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RESEARCH ARTICLE

Independently specified *Atoh1* domains define novel developmental compartments in rhombomere 1

Mary J. Green[1], Anna M. Myat[1], Brian A. Emmenegger[2], Robert J. Wechsler-Reya[2,*], Leigh J. Wilson[1] and Richard J. T. Wingate[1,‡]

ABSTRACT

The rhombic lip gives rise to neuronal populations that contribute to cerebellar, proprioceptive and interoceptive networks. Cell production depends on the expression of the basic helix-loop-helix (bHLH) transcription factor *Atoh1*. In rhombomere 1, *Atoh1*-positive cells give rise to both cerebellar neurons and extra-cerebellar nuclei in ventral hindbrain. The origin of this cellular diversity has previously been attributed to temporal signals rather than spatial patterning. Here, we show that in both chick and mouse the cerebellar *Atoh1* precursor pool is partitioned into initially cryptic spatial domains that reflect the activity of two different organisers: an isthmic *Atoh1* domain, which gives rise to isthmic nuclei, and the rhombic lip, which generates deep cerebellar nuclei and granule cells. We use a combination of *in vitro* explant culture, genetic fate mapping and gene overexpression and knockdown to explore the role of isthmic signalling in patterning these domains. We show that an FGF-dependent isthmic *Atoh1* domain is the origin of distinct populations of *Lhx9*-positive neurons in the extra-cerebellar isthmic nuclei. In the cerebellum, ectopic FGF induces proliferation while blockade reduces the length of the cerebellar rhombic lip. FGF signalling is not required for the specification of cerebellar cell types from the rhombic lip and its upregulation inhibits their production. This suggests that although the isthmus regulates the size of the cerebellar anlage, the downregulation of isthmic FGF signals is required for induction of rhombic lip-derived cerebellar neurons.

KEY WORDS: *Lhx9*, *Otx2*, *Gbx2*, Isthmo-optic nucleus, Deep cerebellar nuclei, FGF receptor knockout, Cerebellum, Chick, Mouse

INTRODUCTION

Recent years have seen a startling conceptual simplification of the genetic programmes underlying the development of the cerebellum and proprioceptive networks. At the heart of this revision has been a series of increasingly complex fate maps that build on Wilhelm Harkmark's original ablation studies in the chick embryo in the 1950s (Harkmark, 1954; Wingate, 2001). More modern anatomical (Wingate and Hatten, 1999; Gilthorpe et al., 2002) and, in particular, genetic fate maps (Rodriguez and Dymecki, 2000; Machold and Fishell, 2005; Wang et al., 2005; Rose et al., 2009) have pinpointed the origin of the glutamatergic precerebellar and cerebellar neurons in the rhombic lip, a thin strip of cells that borders the expanded roof plate of the fourth ventricle. The production of this distinct range of cell types is entirely dependent on the expression of the basic helix-loop-helix (bHLH) transcription factor Atonal 1 (*Atoh1/Math1*) (Ben-Arie et al., 1997; Wang et al., 2005; Rose et al., 2009). These cells form an integrated network that relays proprioceptive information both to the thalamus and cerebellum.

Expression of *Atoh1* in rhombic-lip-derived neurons is both induced by dorsally derived TGFβ signals (Alder et al., 1999; Lee et al., 2000) and actively maintained by the roof plate organiser (Broom et al., 2012). Although *Atoh1* is transient (in all but specialised granule cell precursors of the cerebellum) this expression is sufficient to conditionally drive reporters that have been used to map all rhombic lip derivatives in transgenic mice (Machold and Fishell, 2005; Wang et al., 2005; Rose et al., 2009) and fish (Kani et al., 2010). Increasing resolution in these fate maps has revealed that, for the cerebellar rhombic lip of rhombomere (r) 1, there is a perhaps surprising diversity of neuronal derivatives from the *Atoh1* pool born within a very narrow temporal window, before the generation of cerebellar cell types. In particular, fate maps identify small groups of isthmic nuclei such as the cholinergic parabigeminal nucleus and the dorsal nucleus of the lateral lemniscus that lie outside the cerebellum and at the border of the midbrain (Machold and Fishell, 2005; Rose et al., 2009). These isthmic nuclei play important roles in regulating bilateral correlation of midbrain sensory maps for both vision and audition, respectively (Butler and Hodos, 1996). Isthmic nuclei with divergent anatomical conformations show a fragmented distribution of isthmic nuclear structures across vertebrate phyla. This includes specialised cell groups such as the isthmo-optic nucleus that projects to the retina in birds, some basal fish and a subset of reptiles (Butler and Hodos, 1996). This overall variability highlights different developmental constraints on isthmic rhombic lip derivatives when compared with other, conserved outputs of the *Atoh1* pool in the cerebellum. What are the likely candidates for regulating the diversity of these different derivatives?

A primary determinant of cell fate in the rhombic lip is the temporal patterning cues (Gilthorpe et al., 2002; Machold and Fishell, 2005) through extrinsic signals that are yet to be determined (Wilson and Wingate, 2006). However, a highly diverse set of nuclei in ventral r1 is produced in a very short temporal window, and therefore a temporal signal is not likely to be sufficient to produce such fine grain specificity in different cell types. A possible additional source of patterning information is spatial cues, which can be divided into dorsalising signals from the roof plate boundary organiser (Broom et al., 2012) and rostrocaudal cues originating from the midbrain/hindbrain isthmus. As the linear pool of *Atoh1*-

[1]MRC Centre for Developmental Neurobiology, King's College London, 4th floor New Hunt's House, London SE1 1UL, UK. [2]Department of Pharmacology and Cancer Biology and Preston Robert Tisch Brain Tumor Center, Duke University Medical Center, Durham, NC 27710, USA.
*Present address: Tumor Development Program, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA.

‡Author for correspondence (richard.wingate@kcl.ac.uk)

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expressing cells comprises the most dorsal cell population in the neural tube, variability in dorsoventral patterning can be ruled out as a source of diversity. However, the isthmus, which comprises the anterior boundary of r1, has a profound role on the establishment of the entire region via the action of the secreted morphogen FGF8 (Joyner, 1996; Reifers et al., 1998). This includes restricting the encroachment of *Otx2*-positive midbrain into r1 (Foucher et al., 2006; Sato and Joyner, 2009) and preventing the hypoplasia of the medial [embryonically rostral (Sgaier et al., 2005)] cerebellar vermis, which accompanies downregulation of FGF signalling (Meyers et al., 1998; Chi et al., 2003; Basson et al., 2008). Whether the isthmus also confers rostrocaudal pattern within cerebellar territory is less clear, despite evidence for its highly polarised effects on midbrain maturation (Martinez et al., 1999; Shamim et al., 1999; Zervas et al., 2004). Studies in chick suggest that segmental identity overrides isthmic signalling in inducing rhombic lip cell types (Eddison et al., 2004). However, the sequence of specification of rhombic lip derivatives has not been examined in embryos with attenuated FGF signalling at the isthmus.

To investigate the effects of spatial organisation on *Atoh1* expression and its derivatives in r1, we have contrasted mouse transgenic manipulation of FGF signalling with experiments in the chick embryo model. The latter is amenable to a range of approaches, including an unbiased assessment of the interactions between different tissues through ablation studies in culture and focal targeting of genetic manipulations by *in ovo* electroporation. Through these, we show that the *Atoh1* domain is cryptically organised into two territories that are separately maintained by FGF and roof plate signals. These respective isthmic and rhombic lip domains gives rise to different derivatives: the former giving rise to isthmic nuclei. We propose that this pool of isthmic, FGF-dependent *Atoh1* progenitors may be a conserved feature of r1 patterning, suggesting a re-evaluation of genetic fate maps and a re-definition of 'rhombic lip' by its inductive relationships rather than gene expression alone.

