



Published in final edited form as:

Dev Biol. 2008 June 1; 318(1): 162–171.

Hh and Wnt signaling regulate formation of *olig2*⁺ neurons in the zebrafish cerebellum

Karen A. McFarland¹, Jolanta M. Topczewska², Gilbert Weidinger³, Richard I. Dorsky⁴, and Bruce Appel¹

¹Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA

²Department of Pediatrics, Northwestern University Feinberg School of Medicine CMRC, Chicago IL 60614

³Biotechnological Center, Technical University of Dresden, 01377 Dresden, Germany

⁴Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132-3401

Abstract

The cerebellum, which forms from anterior hindbrain, coordinates motor movements and balance. Sensory input from the periphery is relayed and modulated by cerebellar interneurons, which are organized in layers. The mechanisms that specify the different neurons of the cerebellum and direct its layered organization remain poorly understood. Drawing from investigations of spinal cord, we hypothesized that the embryonic cerebellum is patterned on the dorsoventral axis by opposing morphogens. We tested this using zebrafish. Here we show that expression of *olig2*, which encodes a bHLH transcription factor, marks a distinct subset of neurons with similarities to eurydendroid neurons, the principal efferent neurons of the teleost cerebellum. In combination with other markers, *olig2* reveals a dorsoventral organization of cerebellar neurons in embryos. Disruption of Hedgehog signaling, which patterns the ventral neural tube, produced a two-fold increase in the number of *olig2*⁺ neurons. By contrast, *olig2*⁺ neurons did not develop in embryos deficient for Wnt signaling, which patterns dorsal neural tube, nor did they develop in embryos deficient for both Hedgehog and Wnt signaling. Our data indicate that Hedgehog and Wnt work in opposition across the dorsoventral axis of the cerebellum to regulate formation of *olig2*⁺ neurons. Specifically, we propose that Hedgehog limits the range of Wnt signaling, which is necessary for *olig2*⁺ neuron development.

Keywords

cerebellum; Purkinje neuron; eurydendroid neuron; Hedgehog; Wnt; zebrafish; dorsoventral; *olig2*

Introduction

The cerebellum functions as the main control center for balance and motor function and also contributes to cognitive learning (Fiez, 1996; Holmes, 1939; Ito, 2000; Morton and Bastian, 2004; Raymond et al., 1996; Schutter and van Honk, 2005; Thach et al., 1992). It is radially organized into several distinct layers, which are interconnected by axonal projections and

Corresponding Author: Bruce Appel (e-mail: b.appel@vanderbilt.edu), p: (615) 322-2003 f: (615) 343-6707, Department of Biological Sciences, Vanderbilt University, MRBIII/ U721, 465 21st Avenue South, Nashville, TN 37232, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

synaptic contacts (Altman and Bayer, 1978; Altman and Bayer, 1985; Wang and Zoghbi, 2001). The molecular layer is the outermost and consists of stellate and basket neurons, which make synaptic connections with dendrites of Purkinje neurons. Golgi cells and the cell bodies of Purkinje neurons comprise the Purkinje layer, just below the molecular layer. Purkinje neurons are the chief output neuron of the mammalian cerebellum and their axons project to deep cerebellar nuclei (Altman and Bayer, 1985), which relay signals to various output peduncles that synapse with midbrain structures (thalamus, red nuclei and pre-motocortex), dorsal brainstem or cerebral neocortex (de Zeeuw and Berrebi, 1996). The granule layer, located below the Purkinje layer, consists of granule neurons, which are excitatory cells that also synapse with Purkinje neuron dendrites. In teleosts, Purkinje neurons project within the cerebellum directly onto eurydendroid neurons, located ventral to Purkinje cell bodies, instead of to the deep brainstem. Eurydendroid neurons in turn make connections to the diencephalon and caudal medulla and thus serve the same function as deep cerebellar nuclei in mammalian systems (Diaz-Regueira and Anadon, 2000; Finger, 1978; Ikenaga et al., 2006; Lannoo et al., 1991b).

