Notch signalling stabilises boundary formation at the midbrain-hindbrain organiser

Kyoko Tossell[1],*, Clemens Kiecker[2], Andrea Wizenmann[3], Emily Lang[1] and Carol Irving[1],†

SUMMARY

The midbrain-hindbrain interface gives rise to a boundary of particular importance in CNS development as it forms a local signalling centre, the proper functioning of which is essential for the formation of tectum and cerebellum. Positioning of the mid-hindbrain boundary (MHB) within the neuroepithelium is dependent on the interface of *Otx2* and *Gbx2* expression domains, yet in the absence of either or both of these genes, organiser genes are still expressed, suggesting that other, as yet unknown mechanisms are also involved in MHB establishment. Here, we present evidence for a role for Notch signalling in stabilising cell lineage restriction and regulating organiser gene expression at the MHB. Experimental interference with Notch signalling in the chick embryo disrupts MHB formation, including downregulation of the organiser signal *Fgf8*. Ectopic activation of Notch signalling in cells of the anterior hindbrain results in an exclusion of those cells from rhombomeres 1 and 2, and in a simultaneous clustering along the anterior and posterior boundaries of this area, suggesting that Notch signalling influences cell sorting. These cells ectopically express the boundary marker *Fgf3*. In agreement with a role for Notch signalling in cell sorting, anterior hindbrain cells with activated Notch signalling segregate from normal cells in an aggregation assay. Finally, misexpression of the Notch modulator *Lfng* or the Notch ligand *Ser1* across the MHB leads to a shift in boundary position and loss of restriction of *Fgf8* to the MHB. We propose that differential Notch signalling stabilises the MHB through regulating cell sorting and specifying boundary cell fate.

KEY WORDS: Midbrain-hindbrain boundary, Organiser, Notch signalling, Chick

INTRODUCTION

In the central nervous system (CNS), cells along the length of the neural plate become separated into populations that do not intermingle to define the broad compartments of forebrain, midbrain, hindbrain and spinal cord (Fraser et al., 1990; Kiecker and Lumsden, 2005). Further specialisation of these domains then occurs through the action of local signalling centres. At the interface of midbrain and hindbrain, the midbrain-hindbrain boundary (MHB) is the best characterised boundary in the developing CNS. It acts as a signalling centre to provide a source of planar signals that pattern the neural plate to specify both tectum rostrally and cerebellum caudally (Marin and Puelles, 1994; Martinez et al., 1995; Reifers et al., 1998; Rhinn and Brand, 2001).

The MHB is first visible as a morphological constriction from Hamburger Hamilton stage 10 (HH10) in the chick embryo (Hamburger and Hamilton, 1992). However, the MHB forms at the interface of the expression domains of the homeobox transcription factors *Otx2* and *Gbx2*, which is already detectable in the neural plate at early stages of neural development (Millet et al., 1996b; Wassarman et al., 1997). At this point along the anteroposterior (A-P) axis, a number of MHB organiser genes are induced, including

the transcription factors *Pax2* and *En1/2* and the secreted molecules *Fgf8* and *Wnt1*. Through an interdependent regulatory loop, they become refined into restricted domains at the MHB and are required for formation and maintenance of the MHB (Hidalgo-Sanchez et al., 1999; Wurst and Bally-Cuif, 2001). *Pax2* is a key inducer of *Fgf8* (Ye et al., 2001), which is proposed to be the principal organiser signal because ectopic introduction of FGF8 protein into the neural tube mimics organiser grafts, leading to ectopic tectal and cerebellar structures (Crossley et al., 1996; Irving and Mason, 2000). Conversely, removal of *Fgf8* from the MHB leads to disruption of tectum and cerebellum (Chi et al., 2003; Reifers et al., 1998).

Otx2 and *Gbx2* are key to determining the position of the boundary. Experimentally shifting their expression border using transgenic mice to drive *Gbx2* more anteriorly or *Otx2* more posteriorly results in a corresponding shift in the position of the MHB (Broccoli et al., 1999; Katahira et al., 2000; Millet et al., 1999). Furthermore, differential expression of *Otx2* and *Gbx2* in midbrain and anterior hindbrain cells leads to the initial segregation of these cell types (Sunmonu et al., 2011). Therefore, *Otx2* and *Gbx2* play a key role in MHB formation by creating two adjacent territories of different cell states, at the junction of which a boundary/organiser cell is induced. However, when both of these genes are removed using homologous recombination, MHB organiser genes remain expressed, albeit over a much broader domain (Li and Joyner, 2001; Martinez-Barbera et al., 2001). Thus, it seems that these genes are not required for the induction of MHB genes, but rather serve to refine and restrict their expression, along with the transcriptional repressor *Grg4* (Sugiyama et al., 2000). Recently, differential levels of Notch activation have been reported at the interface of midbrain and hindbrain compartments, suggesting that Notch signalling may also be important in the process of boundary formation there (Yeo et al., 2007).

[1]Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK. [2]MRC Centre for Developmental Neurobiology, King's College London, 4th Floor, New Hunt's House, Guy's Hospital Campus, London SE1 1UL, UK. [3]Institute of Anatomy, University of Tubingen, Osterbergstrasse 3, D-72074 Tubingen, Germany.

*Present address: MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital, London W12 0HS, UK
†Author for correspondence (carol.irving@gmail.com)

The Notch signalling pathway regulates many developmental processes, including neurogenesis, mesoderm segmentation and formation of compartment boundaries in *Drosophila* eye and imaginal discs. These diverse processes share two common themes – that Notch signalling segregates cell lineages from fields of equivalent cells and defines boundaries between distinct cell populations (Lai, 2004). The best example of Notch signalling defining a boundary is at the dorsoventral (DV) boundary of the *Drosophila* wing imaginal disc. This boundary divides the disc along the dorsoventral axis and also functions as a local organiser to pattern surrounding tissues (Artavanis-Tsakonas et al., 1999). Notch is activated in a restricted stripe at the boundary owing to the action of the glycosyltransferase *Fringe* (*Fng*), which acts in the same cells that express the Notch receptor (Bruckner et al., 2000; Moloney et al., 2000). In *Drosophila*, boundaries of *Fng* expression have been shown to determine where Notch is activated (Wu and Rao, 1999). For example, *Fng* is expressed only in dorsal cells at the DV boundary of the wing disc, where it modulates the Notch receptor to be sensitive to *Delta* (expressed only in ventral cells) and insensitive to *Serrate* (expressed only in dorsal cells). Therefore, *Fng* acts as a switch specifically to activate Notch in a narrow band of the cells along the boundary (de Celis et al., 1996; Fleming et al., 1997; Micchelli and Blair, 1999; Fleming, 1998; Moloney et al., 2000). Previous studies have shown that modulation of *Fng* activity allows cells to move across the boundary (Rauskolb et al., 1999), and that this cell behaviour is dependent upon *Fng* activity through Notch signalling (Milan et al., 2001).

Upon ligand activation, the Notch receptor is cleaved to release an intracellular fragment, which itself is a direct transcriptional activator through binding to Suppressor of Hairless [Su(H)] to activate target genes (Bray and Furriols, 2001). At the DV boundary, this leads to a stripe of cells that express the target gene *Wingless* (*Wg*), which acts as a graded morphogen responsible for organising wing growth and patterning (de Celis et al., 1996).

Recent work has also revealed another role for Notch in regulating DV boundary formation; by modulating the actin cytoskeleton, Notch signalling creates a 'fence' of boundary cells with distinctive expression of F-actin and other actin-binding molecules at adherens junctions. This occurs independently of transcription by an unknown mechanism, but it has been proposed that a destabilisation of adherens junctions may lead to de-adhesion and separation of boundary cells from the compartment (Major and Irvine, 2005; Major and Irvine, 2006).

The Notch signalling cascade may also control boundary formation in the vertebrate CNS. In the hindbrain, compartments are initially formed by restriction of cell movement by cell signalling between members of the Eph-Ephrin receptors and ligands, the expression of which is also restricted within specific and alternating rhombomeres (Cooke and Moens, 2002). At the interface of these domains, Notch signalling promotes the segregation of boundary cells from rhombomere compartments and inhibits neurogenesis (Cheng et al., 2004; Pan et al., 2004). These boundary cells are identified by their elongated fan shape, low rate of proliferation, lack of neurogenesis and the expression of a number of molecular markers (Guthrie and Lumsden, 1991; Trokovic et al., 2005).

In the forebrain, the zona limitans intrathalamica (ZLI) is a boundary and local signalling centre that separates and patterns thalamus and prethalamus. Notch signalling has also been implicated at this boundary as the vertebrate *fng* homologue, lunatic fringe (*Lfng*), is expressed in a restricted pattern here, and cells ectopically expressing *Lfng* are sufficient to disrupt ZLI

formation (Zeltser et al., 2001). *Lfng* has also been reported to have a restricted expression at the MHB, further supporting the possibility that Notch signalling may be involved in boundary formation here (Zeltser et al., 2001). The MHB is a lineage restricted boundary in mouse, zebrafish and chick (Jungbluth et al., 2001; Langenberg and Brand, 2005; Zervas et al., 2004) (K.T. and C.I., unpublished). Furthermore, a population of boundary cells with similar properties to those in the hindbrain has been identified in the most posterior midbrain cells and most anterior hindbrain cells (Trokovic et al., 2005).