RESULTS

Atoh1 is expressed in three distinct domains in r1 in chick and mouse

In examining the expression of *Atoh1* in the chick embryo at embryonic day (E) 5, we identified a prominent site of expression in dorsal, isthmic r1 apparently distinct from the rhombic lip, before the formation of an external granule layer (EGL) (Fig. 1A). This isthmic *Atoh1* domain extends from the pial to ventricular surface of the tissue (Fig. 1B) and is bordered rostrally by *Otx2*-positive midbrain (Fig. 1C) and caudally by *Ptf1a*-expressing progenitors in the cerebellar ventricular zone (Fig. 1D). At later stages (E6/7) when *Atoh1*-positive granule cell precursors accumulate in an EGL, the isthmic *Atoh1* domain becomes segregated from the developing cerebellum into a domain that lies rostral to the cerebellar plates (Fig. 1E). Analysis of mouse embryos at an equivalent stage (E14.5) reveals a similar, although markedly smaller domain of *Atoh1* expression abutting the isthmus (Fig. 1F). By E8 in chick, the rostral domain of expression is downregulated and *Atoh1* is exclusively expressed in a distinct EGL (data not shown).

We examined the establishment of the isthmic *Atoh1*-positive domain by a stage by stage expression analysis in both chick (Fig. 1G) and mouse (Fig. 1H). At E4 [Hamburger and Hamilton stage (st.) 24] in chick (Fig. 1G) and at E10.5 in mouse (Fig. 1H), the *Atoh1*-positive rhombic lip in the presumptive cerebellum (r1) is broader than in the hindbrain. Between E4 and E5 (st.24-st.27 in chick) (Fig. 1G), this broad domain of *Atoh1* expression is refined

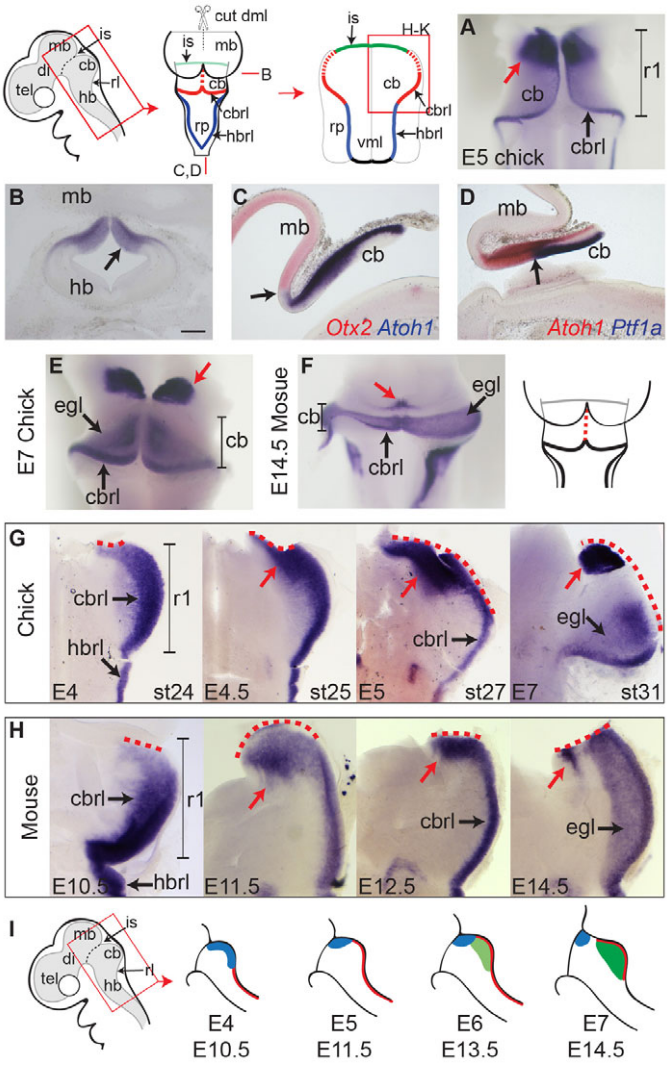

Fig. 1. Distinct Atoh1 domains in r1. (A) In a dorsal view of the cerebellum at E5, *Atoh1* expression is seen in a distinct rostral/isthmic domain (red arrow) and at the cerebellar rhombic lip. (B-D) Coronal (B) and sagittal (C,D) sections at E5 (see schematic, top left) reveal *Atoh1* expression in the isthmic domain extends to the ventricular surface (B, arrow) and abuts the expression of *Otx2* rostrally (C, arrow) and *Ptf1a* (which defines Purkinje cell precursors), caudally (D, arrow). (E,F) Dorsal view of E7 chick (E) and E14.5 mouse (F) cerebellum and rostral hindbrain showing *Atoh1* expression in an isthmic domain (red arrow) segregated from the developing external granule layer of the cerebellum. (G,H) Timecourse of *Atoh1* expression in chick (G) and mouse (H) shown in flat-mounted hindbrain preparations. *Atoh1* expression in the cbrl is initially broader than in the hb rhombic lip (G,H, left) and is subsequently confined to rostral cerebellum (red arrow). A dashed red line indicates the cut edge of the dorsal midline (schematic diagram above). (I) Schematic diagram of hindbrain at different stages indicating the dynamic, broad *Atoh1* domain (blue) in comparison to cerebellar rhombic lip (red) and external granule layer (green) domains of *Atoh1* expression. Scale bars: 200 µm (B-D). cb, cerebellum; cbrl, cerebellar rhombic lip; di, diencephalon; dml, dorsal midline; egl, external granule layer; hb, hindbrain; hbrl, hindbrain rhombic lip; is, midbrain-hindbrain isthmus; mb, midbrain; rl, rhombic lip; rp, roof plate; tel, telencephalon; vml, ventral midline.

to the rostral pole of the cerebellar primordium. In mouse, a similar refinement results in a broad *Atoh1* domain at the fused midline region of the cerebellum at E11.5 and E12.5 and subsequently a spatially separate domain by E14.5 (Fig. 1H). As in chick (Fig. 1G),

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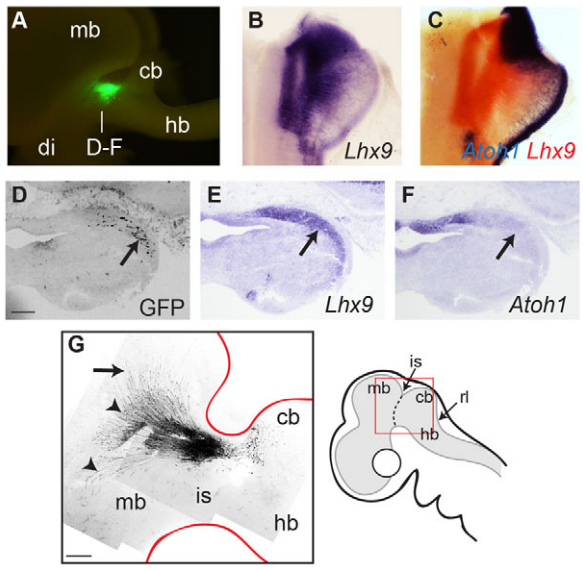

Fig. 2. Isthmic Atoh1 gives rise to specific nuclei. (A) Lateral view of an E7 chick brain showing specific green fluorescent protein (GFP) cell labelling at the isthmus following co-electroporation of *Atoh1-cre* + *lox-stop-lox-gfp* constructs into isthmic *Atoh1* domain at E5. (B,C) *Lhx9* expression at E5 (B, blue; C, red) overlaps isthmic *Atoh1* (C, blue), extending from dorsal/rostral to ventral/caudal rhombomere 1. (D-F) Following electroporation of *Atoh1-cre* + *lox-stop-lox-gfp* at E5, serial coronal sections at E7 show migrated neurons (D, arrow) coincident with *Lhx9* expression (E) lateral to isthmic *Atoh1* (F). (G) Composite confocal image of GFP-labelled cells in the isthmic region (schematic, right) of a flat-mount of the embryo in A: axons extend over the tectum (arrow) and rostrally/ventrally (arrowheads). Scale bars: 200 µm (D-F); 0.5 mm (G). cb, cerebellum; di, diencephalon; hb, hindbrain; is, midbrain-hindbrain isthmus; mb, midbrain.

this isthmic domain in mouse lies outside the cerebellum. Fig.1I summarises this dynamic *Atoh1* expression in the formation of three distinct domains: a broad rostral domain (blue), which is initially seen throughout r1 and becomes refined to an extra-cerebellar territory; the rhombic lip (red); granule cell precursors of the EGL (green).

