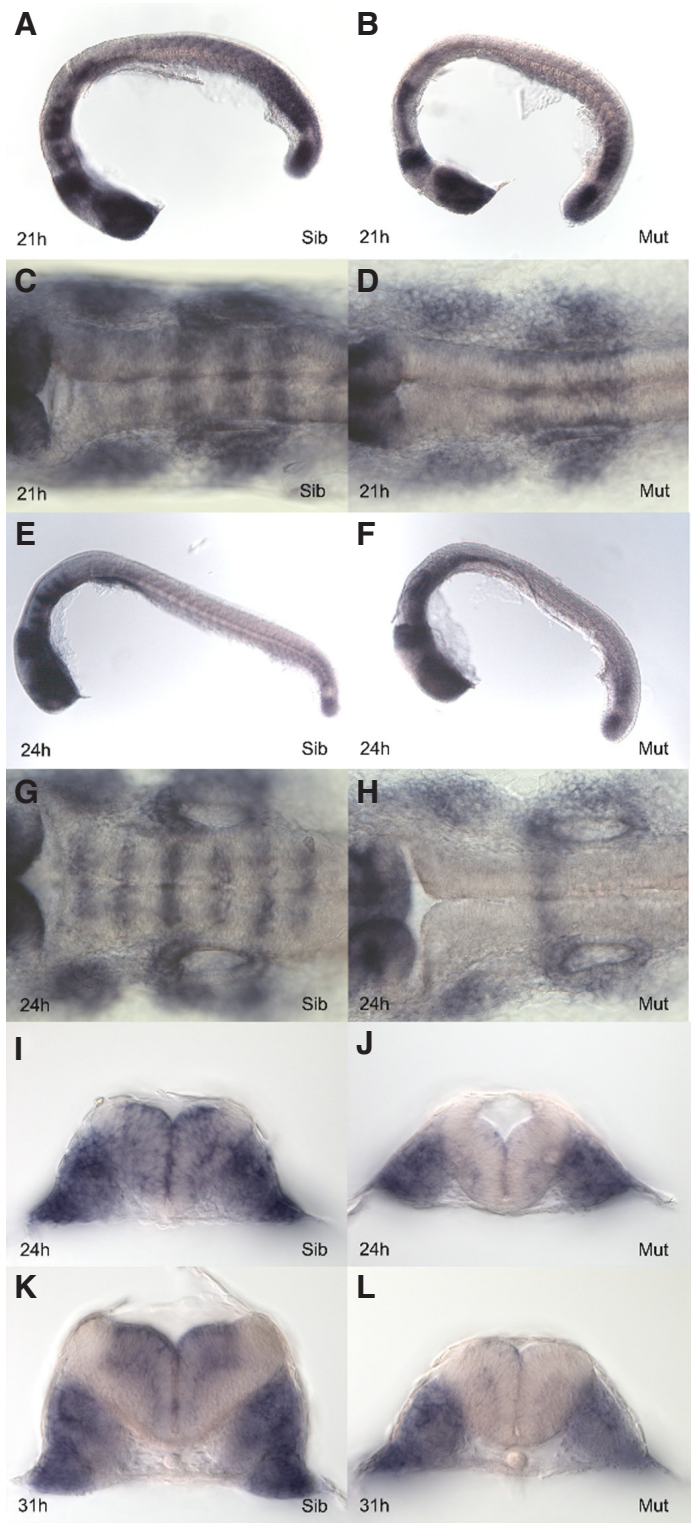
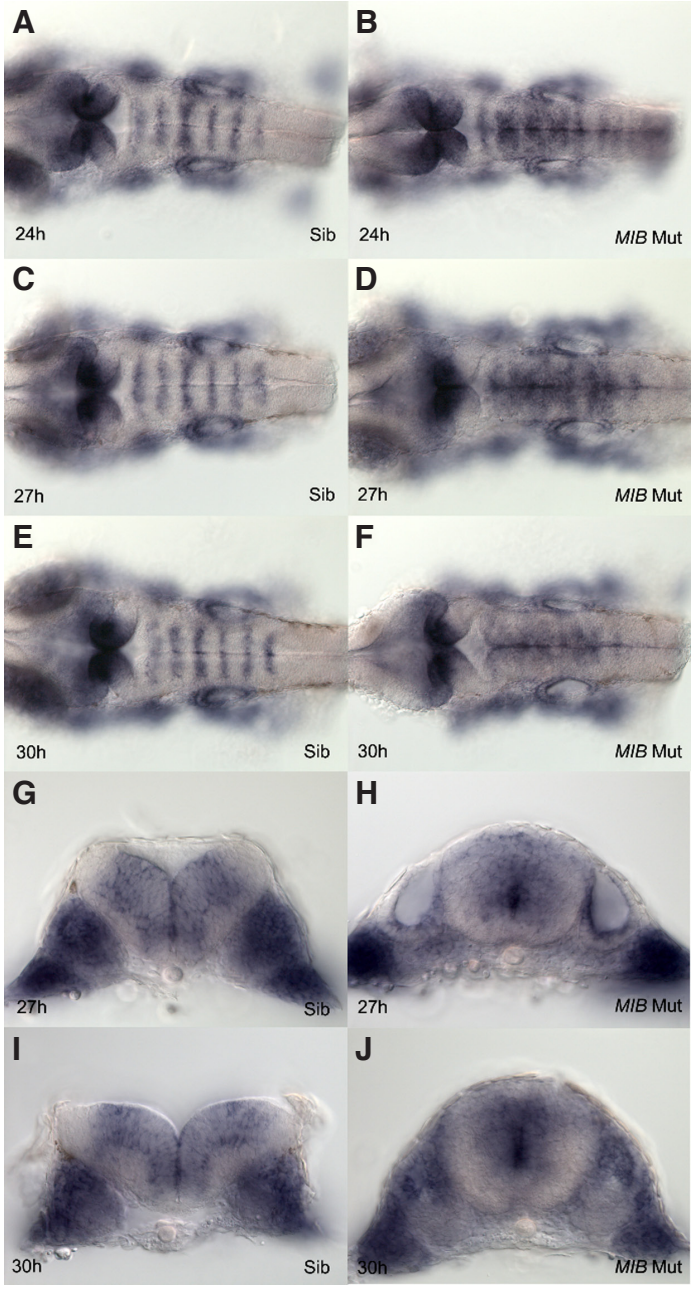