Here, we show that members of the Notch signalling pathway are expressed in a compartment-restricted fashion at the MHB, implicating this pathway in MHB boundary formation. We present evidence for a new role for Notch signalling in the formation of the MHB, both in stabilisation of the boundary and in the regulation of expression of the organiser genes *Fgf8* and *Wnt1*. Activation of Notch signalling within cells also causes them to become excluded from rhombomeres 1 and 2 (r1/2) and clustered at boundaries coinciding with borders of *Lfng* expression. We show that the posterior border of expression of both *Lfng* and *Ser1* at the midbrain-hindbrain interface is important for defining the position of the MHB, and for regulating Notch activation there. Furthermore, activation of Notch signalling induces the boundary cell marker *Fgf3*, suggesting that the role of Notch signalling at the MHB may be to specify boundary cell fate.

MATERIALS AND METHODS
Chick embryos
Fertile chick eggs (Brown Bovan Gold; Henry Stewart and Company) were incubated at 38°C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992).

In ovo electroporation of DNA constructs
HH8+,9 chick embryos were electroporated using fine platinum electrodes and an ElectroSquare Porator ECM 830 (BTX) on the following settings; 25 V, four pulses of 50 ms duration and an interval 950 ms. DNA was injected into the neural tube; electrodes were placed on the vitelline membrane either side of the neural tube.

Notch1 intracellular domain (NICD) linked with green fluorescent protein by an internal ribosomal entry site (IRESGFP) was injected at 1 µg/µl (a kind gift from O. Voiculescu, University of Cambridge, UK). pCAB-IRESGFP was used at 0.5 µg/µl (a kind gift from J. Gilthorpe, Umea University, Sweden). Human serrate 1 (amino acids 1-1222) linked with green fluorescent protein by an internal ribosomal entry site (IRESGFP) was used at 2 µg/µl. Truncated human serrate 1 (amino acids 1-1102) lacking most of intracellular domain was linked with IRESGFP at 2 µg/µl (le Roux et al., 2003). Mouse *Lfng*-IRESGFP was used at 1 µg/µl (a kind gift from O. Cinquin, UC Irvine, CA, USA).

In situ hybridisation and immunohistochemistry
In situ hybridisation used digoxigenin- (DIG) and fluorescein- (FITC)-labelled probes as previously described (Irving and Mason, 2000). Both probes were added simultaneously. Alkaline phosphatase (AP)-conjugated anti-DIG and anti-FITC antibodies (Roche) were added sequentially. DIG probes were detected using NBT:BCIP (Roche); FITC probes were detected using FAST TR/Naphtol AS-MX solution (Sigma). Embryos were fixed in 4% paraformaldehyde for 20 minutes before immunohistochemistry as previously described (Irving et al., 2002). Rabbit anti-eGFP antiserum (Clontech) was used at 1:1000. Secondary anti-rabbit HRP was used at 1:200. Embryos were flat mounted or sectioned as previously described (Irving and Mason, 2000).

TUNEL staining
Whole-mount TUNEL analysis was performed using the manufacturer's protocol. Terminal transferase (recombinant) was obtained from Roche (catalogue number 333574).

Bead implantation

FGF8b was introduced on heparin-coated acrylic beads (SIGMA). Control beads were soaked in PBS. DAPT in DMSO (1:1000) and control beads soaked in PBS with an equivalent amount of DMSO were introduced on Affi-Gel Blue beads (150-200 μm, BioRad). Beads were implanted as previously described (Irving and Mason, 2000).

In vitro short term aggregation assay

The assay and data analysis procedures were performed as described previously (Wizenmann and Lumsden, 1997). Briefly, 17 hours after electroporation (HH9/10) with NICD or GFP alone, embryos were isolated and sorted for GFP expression in r1. Electroporation was targeted to one-half of r1 to obtain a mix of labelled and unlabelled cells. As transfection results in varied levels of labelling within a half domain, rhombomeres that contained less than 50% labelled cells were not used. Typically, rhombomeres contained 70% labelled cells.

Eight to thirteen fluorescent r1s without MHB and of the same developmental stage were isolated and pooled for one experiment. Mesenchyme was completely removed and rhombomeres were dissociated into single cells before cells were incubated on a horizontal shaker for 4-5 hours at 37°C. The cultures were fixed and analysed by confocal microscopy.

Aggregates were classified as 'segregated' when they showed distinct clustering of the GFP-expressing cell population. A cluster was defined as association of at least six cells showing the same fluorescence emission. The percentage of 'segregated' aggregates of one well was expressed as segregation ratio. Experiments with high numbers of dead cells, weak aggregation or incomplete or ambiguous fluorescent labelling were excluded from the analysis.

RESULTS

Genes involved in the Notch signalling pathway are expressed with restricted patterns that coincide with the midbrain-hindbrain boundary (MHB)

In order to establish whether Notch signalling was implicated in the formation of the MHB, we examined the expression patterns of Notch signalling pathway genes during the stages when the MHB forms and is maintained as an organiser centre in the neural tube [from Hamburger and Hamilton (HH) 8-12]. The limits of expression of *Otx2* and *Gbx2* demarcate the future MHB from gastrulation stage (HH6) (Wassarman et al., 1997). At HH10, these expression domains abut with a sharp interface (Fig. 1A). This sharp molecular boundary does not coincide with the visible morphological constriction of the MHB until HH16 due to a caudorostral movement of cells between HH10 and HH16 that repositions the MHB constriction at the *Otx2:Gbx2* boundary (Millet et al., 1996a). From three somites (HH8–) *Fgf8* is expressed at the MHB within the anterior *Gbx2* domain (Fig. 1B) (Hidalgo-Sanchez et al., 1999; Shamim et al., 1999). FGF8 constitutes the major organiser signal and in this study defines an 'MHB organiser cell', as FGF8 protein alone is able to mimic MHB organiser tissue grafts in vivo (Crossley et al., 1996; Irving and Mason, 2000).

The Notch1 receptor itself is ubiquitously expressed throughout the neural tube at these stages (Myat et al., 1996). Hence, we investigated the expression patterns of the ligands and associated Notch pathway genes. We found that the Notch ligand *Serrate1* (*Ser1*) is expressed in the midbrain and hindbrain from r2 onwards, but is excluded from the MHB constriction and from r1 at HH10 (Fig. 1C). Double in situ hybridisation revealed that the posterior limit of *Ser1* expression in the midbrain coincides with that of *Otx2* (Fig. 1D). *Serrate2* (*Ser2*) is expressed in a distinctive stripe at the MHB in a domain complementary to *Ser1* (Fig. 1E). The posterior limit of *Ser2* expression is coincident with the expression of the organiser gene *Fgf8* (Fig. 1F). The *Delta1* (*Dl1*) ligand is strongly expressed in r1 and at much weaker levels in the midbrain (Fig. 1G) (Henrique et al., 1995; Myat et al., 1996). The expression domains of *Dl1* and *Otx2* abut at HH10, indicating that the MHB constitutes an interface between high and low *Dl1* expression (Fig. 1H).

Lfng, a glycosyl transferase known to modulate Notch signalling, is expressed in a pattern highly similar to *Ser1* in the HH10 brain (Fig. 1I). The posterior limit of *Lfng* expression in the midbrain coincides with that of *Otx2* (Fig. 1J). Radical fringe (*Rfng*) is expressed ubiquitously at low levels in this region of the neural tube as has previously been reported in mouse (Fig. 1K) (Johnston et al., 1997). Manic fringe (*Mfng*) is tightly restricted to r3 and r5, but is absent from the MHB, r1 or the midbrain (Fig. 1L).

The activation of the Notch signalling pathway is notoriously difficult to monitor in living cells. We analysed the expression of three potential target genes of the Notch pathway: *Hairy1*, *Hairy2* and *Hes5*. *Hairy1* and *Hairy2* are controlled by Notch signalling during the process of somite segmentation (Jouve et al., 2002; Jouve et al., 2000) and *Hes5* is a target of the Notch pathway in chick neurogenesis (Fior and Henrique, 2005). The expression of both *Hairy1* and *Hairy2* is noticeably upregulated at the MHB at HH10 (Fig. 1M,N). *Hes5* is not expressed in the midbrain, MHB and r1, but domains of *Hes5* expression are detected in the diencephalon and in the posterior hindbrain (r3 onwards; Fig. 1O). The expression of *Hes5* in these domains is punctate, suggesting that *Hes5* expression is more likely to reflect neurogenesis throughout the neural tube, whereas the upregulation of *Hairy1* and *Hairy2* may be indicative of a MHB-specific role for Notch activation.

Thus, Notch pathway genes fall into two groups based on their expression domains at the MHB: those that are expressed rostral to and abutting the border, i.e. expressed in the *Otx2*-positive posterior midbrain; and those that are expressed caudal to and abutting the border, i.e. expressed in the *Gbx2*-positive hindbrain (Fig. 1P). These restricted expression domains with borders at the MHB indicate a possible role for the Notch signalling pathway in boundary formation and this idea is also supported by the upregulation of the Notch target genes *Hairy1* and *Hairy2* at the MHB.