The isthmic *Atoh1* domain gives rise to a distinct pool of *Lhx9*-positive non-cerebellar neurons

To fate map the isthmic *Atoh1* domain, we employed a mouse *Atoh1* enhancer element (Helms et al., 2000) linked to Cre recombinase (Kohl et al., 2012) that conditionally labels cells when co-electroporated with a plasmid containing *gfp* proceeded by a floxed stop-cassette. Rostral r1 was co-electroporated at E5 and examined 2 days later when fluorescent cells were found rostral to the cerebellum at the isthmus (Fig. 2A, *n*=9). The absence of cells labelled in the EGL confirms the specificity of targeting to rostral r1 without electroporating the more caudal region of the r1 rhombic lip. In mouse, non-cerebellar derivatives of the *Atoh1*-positive rhombic lip express *Lhx9* (Rose et al., 2009). In chick, this transcription factor is expressed in a continuous domain in r1 extending from dorsal (rostrally) to ventral (caudally) (Fig. 2B), including the isthmic *Atoh1*-positive domain at E5 (Fig. 2C). Serial coronal sections through this region in electroporated embryos show that neurons labelled by GFP under the *Atoh1* conditional reporter (Fig. 2D) express *Lhx9* (Fig. 2E) and are displaced laterally relative to the isthmic *Atoh1* domain (Fig. 2F). These cells extend axons rostrally over across the optic tectum and a subset of axons turn ventrally/rostrally (Fig. 2G). The position of labelled cells with tectal projections identifies them as neurons of the nucleus isthmi pars parvocellularis (Hunt and Künzle, 1976), which is a part of the isthmic complex involved in integration of visual information. Although we were unable to establish the termination of ventrally/rostrally turning axons, their initial trajectory is characteristic of retinal projections of the isthmo-optic nucleus (McGill et al., 1966; Cowan and Clarke, 1976).

Atoh1 expression at the isthmus and rhombic lip is regulated by the midbrain/hindbrain boundary

The striking, dynamic re-positioning of the isthmic *Atoh1*-positive domain suggests a dependence on proximity to the boundary of the midbrain and hindbrain, which in early development constitutes an important organiser of regional cell fate (Joyner, 1996). To determine whether isthmic *Atoh1* expression is dependent on signalling from the isthmic organiser, we designed a series of *in vitro* explant experiments in which different tissues were selectively and precisely removed (Fig. 3A). Specific removal of roof plate tissue, confirmed by absence of *Gdf7* expression (Fig. 3B), from E6 hindbrain tissue preparations encompassing intact territory from the midbrain-hindbrain isthmus shows the full complement of *Atoh1* expression domains when fixed immediately after dissection (Fig. 3B). Over the course of 32 hours *in vitro*, expression of *Atoh1* in the rhombic lip is selectively abolished (Broom et al., 2012); however, *Atoh1* expression in the isthmic domain is retained (Fig. 3C). By contrast, removing the midbrain at E5 while leaving roof plate intact (Fig. 3D) eliminates isthmic *Atoh1* expression but leaves rhombic lip expression intact after 48 hours in culture (*n*=6). Removal of roof plate and the unilateral midbrain at both E4 (Fig. 3E) and E5 (Fig. 3F) abolishes *Atoh1* expression in the rhombic lip and isthmic domain ipsilateral to midbrain ablation (*n*=35). In a subset of these explants, removal of midbrain territory was confirmed by *in situ* hybridisation for *Otx2* (Fig. 3F). This suggests a model whereby the midbrain/hindbrain boundary maintains *Atoh1* expression in rostral r1 independently of the roof plate. This is further supported by the observation that unilateral midbrain ablation at E3 reduces the breadth of *Atoh1* expression at the rhombic lip before the establishment of a distinct isthmic domain (*n*=6) (Fig. 3G).

Isthmic-dependent features of *Atoh1* expression and rhombic lip length are dependent on FGF signalling

In early embryonic development, the midbrain-hindbrain boundary signalling is mediated by the secreted morphogen FGF8. Induction of *Fgf8* relies on the interaction between *Otx2*-positive midbrain and *Gbx2*-positive hindbrain territory. We therefore examined whether the expression of *Otx2*, *Gbx2* and *Fgf8* beyond E3 support a continued role for this signalling mechanism in later stages of r1 development. From E5, *Otx2* remains uniformly expressed throughout the midbrain, whereas *Gbx2* expression is limited to dorsal r1 (Fig. 4A). At high magnification at E5, the isthmus is bordered by a narrow strip of *Gbx2*-expressing cells in r1 adjacent to the midbrain (Fig. 4B). Correspondingly, *Fgf8* is both expressed at the isthmus but upregulated dorsally, where it extends caudally into dorsal r1 (Fig. 4C), mirroring the expression of *Gbx2*. The caudal extension of *Fgf8* in dorsal r1 overlaps *Atoh1* expression (Fig. 4D) corresponding to the ventricular layer expression of *Atoh1* in the isthmic domain (Fig. 4E). This suggests a functional link between midbrain-hindbrain boundary signalling, *Fgf8* expression and *Atoh1* maintenance. Correspondingly, response to FGF signals, indicated by expression of the downstream effector

Development (2014) doi:10.1242/dev.099119

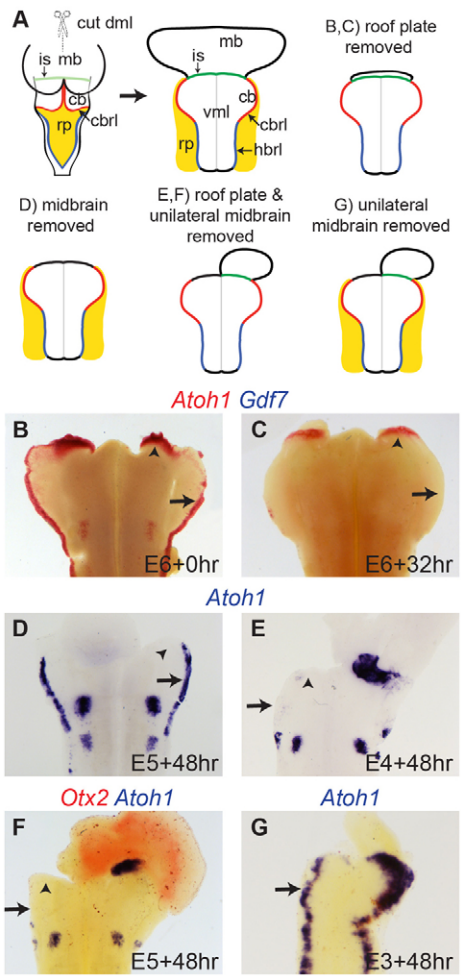

Fig. 3. Atoh1 domains are independently regulated by different organisers. (A) Schematic showing midbrain/hindbrain tissues dissected in flat-mount and different combinations of rp and mb tissue removal. (B) Immediately after dissection (0 hours), E6 tissue with rp ablation but intact mb-hb boundary, shows normal *Atoh1* (red). (C) After 32 hours *in vitro*, *Atoh1* is lost in rl (arrow) but maintained at the isthmus (arrowhead). (D) With rp intact but mb ablated, *Atoh1* is expressed at rl (arrow) but not mb-hb boundary. (E,F) Unilateral midbrain (*Otx2*, red in F) and bilateral rp ablation results in loss of all *Atoh1* ipsilateral to mb ablation. (G) Unilateral ablation of mb alone causes an ipsilateral reduction in width of *Atoh1* expression at the cbrl (arrow). In all images, ventral 'spots' of *Atoh1* are non-rl-derived respiratory paramotor nuclei. cb, cerebellum; cbrl, cerebellar rhombic lip; hbrl, hindbrain rhombic lip; is, midbrain-hindbrain isthmus; mb, midbrain; rp, roof plate; vml, ventral midline.

of FGF signalling, *Sprouty2* (*Spry2* – Mouse Genome Informatics), becomes progressively excluded from caudal r1 from E3 to E5 (Fig. 4F,G).