Fig. 1. *Hdac1* is required to establish and maintain stripes of *erm* expression within the centres of hindbrain rhombomeres 2-7. In situ *hybridisation analysis of* erm *expression in* **(A,C,E,G,I,K)** *wild-type sibling and* **(B,D,F,H,J,L)** *homozygous* hdac1 *mutant embryos, at 21 hpf* **(A-D)**, *24 hpf* **(E-J)** *and 31 hpf* **(K,L)**. *Lateral views show significant reduction of* erm *expression in the hindbrain of* hdac1 *mutant embryos (B,F) compared to wild-type siblings (A,E). Dorsal views of hindbrain are shown in panels C,D,G,H. Transverse sections through rhombomere 5 are shown in panels I,J,K,L. The hindbrain of wild-type sibling embryos (C,G,I,K) exhibits robust stripes of* erm *expression at 21 and 24 hpf that are restricted to rhombomere centres (C, G). Both the ventricular zone and mantle region of the hindbrain express* erm *at 24 hpf, but by 31 hpf* erm *expression is restricted to the ventricular zone. In* hdac1 *mutant embryos* erm *is expressed at 21hpf in a weak, more diffuse domain that encompasses rhombomeres 4, 5 and 6. By 24hpf,* erm *expression is extinguished in all rhombomeres of the* hdac1 *mutant hindbrain apart from rhombomere 4.*

mantle region, which includes all but the lateral-most margins of the hindbrain. At 31 hpf, each stripe of *erm* expression is restricted to a T-shaped domain which encompasses the majority of the ventricular zone, but which excludes its lateral margins as well as the underlying mantle region (Fig. 1). In contrast, transverse sections through rhombomere 5 of *hdac1* mutant embryos reveal a near complete loss of *erm* expression in this rhombomere at both 24hpf and 31hpf. Taken together, these results demonstrate a key role for Hdac1 to maintain *erm* transcription in an expression domain that initially encompasses both the ventricular zone and the mantle region in hindbrain rhombomere centres, but which then becomes restricted to the ventricular zones at rhombomere centres. In addition, we conclude that expression of *erm* in rhombomere 4 is less sensitive to loss of *hdac1* function than is the case for other rhombomeres, because a weak stripe of *erm* transcription persists in *hdac1* mutants when other domains of *erm* transcription in the hindbrain are extinguished.