Notch signalling is required for organiser gene expression at the MHB

To investigate whether the Notch signalling pathway is normally active during, and necessary for, the formation of the MHB boundary, we sought to block Notch signalling in this region of the neural tube. First, we used a chemical inhibitor of γ-secretase activity, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester), which prevents cleavage of the intracellular Notch fragment, thus preventing activation of target genes (Geling et al., 2002). Affi-Gel Blue beads soaked in DAPT were implanted into the region of the neural tube fated to give rise to the MHB at HH stage 8+ (Fig. 2A). After 6, 12 and 24 hours post-implantation, we analysed the effect of blocking Notch signalling on expression of MHB organiser genes. Following control PBS-soaked bead implantation, the organiser gene *Fgf8* was observed normally at the MHB (Fig. 2B; n=12/12 at 6 hours, n=3/3 at 24 hours). By contrast, only 6 hours after DAPT-soaked beads were implanted, *Fgf8* expression was visibly downregulated (data not shown; n=9/11). After 24 hours, *Fgf8* expression was lost entirely from the MHB in embryos treated with DAPT beads (Fig. 2C; n=2/2). The morphological MHB boundary was also absent in
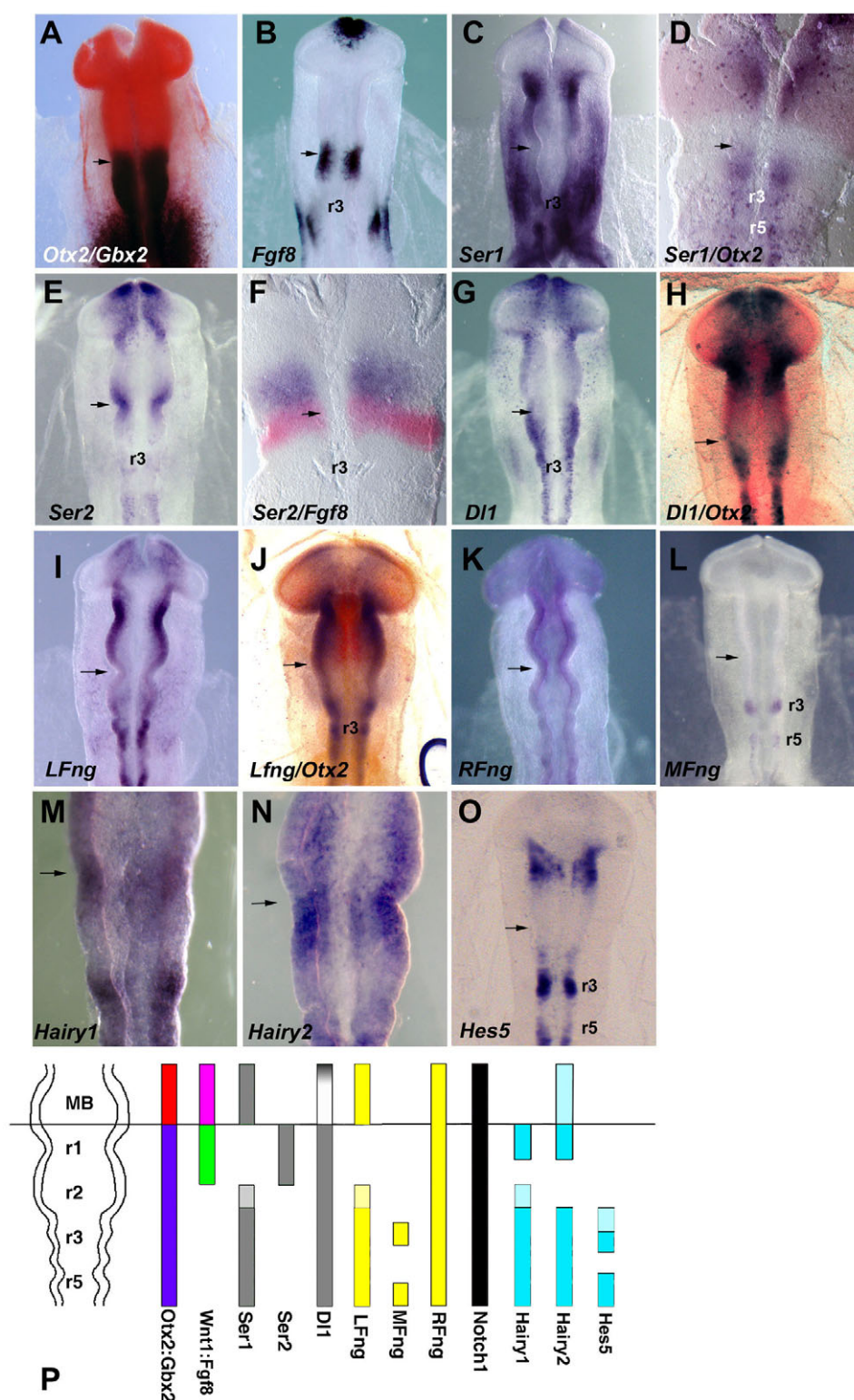
Fig. 1. Notch pathway genes are expressed in restricted patterns at the MHB at HH10. (**A**) In situ hybridisation of *Otx2* (red) and *Gbx2* (blue) indicate the molecular boundary at the MHB. (**B**) *Fgf8* expression indicates the position of the MHB organiser. (**C**) *Ser1* is expressed in the midbrain, diencephalon and posterior hindbrain, but is absent from r1. (**D**) Flat-mounted embryo revealing the posterior limit of expression of *Ser1* (blue) in the midbrain coincides with *Otx2* (red). (**E**) *Ser2* is expressed at the MHB. (**F**) Flat mount of HH12 embryo shows that *Ser2* expression (blue) becomes restricted to the midbrain; its expression abuts *Fgf8* (red). (**G**) *Delta1* is found at high levels in the hindbrain. (**H**) Expression of *Dl1* (blue) and *Otx2* (red) abuts at the MHB. (**I,J**) *Lfng* (blue) is absent from the MHB with a posterior limit of expression in midbrain coinciding with *Otx2* (red). (**K**) *Rfng* is expressed ubiquitously in the neural tube at low level. (**L**) *Mfng* is expressed in r3 and r5. (**M,N**) *Hairy1* and *Hairy2* are upregulated around the MHB. (**O**) *Hes5* is not expressed in the midbrain and r1. Note that Notch pathway genes have expression borders that correspond to the MHB. Black arrows indicate the position of the MHB. (**P**) Summary of gene expression around the MHB. MB, midbrain; r1, rhombomere 1; r3, rhombomere 3; r5, rhombomere 5.

these embryos. *Fgf8* was still present at other sites in the embryo where it is normally expressed, including the branchial arches and nasal placode (Fig. 2C).