To determine whether FGF8 signal transduction is necessary for the *Atoh1* expression in r1, we overexpressed a truncated human FGF receptor, *dn-fgf3c*. This *fgfr3* has been shown to bind and sequester FGF8 ligand but as the receptor lacks an intracellular domain it does not cause a downstream activation of the ERK pathway when electroporated in mouse cortex (Toyoda et al., 2010). Although FGFR3 is not the predominant receptor present in r1 (Walshe and Mason, 2000; Blak et al., 2005; Blak et al., 2007; Saarimäki-Vire et al., 2007), this truncated receptor is expected to act in a dominant-negative manner by competitively inhibiting the other FGF receptors in r1. We first confirmed the function of this construct in chick through electroporation at E2 into the midbrain-hindbrain region. Ectopic *dn-fgfr3c* was sufficient to ablate *Sprouty2* expression at the isthmus at E3 (supplementary material Fig. S1A-C) and induce a caudal expansion of *Otx2*-positive midbrain tissue at the expense of *Gbx2*-positive hindbrain tissue, recapitulating the early effects of a loss of isthmic FGF signalling (Sato and Nakamura, 2004) (supplementary material Fig. S1D-F). Electroporation of *dn-fgfr3c* at E2 results at E5 in a loss of isthmic *Atoh1* expression coincident with *Otx2* upregulation in cerebellum (Fig. 4H). Caudal to the domain of *Otx2* upregulation, *Atoh1* is expressed at the rhombic lip. However, *dn-fgfr3c* suppresses the formation of the prominent isthmic expression domain at the shifted *Otx2-Gbx2* boundary (Fig.4I,J). Because electroporations at E2 result in a significant reorganisation of the midbrain-hindbrain boundary, we performed targeted electroporations of *dn-fgfr3c* into caudal r1 at E3. At this later stage, blockade of FGF signalling can be targeted to caudal r1, leaving the midbrain-hindbrain boundary unaffected. This allowed us to examine the effects of FGF signal blockade independently of changes to isthmic organiser activity. Accordingly, locally disrupted FGF signal transduction results in a reduction, but not complete loss of *Sprouty2* expression in caudal r1 at E4 (supplementary material Fig. S1G,H). Downregulation of *Sprouty2* correlates with a local narrowing of the *Atoh1* expression domain at the rhombic lip at E4 (Fig. 4K,L) and an overall reduction in cerebellar size. To quantify this change we measured the anteroposterior length of the rhombic lip between the isthmus and lateral angle of the rhombic lip and the distance between the ventral midline and most dorsal point of the cerebellum in flat-mounted electroporated embryos. We find that the anteroposterior length of the cerebellar rhombic lip is significantly reduced (Mann–Whitney U-test: $z=-4.28$: $P<0.0001$) compared with the control side of the embryo (Fig. 4M) whereas the dorsoventral dimension shows a smaller but still significant reduction (Mann–Whitney U-test: $z=-2.03$: $P=0.0424$) (Fig. 4N).

Overexpression of *Fgf8b* results in upregulation of *Atoh1*

We next determined whether FGF8 is sufficient to induce *Atoh1* expression by using a full-length mouse *Fgf8b*, the most active isoform of *Fgf8* which accounts for the majority of isthmic FGF function (Sato et al., 2001; Guo et al., 2010). Electroporating *Fgf8b* into the midbrain-hindbrain region at E2/3 is sufficient to upregulate *Sprouty2* (a downstream target of FGF signalling) and transform midbrain territory into r1 (Sato et al., 2001) (supplementary material Fig. S2). When examined at E5, embryos electroporated at E2 with full-length *Fgf8b* displayed high levels of *Atoh1* expression extending into the midbrain (Fig. 5A). In flat-mounted half brain preparations, induction is limited to dorsal neural tube (Fig. 5B) despite extensive electroporation (GFP in Fig. 5B, right), suggesting a dorsoventral restriction in competence to express *Atoh1*. Its expression is highest at the dorsal midline and at the anterior boundary of the ectopic *Atoh1* territory. This is consistent with a transformation of midbrain to an r1/cerebellar fate with a corresponding rostral shift of the midbrain/hindbrain boundary and *Atoh1*-positive isthmic domain.

By electroporating *Fgf8b* at later stages we were able to target ectopic gene expression into caudal r1 rhombic lip and induce a local upregulation of *Sprouty2* at E4 without disrupting the midbrain-hindbrain boundary (Fig. 5C). When electroporated at E3, *Fgf8b* induces a sustained *Atoh1* expression at E5 in streams of migratory cells from the rhombic lip of caudal r1 (Fig. 5D). At high magnification, *Atoh1* expression is absent in cells furthest from the

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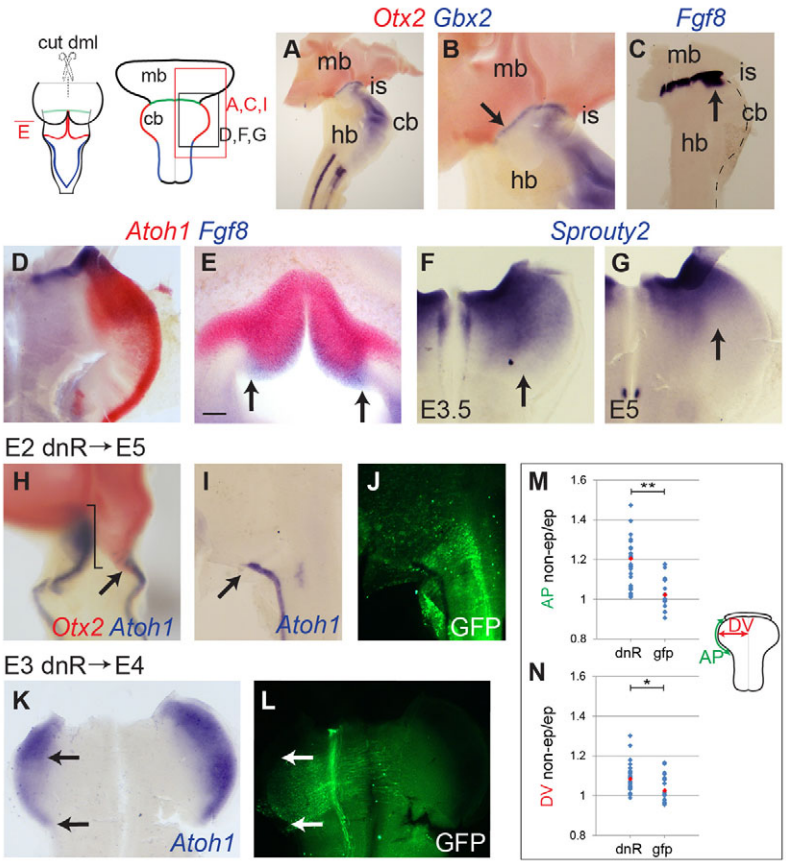