The Notch signalling pathway inhibits expression of the ETS transcription factor gene erm *in the developing embryonic CNS*

Our previous studies demonstrated that Hdac1 promotes the transformation of proliferating progenitors into neurones and oligodendrocytes by activating a programme of neurogenic gene expression that includes proneural and other genes encoding transcription factors (Cunliffe, 2004; Cunliffe and Casaccia-Bonnefil, 2006; Harrison *et al.*, 2011). Moreover, Hdac1 attenuates the expression of the Notch pathway target gene *her6* and opposes the neural progenitor maintenance function of Notch signalling during CNS development. To determine whether Notch signalling regulates *erm* expression in the hindbrain ventricular zone, the expression pattern of *erm* was compared in the hindbrains of wild-type embryos and homozygous *mind bomb* mutants, which lack Notch pathway activity (Itoh *et al.*, 2003; Bingham *et al.*, 2003). This *in situ* hybridisation analysis revealed that *mind bomb* mutant embryos exhibit an expanded domain of *erm* expression in hindbrain rhombomeres 2-7 both anteriorly and posteriorly, towards each rhombomere boundary, at 24, 27 and 30 hpf (Fig. 2). In transverse sections through the hindbrain at the level of rhombomere 5, the expression domain of *erm* in *mind bomb* mutant embryos is centrally located within the hindbrain and excluded from a superficial ring of tissue at the ventral and lateral limits of the hindbrain. By contrast, wild-type sibling embryos exhibited narrow stripes of *erm* expression that were restricted to the ventricular zone at rhombomere centres. Thus, we conclude that in wild-type embryos, Notch signalling represses *erm* expression in the boundary regions of each rhombomere.

erm *expression in the hindbrain ventricular zone is strictly dependent on Hdac1 function and limited by Notch pathway activity*

The opposing activities of Hdac1 and Notch signalling in the regulation of neuronal specification have previously been demonstrated (Cunliffe, 2004). We therefore carried out an epistatic analysis of the relationship between the requirements for *hdac1* function and Notch signalling in the regulation of *erm* expression (Fig. 3). *erm* expression was analysed in 27hpf *mind bomb* mutants and wild-type siblings after microinjection with either an *hdac1*-specific translation-blocking morpholino (Hdac1ATG1) or

Fig. 2. Restriction of *erm* expression to narrow stripes at the centres of rhombomeres 2-7 is dependent on Notch signaling. In situ *hybridisation analysis of* erm *expression in* **(A,C,E,G,I)** *wild-type sibling and* **(B,D,F,H,J)** *homozygous* mind bomb *mutant embryos, at 24 hpf* **(A,B)**, *27 hpf* **(C,D,G,H)** *and 30 hpf* **(E,F,I,J)**. *Dorsal views of hindbrain are shown in panels A-F. Transverse sections through rhombomere 5 are shown in panels G-J. The hindbrain of wild-type sibling embryos (A,C,E) exhibits narrow stripes of* erm *expression at 24, 27 and 30 hpf that are restricted to rhombomere centres. However, in 24, 27 and 30 hpf* mind bomb *mutant embryos,* erm *is expressed in much broader domains within rhombomeres (B,D,F), forming a near-continuous domain of* erm *expression that extends across rhombomeres 2-7. In transverse sections through the hindbrain of 27 and 30 hpf* mind bomb *mutant embryos (H,J), a recognisable ventricular zone cannot be distinguished and* erm *expression is restricted to a central territory within hindbrain tissue, being most intense at the midline. By contrast, in age-matched wild-type sibling embryos (G,I),* erm *expression is restricted to the clearly recognisable ventricular zone.*