In a second, more targeted approach, we used a truncated Ser1 ligand to block Notch signalling. Truncated ligands are able to bind to but not to activate the Notch receptor, and hence block endogenous signalling in a dominant-negative fashion (Hukriede et al., 1997; le Roux et al., 2003). Embryos were electroporated with either truncated Ser1 (Ser1T-GFP) or GFP alone at HH8+ or 9 in the midbrain-hindbrain region of the neural tube. We visualised these cells 24 hours later using a vector where GFP was simultaneously translated via an internal ribosomal entry site (IRES) (Fig. 2D). In control embryos, GFP was observed throughout the neural tube (Fig. 2E), a clear morphological constriction could be seen at the MHB and *Fgf8* was expressed normally there (Fig. 2F; *n*=37/37). By contrast, in embryos electroporated with Ser1T-GFP, *Fgf8* was
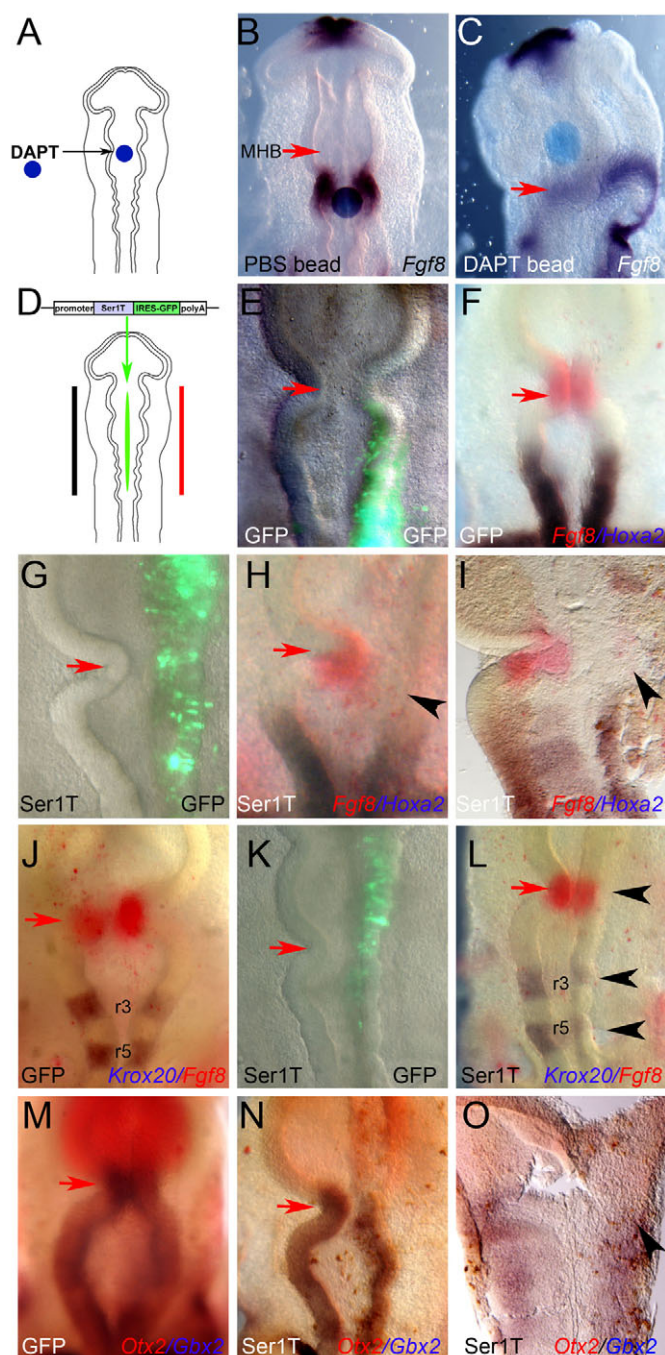
Fig. 2. Notch signalling is required for organiser gene expression at the MHB. (**A**) Schematic representation of bead implantation at HH9. Embryos were analysed 24 hours after bead implantation by in situ hybridisation for *Fgf8*. (**B**) Control bead soaked in PBS (blue). *Fgf8* is expressed normally at the MHB. (**C**) DAPT-soaked bead (blue). *Fgf8* expression is downregulated at the MHB but remains in other sites. (**D**) Schematic representation of the electroporation strategy. Electroporation was carried out at HH8+,9. (**E**) Overlay of GFP fluorescence. (**F**) Electroporation of control GFP followed by in situ hybridisation with *Fgf8* (red) and *Hoxa2* (blue) 24 hours later. (**G-I**) Electroporation of Ser1T-GFP followed by in situ hybridisation (red, blue) and anti-GFP antibody (brown) 24 hours later. (H,I) Expression of *Fgf8* (red) is absent at the MHB (black arrowhead) and *Hoxa2* (blue) is shifted anteriorly on the electroporated side. (**J**) Electroporation of control GFP followed by in situ hybridisation with *Fgf8* (blue) and *Krox20* (red) 24 hours later. (**K**) Overlay of Ser1T-GFP fluorescence. The morphology of the neural tube is flat compared with the control side. (**L**) Expression of *Fgf8* (red) and *Krox20* (blue) are downregulated on the side electroporated with Ser1T-GFP. (**M**) Electroporation of control GFP followed by in situ hybridisation with *Otx2* (red) and *Gbx2* (blue) 24 hours later. (**N,O**) Expression of *Otx2* and *Gbx2* are shifted following electroporation with Ser1T-GFP and the border of expression becomes fuzzy (black arrowheads). Red arrows indicate the position of the MHB. r3, rhombomere 3; r5, rhombomere 5.

dramatically downregulated on the electroporated side compared with the control contralateral side, and in some embryos was completely absent from the MHB (Fig. 2G-I; *n*=17/17). Furthermore, in embryos where both *Fgf8* and *Wnt1* were analysed together, the sharp boundary between their expression domains was lost (data not shown; *n*=6/6). In all cases where truncated *Ser1* was expressed across the MHB, the boundary appeared to be lost and the morphological constriction was absent; the electroporated side of the embryo was markedly straight when compared with the control contralateral side (Fig. 2G). Where Ser1T-GFP extended into the hindbrain, the rhombomere boundaries also appeared to have lost their characteristic constrictions (Fig. 2K,L). Although *Krox20* was observed restricted within rhombomeres 3 and 5 in a normal pattern,

expression was downregulated on the electroporated side (Fig. 2K,L; *n*=6/7) when compared with control embryos electroporated with GFP alone (Fig. 2J; *n*=0/9). We investigated whether the genes involved in initially positioning the boundary, *Otx2* and *Gbx2*, were also affected by blocking Notch signalling through Ser1T-GFP. Their expression interface shifted in a posterior direction; the boundary appeared fuzzy, suggesting there was intermingling of *Otx2*- and *Gbx2*-positive cells at their interface (Fig. 2N,O; *n*=4/5) when compared with both the control side and embryos electroporated with GFP control (Fig. 2M; *n*=0/10). The dominant-negative construct, Ser1T-GFP, is reported to block Notch signalling through all ligands (le Roux et al., 2003). However, as an alternative approach, we repeated the experiments above using a dominant-negative form of *Delta1*, and again observed downregulation of *Fgf8* on the electroporated side compared with the control contralateral side (data not shown; *n*=3/4).

It was possible that the loss of constrictions and molecular markers in the neural tube was due to a loss of neuroepithelial cells. Blocking Notch signalling may cause a failure of lateral inhibition, leading to a decreased progenitor pool and increased neurogenesis (Louvi and Artavanis-Tsakonas, 2006). To examine whether this was occurring, we blocked Notch signalling by electroporating Ser1T-GFP at HH8+,9, then analysed a number of molecular markers of neurogenesis 24 hours later. We did not observe any changes in expression of *Ngn1*, *Ngn2*, *NeuroM*, *NeuroD* or *Delta1* between control and electroporated sides of the embryo, or in control embryos electroporated with GFP alone (see Fig. S1 in the supplementary material; Ngn2 *n*=0/7; NeuroM *n*=0/19; NeuroD *n*=0/9; Delta1 *n*=0/10). This suggests that there is no significant change in neurogenesis at this stage of development, and that the straight neural tubes observed were more likely to be as a result of lack of boundary constrictions rather than a decrease in the number of cells. Together, these results suggest that Notch signalling is required for the proper formation of morphological boundary constrictions in the neural tube and for organiser gene expression at the MHB.
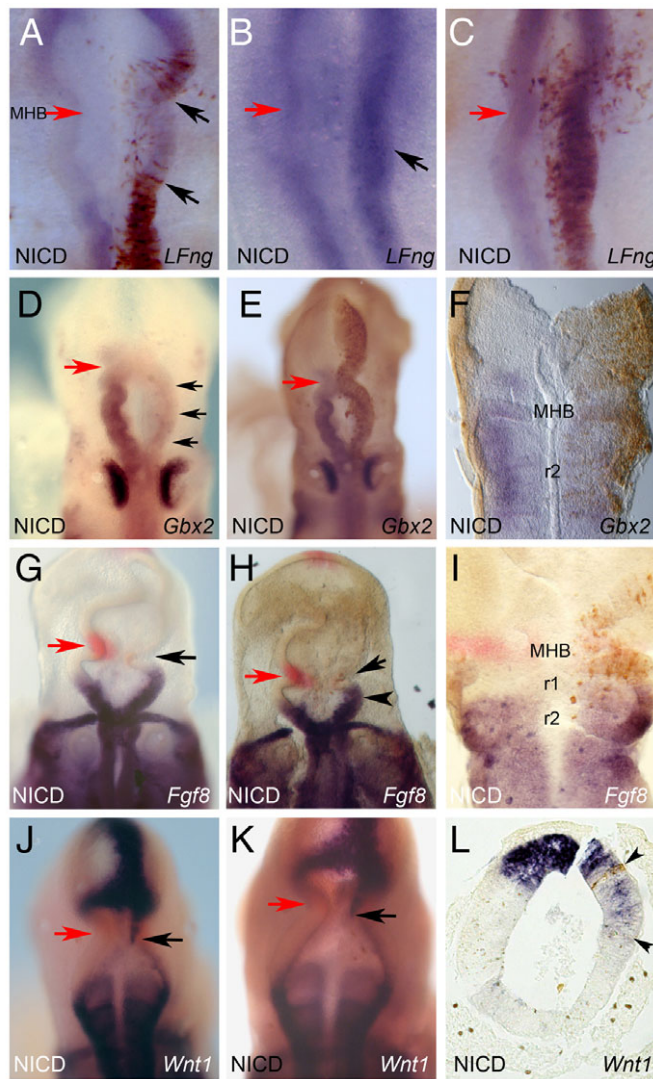
Fig. 3. Expression of MHB organiser genes is disrupted in embryos ectopically expressing high levels of activated Notch. (A-L) Electroporation of NICD-GFP at HH8+,9. (**A-C**) In situ hybridisation of *Lfng*. (A) NICD-GFP-expressing cells are excluded from r1/2 where *Lfng* is normally absent, but are clustered in domains of *Lfng* expression (black arrows). (B,C) *Lfng* expression is upregulated when NICD-GFP cells are seen throughout the r1/2 domain (black arrow). (**D-F**) *Gbx2* (blue). *Gbx2* is downregulated on the electroporated side of the embryo (black arrows). (F) Flat-mount view shows a shift of the *Gbx2* rostral limit. (**G-I**) *Fgf8* and *Hoxa2* (blue). *Fgf8* is downregulated on the electroporated side (black arrows). NICD-GFP-positive cells are present at the MHB where *Fgf8* is downregulated. (I) Flat-mount view shows the anterior border of *Hoxa2* expression is shifted rostrally. (**J-L**) *Wnt1* (blue) and *Hoxa2* (blue). *Wnt1* is expanded dorsally on the electroporated side of the embryo (black arrows). (K) NICD-GFP-positive cells are detected across the MHB. (L) Transverse section of the MHB shows non-cell autonomous ectopic *Wnt1* expression. Black arrowheads indicate the extent of ectopic *Wnt1*. Anti-GFP antibody is in brown. Red arrow indicate the position of the MHB. MHB, mid-hindbrain boundary; r1, rhombomere 1; r2, rhombomere 2.