Fig. 4. FGF signal blockade downregulates *Atoh1*. (A-G) Expression of *Otx2*, *Gbx2* and *Fgf8* at E5 (A-E,G) and E4 (F) in flat-mount (A-D,F,G) and coronal sections (E). (A,B) *Otx2* (red) is expressed throughout mb, whereas *Gbx2* (blue) is expressed in cb and a thin stripe at the isthmus (arrow in B: a high magnification view of A). (C-E) Dorsal expansion of *Fgf8* (arrows) correlates with broader *Atoh1* expression (red). (F,G) *Sprouty2* is expressed in rostral cb that diminished between E4 (F) and E5 (G). (H-J) Co-electroporation of *dn-fgfr3c* (dnR) and *gfp* at E2 causes a caudal shift at E5 (indicated by line) of *Otx2* (red) and ipsilateral downregulation of *Atoh1* (blue). There is no expanded *Atoh1* domain caudal to this shifted boundary (arrows). (K,L) Co-electroporation of *dn-fgfr3c* + *gfp* into caudal r1 at E3 downregulates *Atoh1* and reduces the size of the ipsilateral cerebellar anlage. (M,N) Cerebellar asymmetry at E4 following dnR or GFP electroporation into r1 at E3, measured as the ratio of cbrl length (M) or dorsoventral dimension (N). Each point represents the ratio of unelectroporated:electroporated for a single embryo (median ratio in red). $^{**}P<0.001$, $^{*}P<0.05$ Mann–Whitney U-test. cb, cerebellum; hb, hindbrain; is, midbrain-hindbrain isthmus; mb, midbrain.

rhombic lip, suggesting that FGF-induced upregulation is transient (Fig. 5E, compare left and right). To test whether FGF is sufficient to regulate *Atoh1* in the absence of either roof plate or isthmic signals, we electroporated *Fgf8b* into r1 at E4 and immediately dissected away the midbrain and roof plate tissue before placing the isolated cerebellum in culture (Fig. 5F). After 48 hours *in vitro*, *Atoh1* expression was uniformly abolished at the rhombic lip and in the isthmic domain; however, *Atoh1* was expressed in migrating cells originating from electroporated precursors (n=12/22) (Fig. 5G). This suggests that even when inductive roof plate cues are removed FGF8 signalling is sufficient to induce or maintain *Atoh1* in dorsal r1.

Ectopic FGF signalling drives proliferation and inhibits specification of late-born, cerebellar cell types from the rhombic lip

To explore whether FGF signalling alters the fate of *Atoh1*-positive derivatives, we looked at the gene expression and morphology of rhombic lip derivatives following overexpression of *Fgf8b*. We first examined *Lhx9*, which is expressed in both isthmic nuclei and early-born rhombic lip derivatives. Overexpression of *Fgf8b* at E3 in the rhombic lip migratory stream leads to a disruption in the normally continuous domain of *Lhx9* expression at E6 (Fig. 6A-C). In ventral r1, there are small gaps in *Lhx9* expression (Fig. 6B,C), whereas ectopic *Lhx9* is apparent in streams of migratory cells (Fig. 6A,B, arrowheads). This suggests that *Lhx9* expression is not simply downstream of FGF signalling but secondary to more complex changes in cell fate. We therefore looked in detail at the position and morphology of rhombic lip derivatives expressing ectopic *Fgf8b*.

A control electroporation of *gfp* at E4, cumulatively labels successive cohorts of derivatives of the rhombic lip when examined at E7 in flat-mounted embryos (Fig. 6D), reflecting a birth order that culminates in the production of granule cell precursors (Gilthorpe et al., 2002; Wilson and Wingate, 2006). Co-electroporation of *gfp* and *Fgf8b* into the rhombic lip at E4 results in a massive increase in GFP-labelled cells (Fig. 6E), consistent with increased proliferation (Fig. 6F) but obscuring details of cell morphology. To obtain more specific cell labelling in the presence of ectopic *Fgf8*, we electroporated the conditional *Atoh1* reporter (*Atoh1-cre* and *lox-stop-lox-gfp*) alone (Fig. 6G) or in combination with *Fgf8b* (Fig. 6H) into r1 rhombic lip at E4 and looked at the cell morphology and distribution of rhombic lip derived cells at E7. Following ectopic *Fgf8b* expression we saw an accumulation of cells in ventral r1 in the position of early-born *Lhx9*-positive neurons and a concomitant loss of more dorsal, late-born cell types, such as cells that turn at the lateral edge of the EGL (Gilthorpe et al., 2002) (Fig. 6H). Furthermore, the cerebellar nucleus marker *Tbr1* (Fink et al., 2006) was specifically downregulated at E6 following *Fgf8b* overexpression at E3 (Fig. 6I), indicating a loss of later-born cell types. This raises the possibility that, whereas FGF is required for the allocation of cerebellar territory, late-born cerebellar rhombic lip derivatives can only be produced in its absence. To test whether the specification of rhombic lip derivatives requires FGF signal transduction we next investigated the production of the lineage in a context of reduced FGF signalling in both chick and mouse.

Attenuation of FGF signalling and signal transduction does not affect the specification of rhombic lip derivatives

We examined the expression of *Lhx9* following the electroporation of *dn-fgfr3c* into the rhombic lip at E3. At E6, the distribution of *Lhx9* early-born derivatives is identical on control and electroporated sides of r1 (Fig. 7A). Analysis at E7 of the production

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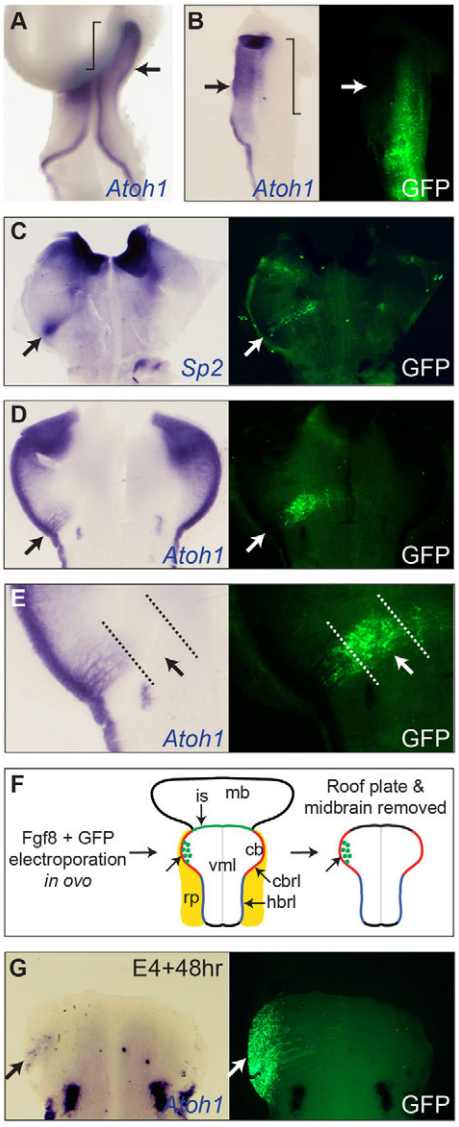

Fig. 5. Ectopic *Fgf8* induces *Atoh1* expression. (A-E) Expression of *Atoh1* at E5 (A,B,D,E) and *Sprouty2* at E4 (C) following co-electroporation of *Fgf8b* + *gfp* at E2 (A,B) and E3 (C-E), in dorsal whole-mount (A) and flat-mount views (B-E). The arrow indicates electroporated right side of the embryo (left of picture in flat-mounts). Note that the fluorescent signal is quenched by *in situ* hybridisation staining in dorsal regions. Electroporation at E2 shifts the isthmus rostrally (A,B, solid line). Electroporation at E3 into caudal cb induces *Sprouty2* (C) and *Atoh1* (D). At high magnification (E), ectopic *Atoh1* proximal to the rl is not maintained in distal derivatives (arrow). (F,G) Electroporation of *Fgf8b* + *gfp* at E4 is sufficient to maintain *Atoh1 in vitro* 48 hours after ablation of rl and mb. cb, cerebellum; cbrl, cerebellar rhombic lip; hbrl, hindbrain rhombic lip; is, midbrain-hindbrain isthmus; mb, midbrain; rp, roof plate; vml, ventral midline.

of successive temporal cohorts following co-electroporation of *dn-fgfr3c* with *gfp* at E4 revealed no difference in cell location or morphology (Fig. 7B). Therefore, autonomous downregulation of FGF signal transduction within rhombic lip progenitors has no apparent effect on the specification of either early-born or late-born derivatives.