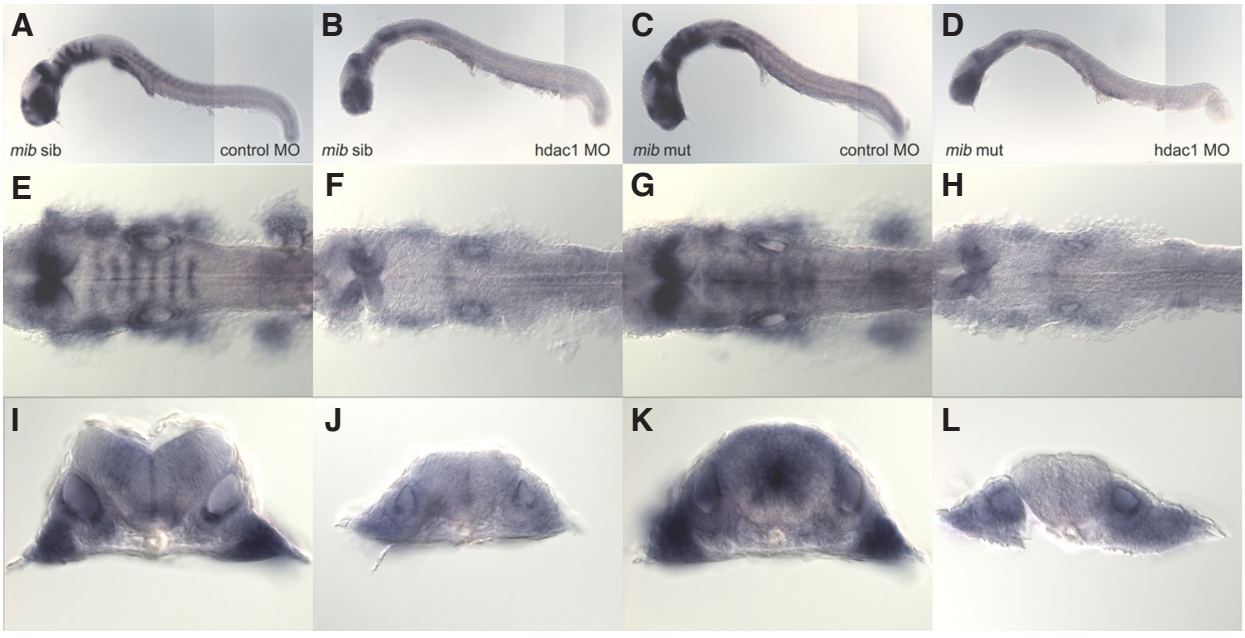


Fig. 3. Hdac1 acts epistatically and in opposition to the inhibitory effect of Notch signalling on *erm* transcription in the hindbrain ventricular zone. In situ *hybridisation analysis of* erm *expression in* **(A,E,I)** *wild-type sibling embyos injected with the hdac1 mismatch control morpholino,* **(B,F,J)** *wild-type sibling embryos injected with the hdac1ATG1 morpholino,* **(C,G,K)** mind bomb *mutant embryos injected with the hdac1 mismatch control morpholino, and* **(D,H,L)** mind bomb *mutant embryos injected with the hdac1ATG1 morpholino. Panels show lateral views (A-D), dorsal views of hindbrain (E-H), and transverse sections through rhombomere 5 (I-L) of 27 hpf embryos. Expression of* erm *is almost completely extinguished in the hindbrain of both hdac1ATG1 morphant embryos (B,F,J) and* mind bomb *mutants microinjected with the hdac1ATG1 morpholino (D,H,L), whereas the* erm *expression domain is expanded in the hindbrain of* mind bomb *mutants (C,G,K), and remains restricted to stripes at rhombomere centres in wild-type siblings microinjected with the hdac1 mismatch control morpholino (A,E,I).*

an *hdac1* mismatch control morpholino (Cunliffe, 2004). Wild-type sibling embryos that were microinjected with the *hdac1* mismatch control morpholino displayed a wild-type *erm* expression pattern in the hindbrain that consists of a series of distinctive stripes within the rhombomere centres. Transverse sections through rhombomere 5 revealed that these stripes of expression were confined to the hindbrain ventricular zone. Wild-type sibling embryos that were microinjected with the Hdac1ATG1 morpholino exhibited a phenotype similar to that of *hdac1* mutant embryos, in which the stripes of *erm* expression in rhombomeres 2-7 were completely extinguished, apart from the persistence of a weak stripe of *erm* transcription in rhombomere 4. Consistent with these observations, transverse sections through rhombomere 5 showed a complete absence of *erm* expression. By contrast, homozygous *mind bomb* mutant embryos microinjected with the *hdac1* mismatch control morpholino displayed a phenotype that was identical to that observed in homozygous *mind bomb* mutants, where the *erm* expression domain within each rhombomere is expanded to fill the entire anterior-posterior extent of the rhombomere, leading to widespread and continuous expression of *erm* throughout the hindbrain (Fig. 3). Intriguingly, *mib* mutant embryos microinjected with the Hdac1ATG1 morpholino developed a phenotype in which *erm* expression was almost completely extinguished within the hindbrain. This phenotype was indistinguishable from that of wild-type embryos injected with the Hdac1ATG1 morpholino and that of *hdac1* mutant embryos, which indicated that the hindbrain phenotype of *hdac1* morphant embryos is epistatic to that of homozygous *mib* mutants. Thus, the derepression of *erm* that occurs on either side