Notch activation regulates specification of the MHB

To investigate further the role of Notch signalling at the MHB, we introduced a dominant-active form of Notch into the anterior hindbrain and posterior midbrain at stages when the MHB

boundary is being established. Despite the complexity of the Notch signalling pathway, it has been reported that the cleaved intracellular fragment of the Notch receptor (NICD) alone is sufficient to activate Notch signalling within cells both in vitro and in vivo (Schroeter et al., 1998; Takke and Campos-Ortega, 1999). We observed two different phenotypes following electroporation of NICD into chick embryo neural tubes at HH stage 8+ or 9, with analysis 24 hours later. First, in some cases NICD-transfected cells were restricted to two domains at the anterior and posterior limits of the electroporation field, which appeared to correlate with clustering to the *Lfng* expression borders (Fig. 3A). Control cells electroporated with GFP alone were observed throughout the midbrain and hindbrain in a continuous manner (data not shown). Second, we observed changes in expression of MHB organiser genes and the Notch modulator *Lfng* in embryos where specific localisation of activated Notch cells was not observed. Strikingly, in those embryos where activated Notch cells were not clustered to the *Lfng*-positive domains, we observed ectopic upregulation of *Lfng* expression within r1/2 (Fig. 3B,C; *n*=16/16).

We analysed *Gbx2* expression, which marks the position of the MHB by expression in hindbrain cells up to and abutting the boundary. *Gbx2* was downregulated throughout the activated Notch electroporated domain such that the rostral boundary of *Gbx2* shifted posteriorly when compared with the control contralateral side and control electroporated embryos (Fig. 3D-F; *n*=11/16). *Fgf8* is normally expressed within *Gbx2*-positive cells in anterior hindbrain. *Fgf8* was also specifically downregulated on the electroporated side of activated Notch embryos when compared with the control contralateral side (Fig. 3G-I; *n*=41/60). *Hoxa2* normally has an anterior limit of expression at the r1/2 boundary (Davenne et al., 1999). In embryos where *Fgf8* was downregulated, a rostral shift of *Hoxa2* expression was observed (Fig. 3G-I; *n*=20/34). The anterior limit of *Hoxa2* expression has previously been shown to be regulated by *Fgf8* at the MHB. Thus, the observed shift in *Hoxa2* expression is most probably due to a lack of *Fgf8*, which normally acts to repress *Hoxa2* in r1 (Irving and Mason, 2000).

In *Drosophila*, *wingless* has been shown to be a target of Notch signalling at the DV boundary of the wing disc (Couso et al., 1995; de Celis et al., 1996; Kim et al., 1995). *Wnt1* is normally expressed in midbrain cells on the anterior side of the MHB. In embryos ectopically expressing activated Notch, *Wnt1*-expressing cells could be observed extending into the anterior hindbrain, throughout the *Fgf8* domain (Fig. 3J-L; *n*=15/21). At the MHB, induction of *Wnt1* by activated Notch cells did not appear to be cell autonomous, as ectopic *Wnt1* expression was observed at a distance from the electroporated cells (Fig. 3L). Thus, high levels of activated Notch appear to repress anterior hindbrain fate (posterior MHB) and promote posterior midbrain fate (anterior MHB).

Notch activation causes cells to be excluded from the metencephalon (r1/2) and clustered at boundaries

To further investigate the cell localisation phenotype, we again electroporated NICD into chick embryo neural tubes at HH8+,9. 24 hours after electroporation, control cells electroporated with GFP alone were observed throughout the midbrain and hindbrain in a continuous manner (*n*=21/21) (Fig. 4A). By contrast, two phenotypes were caused by electroporation of NICD, dependent on the density of electroporated cells: high numbers of electroporated cells caused molecular changes within r1/2, whereas low numbers of electroporated cells caused specific exclusion from r1/2 such that
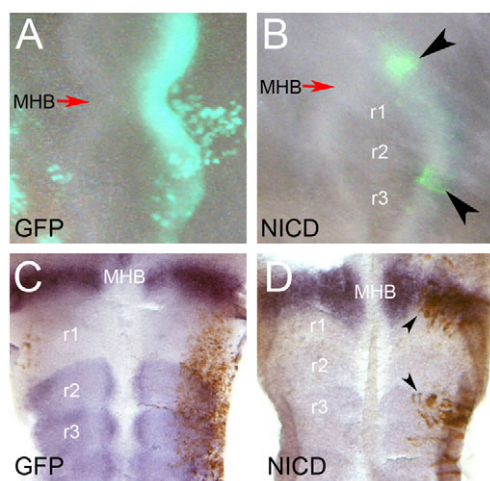
Fig. 4. Cells expressing activated Notch are excluded from metencephalon (r1,2). (**A**,**B**) Dorsal view of GFP fluorescence. (**C**,**D**) Flat-mounted neural tube opened along the dorsal midline. In situ hybridisation marks the MHB with *Fgf8* (blue) and posterior rhombomeres from r2 with *Hoxa2* (blue). Immunohistochemistry marks GFP protein (brown). (A,C) Control GFP-expressing cells in a continuous line within the neural tube 24 hours post-electroporation. (B,D) NICD-GFP expressing cells are found clustered at the MHB and posterior from r2/r3 boundary (black arrowheads). MHB, mid-hindbrain boundary; r2, rhombomere 2; r3, rhombomere 3.

Fig. 5. R1 cells expressing NICD separate from others. (**A-D**) Aggregates of r1 cells mixed with cells expressing either NICD (A,B) or GFP (C,D). (A,C) Overlay of fluorescence with entire aggregate. (B,D) Transfected fluorescent cells alone. (A,B) Aggregates containing cells transfected with NICD, which segregated from the untransfected r1 cells. (C,D) Aggregates containing cells transfected with GFP. GFP-positive cells mix freely with GFP-negative r1 cells. (**E**) Quantitative analysis of aggregates containing different cell populations. The histogram depicts the segregation ratio of aggregates (percentage of segregated versus mixed aggregates) formed by GFP- or NICD-expressing cells, and untransfected r1 cells. GFP and NICD cells show different segregation ratios (25 versus 58) that are significantly different from each other (χ^2; $P \le 0.01$). Data are mean±s.e.m.

NICD-transfected cells became restricted to two domains at the anterior and posterior limits of the metencephalon (r1/2) (Fig. 4B). An analysis of *Fgf8* expression (MHB) and *Hoxa2* expression (the anterior limit of expression of which demarcates the r1/2 boundary) of such embryos confirmed that NICD-expressing cells were not observed in r1 or r2, but were restricted to the boundaries anterior and posterior to this domain (Fig. 4D; *n*=30/55). These data suggest that the coverage of activated Notch in a field of cells is important in determining cellular fate. It is possible that ectopically expressing activated Notch in a sparse population of cells causes repositioning, whereas activating Notch signalling in a dense population of cells causes cell fate changes.

Two possible explanations could account for the exclusion of NICD cells from r1/2. First, cells containing activated Notch may become apoptotic and die. Second, NICD-electroporated cells may acquire different properties from their non-transfected neighbours and move to regions of the neural tube where Notch is normally active and cells display similar cell surface properties, as previously described in the zebrafish hindbrain (Cheng et al., 2004). To distinguish between these possibilities, we analysed embryos using TUNEL staining to detect apoptotic cells. In embryos where both activated Notch and GFP were introduced at HH8+ or 9, no increase in apoptosis was observed 24 hours later (*n*=53/64) when compared with control embryos containing GFP alone (*n*=41/44; see Fig. S2A-F in the supplementary material). In fact, NICD appeared to decrease the number of apoptotic cells, consistent with the known role of Notch in maintaining the undifferentiated state of cells (see Fig. S2E in the supplementary material) (Miele and Osborne, 1999). Similar results were observed in embryos at 6 and 12 hours post-electroporation (data not shown).

These results indicate that the absence of NICD-electroporated cells from r1 and r2 is not due to cell death, but instead favour the hypothesis that cells are actively being excluded from this domain.
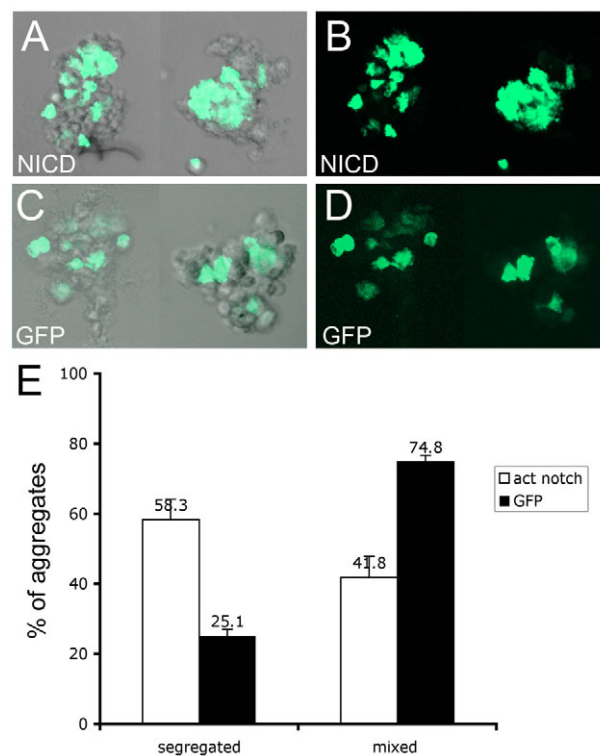
Furthermore, this led us to hypothesise that the clustering of activated Notch cells at the MHB and r2/3 boundary could reflect a requirement for Notch signalling at these boundaries.