We further examined the molecular specification of rhombic lip derivatives in mice, where the precise sequence of neuronal production has been examined in a series of genetic fate maps (Machold and Fishell, 2005; Fink et al., 2006; Rose et al., 2009; Hagan and Zervas, 2012). We analysed a mutant in which cells of the *Atoh1* lineage are unable to transduce FGF signals. These mice were produced by crossing mice expressing three floxed/null FGF receptor alleles: $fgfr1^{flox/flox}$; $fgfr2^{flox/flox}$; $fgfr4^{-/-}$ with mice expressing Cre recombinase under the control of the *Math1/Atoh1*. Loss of FGFR1 and FGFR2 in r1 is sufficient to phenocopy loss of FGF signalling in r1 (Saarimäki-Vire et al., 2007) and although FGFR3 is expressed in the most caudal region of r1 (Blak et al., 2005), it is not required for normal cerebellar development, and cannot compensate for the loss of the other FGF receptors (Blak et al., 2007). The conditional triple knockout (CTKO) mice show a superficially normal cerebellum size and developmental timecourse (Emmenegger et al., 2013). At P4, brains from these animals show normal development of the EGL (Fig. 7C) and fastigial (Fig. 7D) and dentate (Fig. 7E) cerebellar nuclei shown by expression of *Atoh1*, *Tbr1* and *Lhx9*, respectively. Early-born, extra-cerebellar rhombic lip derivatives in isthmic and ventral r1 are also unchanged (Fig. 7E).

The lack of effect of cell-autonomous downregulation of FGF signal transduction in both chick and mouse cerebellum contrasts with the dramatic non-autonomous effects of attenuated isthmic signalling on cerebellar morphogenesis (Meyers et al., 1998; Chi et al., 2003). Our observations suggest that FGF is not required for cell specification at the rhombic lip. By contrast, a previous study (Basson et al., 2008) suggests that cell-non-autonomous loss of isthmic signalling leads not only to the loss of medial cerebellum (vermis) but also the most medial, fastigial cerebellar nucleus. We therefore investigated whether cell specification and morphogenesis are indeed independently regulated in the $Fgf8^{neo/neo}$ cerebellar hypomorph (Meyers et al., 1998). This mouse shows a pronounced loss of medial cerebellar tissue (Fig. 8A,B) (Meyers et al., 1998; Chi et al., 2003). In the residual, lateral cerebellum, *in situ* hybridisation for *Atoh1* (Fig. 8C,D), *Tbr1* (Fig. 8E,F) and *Lhx9* (Fig. 8G,H), respectively, show an intact EGL, fastigial and dentate cerebellar nuclei and early-born population of isthmic and ventral non-cerebellar rhombic lip derivatives at E16.5. Thus, the sequence of cell fate allocation occurs normally at the rhombic lip despite a non-autonomous reduction in FGF signalling and accompanying cerebellar size reduction. However, it was not possible to determine whether individual nuclei within the early-born cohorts were present, owing to the lack of specific cell markers and significant changes in the anatomical structures. We conclude that ectopic FGF signalling not only prevents the production of late-born cell types from the rhombic lip, but also that FGF is not required to mediate the specification of any cell types from the cerebellar rhombic lip.

DISCUSSION

In this study, we show that the initially contiguous expression of *Atoh1* in dorsal r1 in chick and mouse is composed of two cryptic progenitor domains that can be distinguished by their differential dependency on two organisers: the midbrain-hindbrain isthmus and the roof plate boundary. These domains give rise to isthmic nuclei and rhombic lip derivatives, respectively. FGF-mediated isthmic signalling is required for the maintenance of a rostral *Atoh1*-positive progenitor pool, independent of roof plate signals. FGF signalling also influences the size of the cerebellar anlage by increasing the length of the rhombic lip in r1. However, FGF signalling is not required for the patterning of rhombic lip derivatives and inhibits their production when ectopically upregulated. Differently regulated *Atoh1* domains thus give rise to discrete functional elements of the proprioceptive/interoceptive network.

Development (2014) doi:10.1242/dev.099119

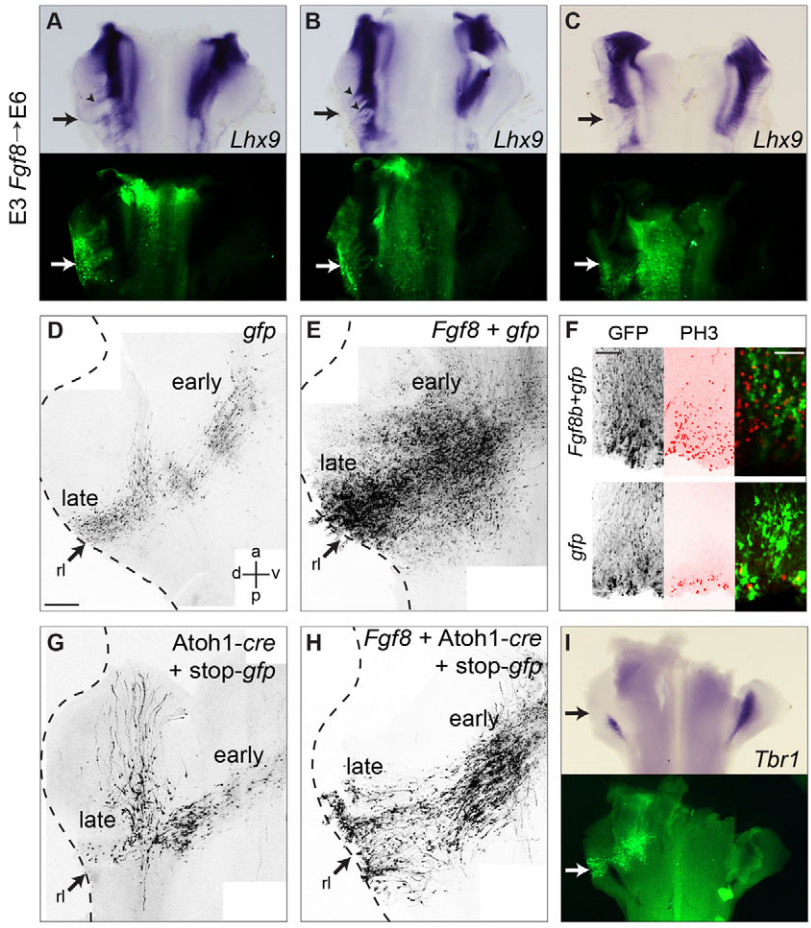

Fig. 6. FGF overexpression supresses cerebellar cell fates. (A-C) Electroporation of *Fgf8b* + *gfp* at E3 (arrow) disrupts *Lhx9* at E6, producing both ectopic expression (arrowheads) and gaps (arrows). (D,E) Electroporation of *gfp* (D) or *Fgf8b* + *gfp* (E) at E4 produces markedly different numbers of GFP-labelled cells. (F) Electroporation of *Fgf8b* + *gfp* at E3 cell-non-autonomously induces at E5 ectopic proliferation (PH3 in red). (G) Electroporation of *Atoh1-cre* + stop-*gfp* into rl at E3 (arrow) labels rhombic lip derivatives at E7. (H,I) Co-electroporated *Fgf8b* increases the proportion of ventral derivatives (H) and downregulates the deep cerebellar nucleus marker *Tbr1* (I). Scale bars: 200 µm (D,E,G,H); 100 µm (F, left and middle); 50 µm (F, right). rl, rhombic lip.