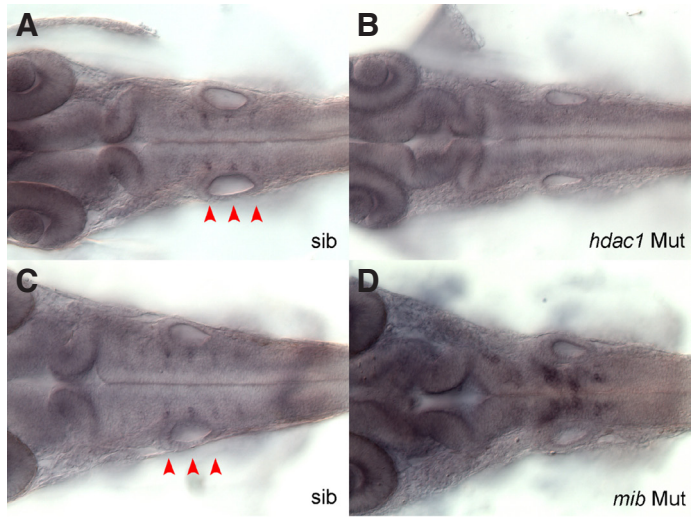


Fig. 4. Expression of *fgf20a* in the hindbrain is restricted to rhombomere centres by the opposing actions of Hdac1 and Notch signalling. In situ *hybridisation analysis of* fgf20a *expression in* **(A,C)** *wild-type sibling,* **(B)** hdac1 *mutant and* **(D)** mind bomb *mutant embryos at 27hpf (A-D). Dorsal views of hindbrain are shown in each panel. Wild-type sibling embryos (A,C) exhibit weak, narrow stripes of* fgf20a *expression in the hindbrain at 27 hpf that are localised to rhombomere centres (signals in rhombomeres 4, 5 and 6 are indicated by red arrowheads).* fgf20a *expression is extinguished in the hindbrain of* hdac1 *mutant embryos (B). By contrast,* mind bomb *mutant embryos exhibit increased and ectopic expression of* fgf20a *in a broad domain within the hindbrain that encompasses multiple adjacent rhombomeres (D).*

of rhombomere centres in *mind bomb* mutant embryos, like the restricted expression of *erm* at rhombomere centres in wild-type embryos, is strictly dependent on *hdac1* function.

Taken together, our results indicate that expression of *erm* in the hindbrain ventricular zone is facilitated by Hdac1 and inhibited by Notch signalling. Moreover, the results of our epistasis experiments imply that Notch signalling attenuates Hdac1-driven transcription of *erm* in the ventricular zone specifically at rhombomere boundaries, thereby restricting *erm* expression to a narrow stripe of ventricular zone tissue at each rhombomere centre.

Expression of* fgf20a *in the hindbrain requires Hdac1 function and is restricted to rhombomere centres by Notch signalling

Expression of *erm* in the hindbrain ventricular zone is strictly dependent on *fgf20a* function in a subset of neurones within the underlying mantle region (Gonzalez-Quevedo *et al.*, 2010). We therefore sought to determine whether, as for *erm,* the expression pattern of *fgf20a* in the hindbrain is influenced by Hdac1 function and Notch signalling. Fig. 4 shows that *fgf20a* expression is extinguished in the hindbrain of *hdac1* mutant embryos. By contrast, the hindbrains of *mind bomb* mutant embryos exhibit increased and ectopic expression of *fgf20a,* giving rise to an extensive domain of strong *fgf20a* expression that extends across rhombomeres 4, 5 and 6. Taken together with our analysis of *erm* expression in *hdac1* and *mind bomb* mutant embryos, these observations suggest that the gene expression changes observed for *erm* in *hdac1* mutant and *mind bomb* mutant embryos are likely to be consequences of the effects of these mutations on transcription of *fgf20a* in mantle region neurones.