Notch activation causes cell adhesive differences in r1 cells

To test whether NICD-expressing r1 cells actively separate from other cells, we performed an in vitro short-term cell re-aggregation assay (Wizenmann and Lumsden, 1997). R1s, of which one half was transfected with NICD, were dissociated and then allowed to aggregate and segregate for 4-5 hours. The resulting aggregates were analysed by microscopy and classified as either segregated or mixed. Aggregates were classified as 'segregated' when they showed distinct clustering of the GFP-expressing cell population. A cluster was defined as association of at least six cells showing the same fluorescence emission (Fig. 5). Control aggregation cultures, where r1 cells expressed GFP alone, revealed a baseline segregation ratio of 25% (*n*=175; Fig. 5C,D,E.). Aggregates of cells transfected with NICD showed a segregation ratio of 58% (*n*=176; Fig. 5A,B,E). The segregation ratio of NICD-positive cells was
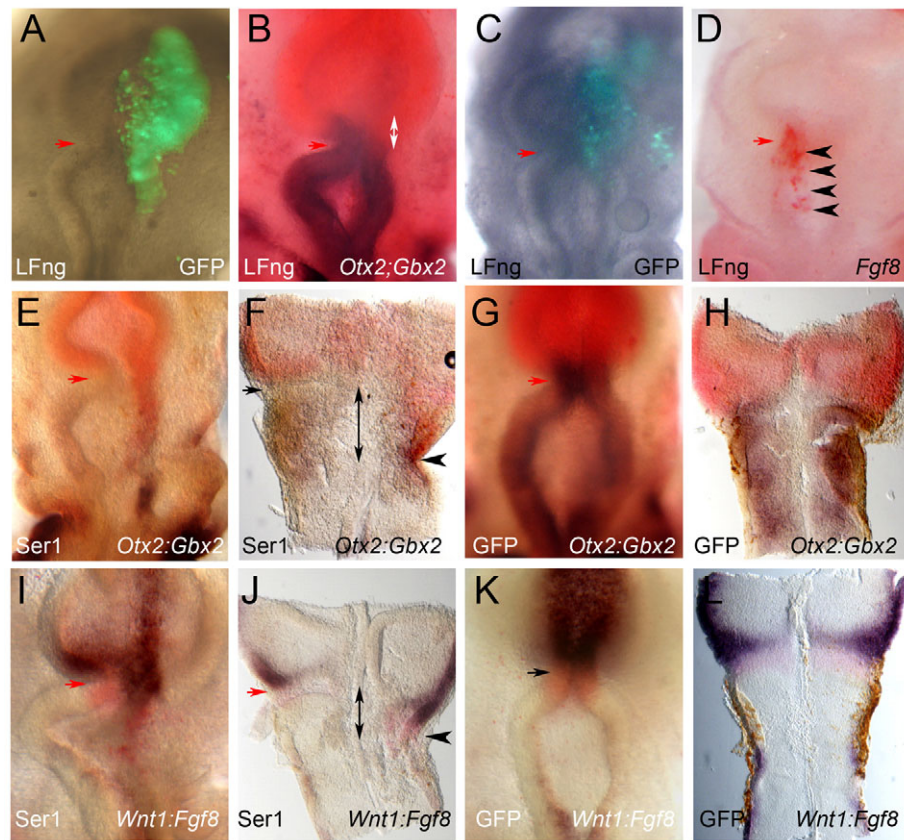
DEVELOPMENT

Fig. 6. Disrupting the *Lfng* or *Ser1* molecular border causes disruption of the MHB boundary. Embryos were electroporated at HH8+,9 with (A-D) *Lfng*-GFP, (E,F,I,J) Ser1-GFP and (G,H,K,L) GFP control. (A,C) Overlay of GFP fluorescence. (**A**,**B**) *Lfng*-GFP electroporated across the MHB shifts the boundary of *Otx2* (red) and *Gbx2* (blue) expression (white double-headed arrow). (**C**,**D**) *Fgf8* (red)-expressing cells are not restricted at the MHB but are seen throughout r1 following electroporation of *Lfng*-GFP across the MHB (black arrowheads). (**E**,**F**) Ser1-GFP electroporated across the MHB shifts the boundary of *Otx2* (red) and *Gbx2* (blue) expression when compared with the control contralateral side. Note a shifted morphological constriction can be seen (black arrowhead) at the new *Otx2:Gbx2* interface. (**G**,**H**) Normal *Otx2:Gbx2* expression and morphological constriction in control GFP embryos. Black double-headed arrow indicates shift of both the *Otx2:Gbx2* interface and the morphological boundary. (**I**,**J**) *Fgf8* (red) and *Wnt1* (blue) expression shifts to a new morphological constriction in Ser1-GFP electroporated embryos when compared with control contralateral side (black arrowhead). Black double-headed arrow indicates shift of both the *Wnt1:Fgf8* interface and the morphological boundary. (**K**,**L**) Normal expression of *Fgf8* (red) and *Wnt1* (blue) in a tightly restricted stripe at the MHB in control GFP embryos. Red arrow indicates the position of the MHB.

significantly higher than that of the GFP-positive cells (χ^2; $P \leq 0.01$). Thus, cells of r1/r2 that express activated Notch seem to develop different adhesive/surface properties to untransfected r1/r2 cells. These results support a hypothesis that activated Notch could lead to activation of target genes responsible for affinity differences between boundary and non-boundary cells. Thus, cells containing active Notch within the r1/r2 compartment would either preferentially move to boundary regions or be excluded from non-boundary regions.

Restricted *Lfng* and *Ser1* expression is required to maintain the MHB and stabilise the formation of the organiser at the boundary

Both *Lfng* and *Ser1* are expressed in the midbrain with a posterior border of expression coincident with that of *Otx2* at the MHB (Fig. 1D,F). In *Drosophila*, boundaries of *Fng* and Notch ligand expression have been shown to determine where Notch is activated (de Celis et al., 1996; Fleming et al., 1997; Micchelli and Blair, 1999; Wu and Rao, 1999). We hypothesised that the limits of expression of *Lfng* and *Ser1* at the MHB may similarly signify a

zone of active Notch signalling, causing cells expressing NICD to relocate to this domain where the surrounding cells share similar cell-surface properties, i.e. a field of cells where Notch is active. Indeed, the location of NICD-expressing cells correlated with the edges of *Lfng*-positive domains. Furthermore, NICD cells were excluded from the r1/2 domain which does not express *Lfng* (Fig. 3A).

To test the hypothesis that the border of *Lfng* and *Ser1* expression is important for determining where Notch is active and the hence position of the MHB boundary, we perturbed the sharp expression border of both genes by ectopic expression of *Lfng* or *Ser1* across the expression domain and into r1/2 and examined the MHB boundary markers *Otx2* and *Gbx2*, and the organiser gene *Fgf8*. Introduction of ectopic and mosaic expression of *Lfng* throughout the MHB and into r1/2 at HH8+,9 resulted in a dramatic shift of the *Otx2:Gbx2* boundary on the electroporated side when compared with the control contralateral side. *Otx2*-positive cells were observed more caudally and the boundary appeared less well defined than the control contralateral side. These cells were always observed within the field of cells ectopically

expressing *Lfng* (Fig. 6A,B; *n*=5/6). To determine what effect this might have on the organiser itself, we next looked at *Fgf8* expression following *Lfng* misexpression. In control embryos, *Fgf8* was expressed in a restricted stripe in anterior r1 (*n*=30/30). Following ectopic expression of *Lfng* across the MHB, *Fgf8*-positive cells appeared to lose their restriction to the boundary and were observed scattered throughout r1 (Fig. 6C,D; *n*=5/7).

Ectopic expression of *Ser1* throughout the MHB and anterior hindbrain again resulted in a dramatic shift of the *Otx2:Gbx2* boundary on the electroporated side when compared with the control contralateral side. A new morphological boundary was apparent at the new *Otx2:Gbx2* boundary (Fig. 6E,F; *n*=6/6). Embryos electroporated with control GFP alone maintained a sharp *Otx2:Gbx2* and morphological boundary in all cases (Fig. 6G,H; *n*=14/14). We also saw a corresponding shift in *Fgf8* and *Wnt1* expression following *Ser1* misexpression. Again, the morphological boundary shifted caudally on the electroporated side, and the *Wnt1* and *Fgf8* expression stripes either aligned to the new constriction or expanded to occupy the space between the levels of the original and new constrictions (Fig. 6I,J; *n*=26/26). Embryos electroporated with control GFP alone maintained sharp *Wnt1:Fgf8* stripes and morphological boundary in all cases (Fig. 6K,L; *n*=17/17). These results suggest that the posterior border of both *Lfng* and *Ser1* expression at the posterior midbrain compartment is important for determining where the MHB boundary will form, at both a molecular and morphological level.