Isthmic function and cerebellar development

The isthmus has been shown to have a profound influence on cerebellum development by both regulating the boundaries of midbrain territory (Sato et al., 2001; Sato and Nakamura, 2004; Foucher et al., 2006) and the size of dorsal r1, from which the cerebellum will develop (Irving and Mason, 2000; Sato and Joyner, 2009). Our results support a model of declining influence of isthmic-derived FGF signals on cerebellum growth (Sato and Joyner, 2009) and we show that the interface between *Otx2*- and *Gbx2*-expressing cells at the isthmus becomes progressively dorsalised (Fig. 4A-C) and, correspondingly, responses to isthmic signalling (indicated by *Sprouty2*) become restricted to rostral r1 (Fig. 4F,G). Despite this reduced influence, blockade of FGF responses using a dominant-negative receptor *fgfr3c*, in chick, results in significant changes in cerebellar size (Fig.4K-N), independent of any change to the integrity or position of the isthmus itself. This indicates a role for FGF in regulating proliferation and specifically the linear extent of the rhombic lip, beyond the initial establishment of r1 territory.

That a normal cerebellar morphology and complement of rhombic lip derivatives is displayed in the Math1-CTKO mice suggests that the effects of FGF signalling occur before the allocation of cells to the *Math1*-positive lineage from an as yet unidentified stem cell pool (Machold and Fishell, 2005; Wingate, 2005). This observation is confirmed by the fact that alterations to rhombic lip 'length' appear to be independent of a normal orderly sequence of cell production in chick *dn-fgfr3* electroporated embryos and in a mouse *Fgf8* hypomorph. However, whilst FGF is required for the correct establishment of r1 territory and size, altered gene expression and cell fate following *Fgf8* overexpression suggest that FGF signalling, although not required at the rhombic lip, can override its normal programme of cell specification when ectopically expressed. Therefore the declining influence of isthmic FGF8 is permissive for the generation of cerebellar cell types. This is supported by evidence that FGF signalling must be suppressed to allow ectopic cerebellar differentiation following the transformation of the mesencephalon to a metencephalic fate (Suzuki-Hirano et al., 2010).

When combined with previous studies, our observations suggest a three-stage model for avian and mammalian cerebellar growth: an FGF-dependent regulation and maintenance of r1 territorial boundaries (Irving and Mason, 2000; Sato et al., 2001; Sato and Nakamura, 2004; Foucher et al., 2006; Sato and Joyner, 2009), an extended intermediate FGF-dependent phase of expansion of the progenitor pool independent of cell specification, and finally a late Shh-dependent expansion of the cerebellar cell surface through the FGF-independent transit amplification of granule cell precursors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Klein et al., 2005; Corrales et al., 2006), which is dependent on sustained *Atoh1* expression (Ben-Arie et al., 1997; Flora et al., 2009). The intermediate, FGF-dependent growth phase, explored in this study, may be particularly significant for cerebellar development in non-mammalian vertebrates where Shh-responsive granule cell precursors are absent (Chaplin et al., 2010; Kani et al., 2010). The developing cerebellum in embryonic basal vertebrates such as the embryonic shark (Chaplin et al., 2010) and basal ray-finned fish (T. Butts and R.J.T.W., unpublished observations) display a highly elongated cerebellar rhombic lip. In the zebrafish, similarly,

Development (2014) doi:10.1242/dev.099119

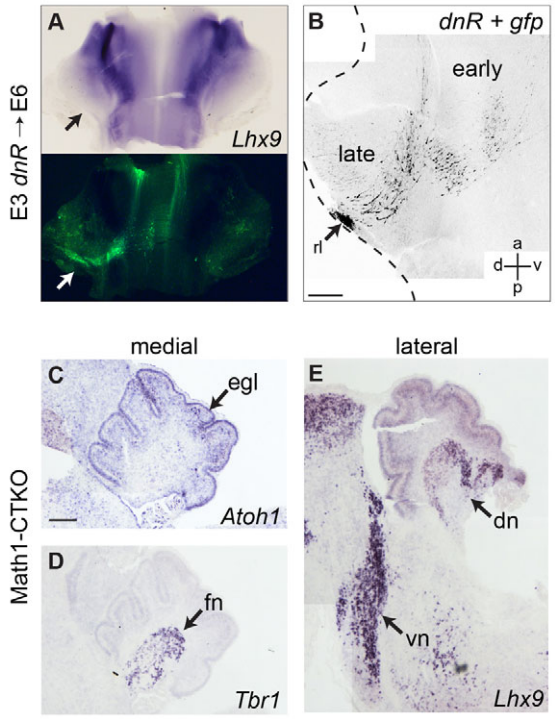

Fig. 7. FGF signal blockade does not affect cell fate allocation at the rhombic lip. (A,B) Electroporation of *dn-fgfr3c +gfp* (arrow) at E3 (A) and E4 (B) does not affect either *Lhx9* expression at E6 (A) or cell labelling at E7 (B). (C-E) *Atoh1* (C), *Tbr1* (D) and *Lhx9* (E) are normal in P4 Math1-CTKO mice, seen in medial (C,D) and lateral (E) sagittal section. Scale bars: 200 µm. dn, dentate nucleus; egl, external granule layer; fn, fastigial nucleus; rl, rhombic lip; vn, ventral nuclei.

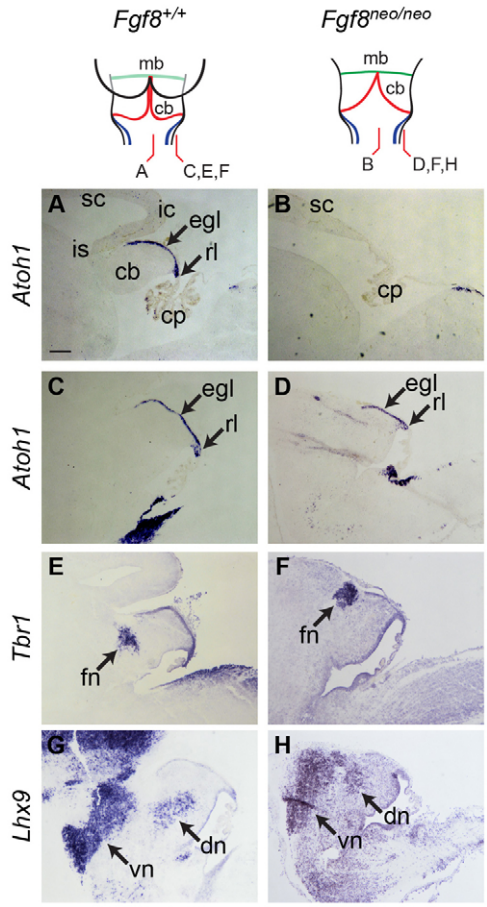

Fig. 8. *Fgf8* hypomorphs show a normal range of rhombic lip derivatives. *Atoh1*, *Tbr1* and *Lhx9* expression in sagittal sections of E16.5 *Fgf8*$^{neo/neo}$ mice (right) and wild-type littermates (left) reveals normal, if misplaced, rl derivative markers in mutants, despite the overall loss of medial cerebellar territory (B). Scale bar: 200 µm. cb, cerebellum; cp, choroid plexus; dn, dentate nucleus; egl, external granule layer; fn, fastigial nucleus; ic, inferior colliculus; is, midbrain-hindbrain isthmus; mb, midbrain; rl, rhombic lip; sc, superior colliculus; vn, ventral nuclei.

cerebellar growth is driven by cell division at the isthmic apex of the cerebellar rhombic lip: the valvulus (Kaslin et al., 2009; Chaplin et al., 2010; Kani et al., 2010).