Discussion

Previous studies of the function of Hdac1 in development of the zebrafish nervous system have revealed important roles for this epigenetic regulator in facilitating the transformation of neural progenitors into neurones and oligodendrocytes. Hdac1 promotes transcription of a core programme of neurogenic regulators, including proneural proteins and other bHLH transcription factors (Cunliffe, 2004; Cunliffe and Casaccia-Bonnefil, 2006; Harrison *et al.*, 2011), and it also attenuates transcription of neural progenitor marker genes, such as the Notch target *her6* (Cunliffe, 2004; Harrison *et al.*, 2011). In the zebrafish hindbrain, Hdac1 functions by rendering neural progenitors competent to respond to the Hedgehog signalling pathway, thereby directing their differentiation into postmitotic neurones (Cunliffe, 2004). Intriguingly, whilst hindbrain neurogenesis and neuronal patterning is severely defective in *hdac1* mutant embryos, the primary patterning processes that establish rhombomeres and define their identities are relatively unaffected by loss of *hdac1* function (Cunliffe, 2004). The spatial control of neurogenesis within rhombomeres is regulated by mechanisms involving Notch-mediated inhibition of neuronal differentiation at rhombomere boundaries (Cheng *et al.*, 2004), and by FGF signalling from *fgf20a*-expressing neurons in the hindbrain mantle region (Gonzalez-Quevedo *et al.*, 2010), which inhibits neurogenesis in the overlying tissue at rhombomere centres. Thus, the domains within which neuronal specification occurs in the hindbrain are defined by the activities of two distinct inhibitory signalling pathways. Here we show that expression of the FGF-regulated gene *erm* in the non-neurogenic central region of each rhombomere, and expression of *fgf20a* in the underlying mantle region, are strictly dependent on the function of Hdac1. These observations are consistent with the known role of Hdac1 as a key regulator of a core neurogenic programme in the zebrafish CNS, since the proneural gene expression that underlies neuronal differentiation in the mantle region, and expression of other markers of post-mitotic neurones, are almost completely extinguished in *hdac1* mutant embryos (Cunliffe, 2004; Harrison *et al.*, 2011). The restriction of *erm* expression to the ventricular zone at rhombomere centres implies a specific function for the Erm transcription factor in neural progenitors, which is consistent with other studies showing a close correlation between loss of *erm* expression and ectopic neurogenesis at rhombomere centres in homozygous *fgf20a* mutant embryos (Gonzalez-Quevedo *et al.*, 2010). Further studies will aim to explore the role of Erm in regulation of neural progenitor behaviour at rhombomere centres. Our results show that loss of Notch pathway activity expands the expression domains of both *erm* and *fgf20a* in the hindbrain. The expanded domain of *erm* transcription observed in the *mind bomb* mutant hindbrain is likely to be a consequence of the increased *fgf20a* expression that results from the ectopic and precocious induction of neuronal differentiation that occurs in *mind bomb* mutants. Thus, we infer that Notch signalling most likely inhibits *erm* transcription in the ventricular zone by inhibiting *fgf20a* transcription in the underlying mantle region at the anterior and posterior ends of each rhombomere. Such a possibility is also consistent with the additional observation that derepression of *erm* expression in the *mind bomb* mutant hindbrain is strictly dependent on neurogenesis-promoting Hdac1 (Fig. 3), implying that Hdac1-dependent feedback from neurons in the mantle region, in the form of secreted Fgf20a protein, induces *erm* expression in the overlying ventricular zone. Future studies will aim to investigate the relationships between the Notch and FGF signalling pathways and the role of Hdac1 in mediating interactions between these two pathways in neural progenitors and their differentiated neuronal progeny.

Materials and Methods

Zebrafish stocks

hdac1^{hi1618} and *mind bomb*^{ta52b} mutant zebrafish were maintained at the University of Sheffield. Animal care and use was in accordance with the UK Animals (Scientific Procedures) Act 1986.

Microinjection of morpholinos

Morpholino sequences were as follows: Hdac1ATG1: 5'-ttg ttc ctt gag aac tca gcg cca t-3'; Hdac1 Mismatch control: 5'-ttg ctc gtt gag aac tct gca cca t-3'.

1-2 nl of 0.3 mM morpholino solution in milli-Q water was microinjected into embryos at the 1-2-cell stage. Embryos were maintained in E3 culture medium at 28.5ºC until required for fixation overnight in 4% paraformaldehyde at 4ºC, then subsequently dehydrated in methanol and stored at -20ºC until required for *in situ* hybridisation.

In situ *hybridisation analysis of gene expression*

Digoxigenin-labelled RNA 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 *erm* probe utilised are available on request.

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