To further investigate our hypothesis that Notch might be activated at the border of *Lfng* and *Ser1* expression, we misexpressed mouse *Lfng* and analysed embryos for changes in chick *Lfng* expression using a probe that does not recognise the mouse *Lfng* electroporation construct. *Lfng* is both a direct target of Notch, through its CBF1/RBP-J binding sites [and we observed upregulation of *Lfng* by NICD (Fig. 3B,C)] (Cole et al., 2002; Morales et al., 2002) and acts as a glycosyltransferase to modify Notch to either promote or suppress signalling (Chen et al., 2001; Chen et al., 2005; Dale et al., 2003; Moloney et al., 2000; Rampal et al., 2005). Twenty-four hours electroporation of mouse *Lfng* into the neural tube at HH8+,9, chick *Lfng* was upregulated on the electroporated side compared with the control contra-lateral side (Fig. 7A,B; *n*=6/14). *Lfng* expression was normal in embryos electroporated with GFP alone (Fig. 7C,D; *n*=9/9). This suggests that Lfng acts positively to promote Notch signalling in this region.

MHB genes do not regulate Notch pathway genes

Misexpression of Notch ligands, the modulator Lfng or Notch itself all perturb MHB gene expression (namely *Otx2*, *Gbx2*, *Fgf8* and *Wnt1*), suggesting that Notch signalling is either upstream of MHB gene expression or linked in a crossregulatory loop. To investigate in more detail how expression of Notch pathway genes relates to the MHB gene regulatory network, we used FGF8b-coated beads to identify any crossregulation; ectopic FGF8b protein in midbrain induces MHB organiser around it and reorganises MHB genes, i.e. *Fgf8* and *Gbx2* are upregulated and *Otx2* is downregulated around the bead (Crossley et al., 1996; Irving and Mason, 2000; Shamim and Mason, 1999). Either FGF8- or PBS-soaked beads were inserted into the neural tube at HH8+,9 and 24 hours later embryos were analysed for expression of *Lfng*, *Ser1*, *Ser2*, *Delta1*, *Notch1* and *Fgf8* (positive control). *Fgf8* was observed ectopically expressed around the bead as has previously been reported (Crossley et al., 1996; Irving and Mason, 2000; Shamim and Mason, 1999). No change in *Fgf8* expression was observed using PBS control beads. We did not observe any change in gene
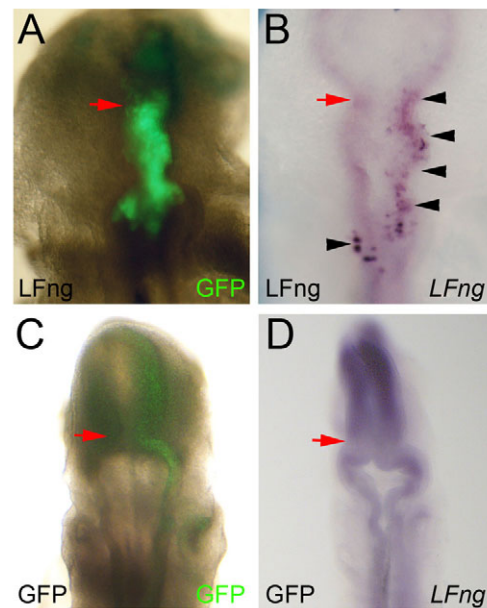

Fig. 7. Misexpression of mouse *Lfng* at the MHB positively regulates chick *Lfng* expression. (A,C) Overlay of GFP fluorescence. (B,D) In situ hybridisation for *Lfng* (blue). (**A**,**B**) Electroporation of Lfng-GFP in the neural tube causes upregulation of *Lfng* expression (blue) within the electroporated domain (black arrowheads). (**C**,**D**) No change in *Lfng* expression following electroporation of GFP control. Red arrow indicates the position of the MHB.

expression of Notch pathway genes with either FGF8- or PBS-soaked beads (Table 1). This suggests that during the boundary formation phase of MHB development, Notch signalling is upstream of MHB genes.

Notch signalling at the MHB specifies boundary cell fate

Specialised boundary cells arise between compartments, once a stable interface has been established between them. One explanation for the clustering of activated Notch cells out of r1/2 and into boundary regions is that those cells have become respecified to a boundary cell fate. Subsequent expression of boundary-specific adhesion molecules could lead to the cell-sorting phenomena observed (Figs 4 and 5). We therefore investigated the effect of activating Notch signalling on the boundary cell marker, *Fgf3* (Mahmood et al., 1995). In embryos electroporated with GFP control, *Fgf3* was expressed normally in a narrow stripe at the MHB, as well as in rhombomere boundaries posteriorly and in r4/5

Table 1. FGF8-soaked beads inserted into midbrain have no effect on expression of Notch pathway genes

Gene	FGF8-soaked bead	PBS-soaked bead
Lunatic fringe	0/11	0/5
Ser1	0/12	0/6
Ser2	0/6	0/3
Delta1	0/6	0/3
Notch1	0/3	0/3
Fgf8	2/2	0/2

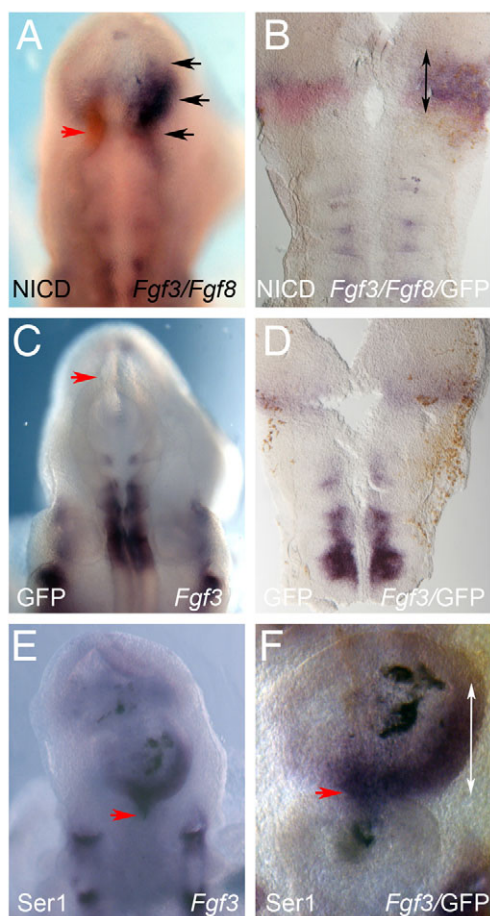The number of embryos showing a change in gene expression following FGF8 or control treatment.

Fig. 8. Notch signalling induces the boundary marker *Fgf3*. Electroporation of (A,B) NICD, (C,D) GFP control, (E,F) Ser1 at HH8+/9 followed by in situ hybridisation for *Fgf3* (blue) and *Fgf8* (red) and immunohistochemistry with anti-GFP (brown) after 24 hours incubation. (B,D) Flat-mounted neural tubes. (F) High-magnification image of MHB region of embryo shown in E. (**A,B**) *Fgf3* expression is expanded into the midbrain on the side electroporated with NICD (black arrows in A, double-headed arrow in B). (**C,D**) No change in *Fgf3* expression following control electroporations. (**E,F**) *Fgf3* expression is expanded into the midbrain on the side electroporated with Ser1 (double-headed arrow in F). Red arrows, MHB.

Fig. 9. A comparative model for Notch signalling in boundary formation in the *Drosophila* wing imaginal disc and at the MHB. (Left) *Fng* is expressed in dorsal cells of the *Drosophila* wing disc, where it modulates the Notch receptor to be sensitive to Delta (expressed only in ventral cells) and insensitive to Serrate (expressed only in dorsal cells), resulting in a narrow stripe of Notch activation along the DV compartment boundary. Notch activation (*) leads to *wingless* expression that regulates wing patterning and outgrowth. (Right) *Lfng* and *Ser1* are expressed on one sid of the MHB, in the *Otx2*-positive midbrain, whereas *Dl1* is expressed on the other side, in the *Gbx2*-positive hindbrain, suggesting a narrow stripe of Notch activation (*) along the MHB that results in the induction of *Wnt1* and in the positional refinement of *Fgf8* expression. Note that Notch signalling is required, but is not sufficient, for *Fgf8* induction (dashed arrow).

(Fig. 8A,B; *n*=36/36). By contrast, in embryos electroporated with NICD, *Fgf3* was expanded into the midbrain on the electroporated side (Fig. 8C,D; *n*=9/11). Ectopic expression of Ser1-GFP in the neural tube also resulted in an expansion of *Fgf3* on the electroporated side when compared with the control contralateral side (Fig. 8E,F; *n*=17/25). Expansion of *Fgf3* at hindbrain boundaries was also observed (data not shown) and in two cases, ectopic stripes of *Fgf3* were observed throughout r1/2 (data not shown). This data suggests that the function of Notch signalling at the MHB may be to specify boundary cell fate.