Two different modes of *Atoh1* regulation define different progenitor territories

Just as the teleost proliferative node or valvulus remains juxtaposed to the isthmus as the cerebellum grows, we describe a distinct isthmic *Atoh1* domain that is dynamically repositioned with respect to, and ultimately excluded from, the growing cerebellum. In contrast to *Atoh1* expression at the rhombic lip, which is both induced (Alder et al., 1996; Lee et al., 2000) and maintained (Broom et al., 2012) by roof plate signals, maintenance of the isthmic domain is exclusively dependent on the isthmic organiser via FGF signalling. Fate mapping in chick using targeted electroporation of a *Atoh1-Cre* enhancer reveals this domain as the origin of the nucleus isthmi pars parvocellularis, which projects to the tectum, and the presumptive isthmo-optic nucleus, which are born at E5 and (Clarke, 1982; Puelles and Martinez-de-la-Torre, 1987; Hellmann et al., 2001) and E6/E7 (Clarke, 1982), respectively. Mammals lack a late-born isthmo-optic nucleus and correspondingly, the isthmic domain of *Atoh1* expression is smaller in mouse than in chick. However, the parabigeminal nucleus, which genetic fate maps have identified as a product of *Atoh1*-positive progenitors and hence a presumptive rhombic lip derivative (Machold and Fishell, 2005; Rose et al., 2009), is functionally homologous to the earlier-born isthmic nuclei of the chick. Both have reciprocal topographic projections to the visual midbrain and monitor and modulate bilateral sensory representation (Butler and Hodos, 1996). Early-born neurons of the dorsal nucleus of the lateral lemniscus fulfil a similar anatomical and functional role in the auditory system. Again, their neurons are also attributed to the different progenitor pools in chick (isthmus) and mouse (rhombic lip). This developmental homology between nuclei in mouse and chick demonstrates that previous genetic fate maps of this region in mouse (Machold and Fishell, 2005; Wang et al., 2005) failed to discriminate between cryptic isthmic and rhombic lip domains of *Atoh1* expression. Thus, a diversity that has previously been explained in terms of homoplasy (multiple different developmental origins coupled with functional convergence) (Butler and Hodos, 1996) may be resolved by the presence of a conserved isthmic *Atoh1* compartment that is contiguous with the rhombic lip.

Our experimental demonstration in chick of different regulatory constraints on *Atoh1* expression suggests that FGF signalling is involved in its maintenance. A possible mechanism is the blockade of the auto-inhibitory feedback loop that normally attenuates *Atoh1* expression via ATOH1 protein (Gazit et al., 2004). This negative feedback is uncoupled in specific developmental contexts: in the

Development (2014) doi:10.1242/dev.099119

EGL to promote transit amplification (Gazit et al., 2004) and in Zic1-mediated regulation of the rhombic lip progenitor pool (Ebert et al., 2003). Such a model is consistent with our observations in chick that isthmic signalling can alter the breadth of *Atoh1* expression in the rhombic lip but is not required for its induction. Correspondingly, *Fgf8b* overexpression maintains only a relatively weak expression of *Atoh1* in the absence of roof plate. A parsimonious model for the origin of an *Atoh1* isthmic domain is that FGF selectively and regionally sustains *Atoh1* expression in a progressively rostralised pool of *Atoh1* progenitors induced by the roof plate.

We show that an absence of FGF signalling at the rhombic lip does not alter the generation of successive temporal cell fates. Although it is not clear what specific instructive cues determine cell fate, particularly of the late born cerebellar nuclei and cerebellar granule cells, the fourth ventricle roof plate is a possible source for these patterning cues (Wilson and Wingate, 2006; Broom et al., 2012). Furthermore, we show that the lack of FGF signalling in caudal r1 at later stages is permissive in generating cerebellar cell types and accordingly the prolonged presence of FGF signalling at the isthmus is likely to be important in reserving this territory as non-cerebellar. This region is comparatively larger in chick, which displays an additional isthmic nucleus but lacks a cerebellar vermis. Correspondingly, the mouse has a smaller isthmic domain and a rostrally extended rhombic lip. This suggests that FGF signalling determines the proportion of rostral r1 that is allocated to cerebellum. It also implies that the cerebellar vermis, which is derived from rostral rhombic lip (Sgaier et al., 2005), may have evolved through a reduction in the influence of the isthmus. This is an unexpected inference given that FGF8 mutants display vermal hypoplasia (Meyers et al., 1998; Chi et al., 2003) and highlights the importance of separating early territorial readjustments of the midbrain-hindbrain boundary (resulting in loss of rostral tissue) from the later roles of isthmic signalling that we demonstrate here. At these later stages of cerebellar specification, discrete domains of *Atoh1* expression are defined by their differential dependence on discrete local organisers, with FGF signalling both influencing the size of cerebellum and sub-regionalisation of rhombomere 1.

MATERIALS AND METHODS

In ovo and *in vitro* manipulations

Fertilised wild-type eggs (Henry Stewart, UK) were incubated at 38°C for 2 to 6 days. For explant cultures, embryos were removed from eggs and the midbrain/hindbrain region of the neural tube was dissected in Tyrode's solution. Tissue was bisected along the dorsal midline and roof plate or midbrain tissue was removed by dissection. Explants were cultured pial surface uppermost on 0.4 µm inserts (Millicell-CM, Millipore) for 2 days (37°C/6% CO_2) as per Broom et al. (Broom et al., 2012). For electroporations, the neural tube in the region of r1 was injected with ~100-200 nl of DNA plasmids mixed to equal concentrations, giving a final concentration of 0.8-2 µg/µl of each plasmid: Atoh1-cre (Kohl et al., 2012), pFlox-pA-EGFP (lox-stop-lox-GFP) and pCX-Cre (Morin et al., 2007), pEFX-dn-fgfr3c and pEFX-Fgf8 (Toyoda et al., 2010), pCAβ-eGFP-m5 (Yaneza et al., 2002). Electroporated embryos were incubated for a further 1-3 days at 38°C.

Mouse embryos

CTKO mice (*Math1-cre*; *fgfr1*$^{flox/flox}$; *fgfr2*$^{flox/flox}$; *fgfr4*$^{-/-}$) were generated and genotyped as per Emmenegger et al. (Emmenegger et al., 2013). *Fgf8*$^{neo/neo}$ embryos were donated by Albert Basson (King's College London, UK) and obtained from crosses of *Fgf8*$^{neo/+}$ mice as previously described (Meyers et al., 1998). Wild-type littermates were used as controls.

Histology and photomicroscopy

Embryos were fixed in 4% (w/v) paraformaldehyde (in phosphate-buffered saline) and either dissected or processed for cryostat sectioning. Tissue was stained by *in situ* hybridisation (Myat et al., 1996) with digoxygenin- or fluorescein-labelled (Roche) riboprobes for: *Atoh1* (Wilson and Wingate, 2006), *Otx2* (Millet et al., 1996), *Ptf1a* (ChEST1028o4), *Lhx9* (Alessio Delogu, King's College London, UK), *Gdf7* (Broom et al., 2012), *Gbx2* (Kiecker and Lumsden, 2004), *Fgf8* and *Sprouty2* (Chambers et al., 2000); and mouse probes for: *Atoh1* (Albert Basson, King's College London, UK), *Lhx9* and *Tbr1* (Alessio Delogu, King's College London, UK). Following *in situ* hybridisation, GFP signal was amplified immunohistochemically with an anti-GFP antibody (IgG 1:100, Invitrogen) and mitotic cells were detected with an anti-phospho-histone H3 antibody (1:100, NEB). Some whole-mounts were further processed for vibratome sectioning. Digital brightfield and fluorescence images were acquired on either stereo (Leica MZFLIII) or compound (Nikon Elipse80i) microscopes equipped with epifluorescence or by laser scanning confocal microscopy (Olympus AX70).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.J.G. and R.J.T.W. planned the study and prepared the manuscript. M.J.G., A.M.M. and L.J.W. characterised gene expression. M.J.G. carried out chick experiments. B.A.E. and R.J.W.-R. generated the CTKO mouse line.

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Supplementary material

Supplementary material available online at
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