DISCUSSION
Current models for boundary formation at the MHB

It is a developmental principle that local organisers form along, and are stabilised by, cell-lineage restriction boundaries (Garcia-Bellido et al., 1973; Kiecker and Lumsden, 2005). Here, we have
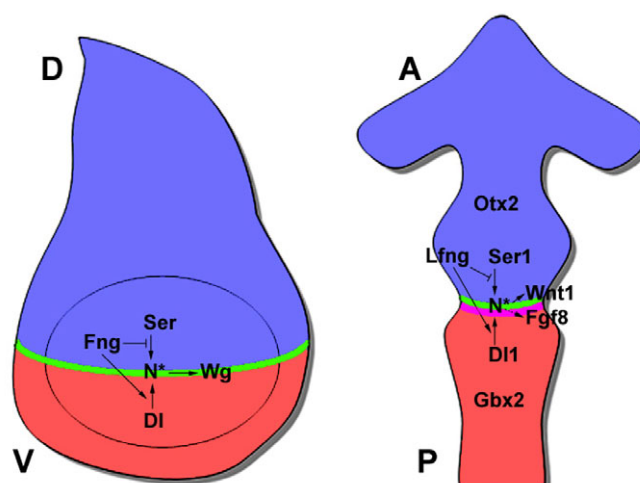
investigated how cell lineage restriction between the midbrain and hindbrain is established, in order to understand how the MHB organiser forms there. Notch activation at the interface between the midbrain and hindbrain has been demonstrated previously in the zebrafish embryo: Yeo et al. used the Hes4 regulatory elements to identify the spatio-temporal pattern of Notch signalling in the CNS, revealing elevated Notch activation in midbrain but none in r1/2 (Yeo et al., 2007). We have now demonstrated that Notch signalling is important in the process of boundary formation between these two compartments.

Previous studies on MHB formation have focused on the transcription factors *Otx2* and *Gbx2* as the interface of their expression domains demarcates the position of the boundary from early stages of neural development (Millet et al., 1996a; Wassarman et al., 1997). Misexpression studies have revealed that the position of the MHB along the anteroposterior axis of the neural tube is dependent on this expression border (Broccoli et al., 1999; Katahira et al., 2000; Millet et al., 1999). However, in the absence of both of these genes, MHB organiser genes remain expressed, although their expression is not restricted to the boundary, indicating that these genes are important for the spatial refinement, rather than the induction, of genes associated with the organiser (Li and Joyner, 2001; Martinez-Barbera et al., 2001). Recent work has shown that differential expression of *Otx2* and *Gbx2* contributes to the segregation of midbrain and hindbrain cells, yet the mechanisms by which these two genes determine the position of the boundary are unclear, particularly as they function as transcriptional repressors (Glavic et al., 2002; Sunmonu et al., 2011).

The Fng/Ser1 border determines where the MHB will form by activating Notch signalling

Fng expression domains coincide with boundary formation in a number of diverse tissues. In *Drosophila*, this includes the DV boundary in the eye and the DV boundary in the wing imaginal disc (Babcock et al., 1998; Rauskolb et al., 1999). In vertebrates, fringe genes are involved in boundary formation at the apical ectodermal ridge (AER) in the limb bud, during somitogenesis, in the ZLI in the forebrain and in rhombomere boundaries in the hindbrain (Amoyel et al., 2005; Dale et al., 2003; Laufer et al., 1997; Pan et al., 2004; Zeltser et al., 2001). The positioning of a sharp border between *Fng*-expressing and *Fng*-nonexpressing cells is important: in the *Drosophila* eye, a new equator forms wherever there is an interaction between cells expressing different levels of *Fng* (Dominguez and de Celis, 1998). Furthermore, ectopically expressing *Fng* in the avian forebrain disrupts ZLI formation (Zeltser et al., 2001). We have shown that the posterior borders of *Lfng* and *Ser1* expression, as well as the anterior borders of *Dl1* and *Ser2*, coincide with the MHB boundary. Ectopic expression of *Lfng* or an activated form of *Ser1* posteriorly, across the *Otx2/Gbx2* border, resulted in either a shift of the MHB or in a complete breakdown of restriction of the organiser gene *Fgf8* to the MHB.

In the *Drosophila* wing imaginal disc, *Fng*, which is exclusively expressed in the dorsal compartment, reduces the sensitivity of Notch to Serrate (dorsal) and increases its sensitivity to Delta (ventral), resulting in a narrow stripe of Notch pathway activation along the dorsoventral compartment boundary (Panin et al., 1997). Ectopic *Fng*-positive clones in the ventral compartment induce *wg* expression at their interface (through activation of Notch), but do not sort towards the boundary. This suggests that *Fng* clones located at the boundary redefine it by activating Notch signalling leading to *wg* expression, rather than sorting across it (Micchelli and Blair, 1999; Rauskolb et al., 1999). Our expression analysis of *Dl1*, *Ser1* and *Lfng* has revealed a distribution of these factors around the MHB that is almost identical to that of their orthologues in the *Drosophila* wing disc. Based on their expression patterns, we propose a model where Lfng (expressed anterior to the MHB) modulates the affinity of Notch for Dl1 (posterior to the MHB) and Ser1 (anterior to the MHB), resulting in a narrow stripe of Notch pathway activation at the MHB. Furthermore, new evidence suggests that Ser2 acts as a Dl-like Notch ligand. Lfng potentiates signalling through both Dl1 and Ser2, but inhibits signalling through Ser1 (Van de Walle et al., 2011). As we observe Ser2 expressed in the same domain as Dl1 it is possible that these two ligands are acting in concert to activate Notch at the boundary. We have also discovered a positive-feedback loop with Lfng upregulating its own expression, suggesting that Notch activation potentiates itself at the MHB, possibly resulting in a stabilisation of boundary fate (Fig. 9).

Lfng has been suggested to directly induce cell sorting in the chick forebrain (Zeltser et al., 2001). We did not observe cells expressing ectopic *Lfng* sorting to the MHB in a similar manner to those expressing activated Notch, although our data suggest that Lfng increases Notch activity. This may be either due to a difference in the level of Notch activation, or due to the direct activation of Notch target genes by NICD, whereas Lfng merely potentiates the activation of Notch but still requires a Notch-ligand interaction. Our findings are therefore in keeping with observations in *Drosophila* that ectopic expression of *Fng* is not sufficient to drive cells to sort. Further support for this hypothesis comes from the finding that, as at the DV boundary in the *Drosophila* wing

disc, cell adhesion at the MHB is mediated independently of *Fng* by members of the leucine-rich repeat (LRR) family (Milan et al., 2005; Milan et al., 2001; Tossell et al., 2011).

Notch specifies boundary cell fate

Our findings provide evidence that activation of Notch in the neural tube results in specification of boundary cell fate. Ectopic expression of the boundary cell marker *Fgf3* was observed following ectopic activation of the Notch pathway. Furthermore, ectopically activated Notch cells were found clustered at boundaries. One possibility is that respecification to a boundary cell fate by activation of Notch regulates a cell affinity difference between rhombomere compartment (non-boundary) cells and those at the border of *Lfng* expression domains (boundary cells), which causes cells to segregate to the boundary region. Our finding that cells containing activated Notch preferentially clump together in an aggregation assay supports this. However, the proteins responsible for any affinity change remain unknown.

The number of ectopically activated Notch cells within the electroporated field determined whether cells became relocated within the neural tube, or whether they caused cell fate changes within the ectopic domain. In embryos where cell fate changes were observed, it is possible that sufficient cells had been driven to express activated Notch that they created a new environment due to a community effect (Gurdon et al., 1993). Indeed, counting the total number of electroporated cells per rhombomeric segment revealed that when large numbers of activated Notch cells were present, only the cell fate change phenotype was observed. This suggests that when the number of ectopically activated Notch-expressing cells is small and they are distributed with a low density within the tissue, cells relocate to the morphological boundary, i.e. their preferred environment. When greater numbers of ectopically activated Notch-expressing cells are present and they are closely packed with neighbouring cells sharing similar cell-surface properties, a community effect would result in a new stable environment created by the cells themselves, so that they have no need to relocate. Alternatively, it is possible that the dose of Notch activation is crucial in determining cellular outcome as observed in other processes that use Notch signalling (Louvi and Artavanis-Tsakonas, 2006). Despite a controlled amount of DNA injected into the neural tube, electroporation does not allow for precise quantity control.

A model of boundary and organiser formation at the MHB

Taken together, our results support a model where Notch is activated in a narrow band at the midbrain-hindbrain interface, which is determined by the border of *Fng/Dl1* expression in a similar manner to that at the *Drosophila* DV boundary in the wing disc (de Celis and Bray, 1997; Micchelli and Blair, 1999; Rauskolb et al., 1999). The primary role of Notch at the MHB may be to specify boundary cell fate: activated Notch induced expression of the boundary marker *Fgf3*. Because manipulating Notch signalling disrupts the *Otx2:Gbx2* expression interface, yet misexpression of FGF8 protein has no effect on Notch genes, we propose that Notch is upstream of MHB organiser formation in a molecular cascade, at least with respect to the establishment of stable boundary. Notch may directly regulate organiser genes, or the changes that we have observed may be due to a secondary effect of disrupting boundary specification. Once induced, MHB organiser genes become interdependent through complex crossregulatory networks that stabilise and maintain the organiser (Rhinn and Brand, 2001; Wurst

and Bally-Cuif, 2001). In particular, *Fgf8* has been shown to be important in maintaining lineage restriction at the boundary (Sunmonu et al., 2011).

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