Of these cerebellar features, the development of granule neurons is perhaps best understood. Granule neuron progenitors arise from the rhombic lip, a specialized proliferative zone along the dorsolateral rhombencephalon. Guided, at least in part, by Netrin1 and Slit2, they migrate over the surface of the cerebellum to form a transient external granule layer (Gilthorpe et al., 2002). Subsequently, signals secreted by Purkinje neurons cause granule neuron progenitors to proliferate and migrate into the cerebellum to form the inner granule layer (Dahmane and Ruiz i Altaba, 1999; Traiffort et al., 1998).

Many other neurons of the cerebellum, including Purkinje neurons, stellate, basket and Golgi neurons, apparently originate within the proliferative ventricular zone (VZ) (Altman and Bayer, 1978; Altman and Bayer, 1985). Retrospective lineage analysis in mouse and chick showed that some clones included Purkinje neurons, molecular layer neurons and glia (Lin and Cepko, 1999; Mathis et al., 1997). Thus, many cerebellar VZ precursors are multipotent, producing distinct cell types that occupy different layers. Although several genes that are important for Purkinje neuron migration and maintenance have been described (Wang and Zoghbi, 2001), very little is known about the mechanisms that specify cerebellar cell type and determine the layered organization of the cerebellum.

Here we describe experiments designed to investigate mechanisms of cerebellar patterning during development, using zebrafish as a model system. We hypothesized that signals that pattern the dorsoventral (DV) axis of the neural tube influence the fate of cells that arise from the cerebellar VZ, similar to neuronal specification in the spinal cord. Consistent with this, we found that Hedgehog (Hh) signaling, which is active in ventral neural tube, limits the number of neurons that express *olig2*. Expansion in *olig2*⁺ neuron number resulting from loss of Hh signaling was accompanied by an expansion of *wnt1* expression. Conversely, embryos deficient for Wnt signaling failed to form *olig2*⁺ neurons, even in the absence of Hh signaling. Our data support a model in which Wnt signaling, limited to dorsal cerebellum by Hh, is necessary for *olig2*⁺ neuron specification.

Materials and methods

Zebrafish staging and strains

Embryos were produced by pairwise matings and raised at 28.5°C, then staged according to hours post fertilization (hpf) and days post fertilization (dpf) in addition to morphological criteria (Kimmel et al., 1995). Mutant alleles included *smo*^{b641} (Barresi et al., 2000; Varga et al., 2001) and *Df(LG01:lef1)^{x8}* (Phillips et al., 2006). Transgenic alleles included *Tg(olig2:egfp)^{vu12}* (Shin et al., 2003) and *Tg(hsp70:dkk1-GFP)^{w32}* (Stoick-Cooper et al., 2007).

In situ RNA hybridization

In situ RNA hybridization was performed as described previously (Hauptmann and Gerster, 2000). Antisense RNA probes included *olig2* (Park et al., 2002), *ptc1* (Concordet et al., 1996), *shh* (Krauss et al., 1993), *atoh1a* (Kim et al., 1997) and *wnt1* (Molven et al., 1991). Hybridization was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase, followed by a color reaction using a solution of BM Purple AP Substrate (Roche Diagnostics). All embryos for sectioning were embedded in 1.5% agar/5% sucrose and frozen in 2-methyl-butane chilled by immersion in liquid nitrogen. Sections of 10 μm thickness were obtained using a cryostat microtome. Whole embryos were deyolked for imaging and placed in 75% glycerol solution on bridged slides and coverslipped. Images were collected using a QImaging Retiga Exi color CCD camera mounted on an Olympus AX70 compound microscope and imported into Adobe Photoshop. All image manipulations were restricted to adjustment of levels, curves, saturation, hue and color balance.

Immunohistochemistry

We used the following primary antibodies for immunohistochemistry on fixed embryos and larvae: mouse anti-Zebrin II (1:1000, gift of Dr. R Hawkes) (Brochu et al., 1990), mouse anti-HuC/D (16A11, 1:100, Molecular Probes) (Marusich et al., 1994), rabbit anti-Calretinin (1:1000, Swant Products) (Schwaller et al., 1993), mouse anti-Parvalbumin (1:1000, Chemicon) (Porteros et al., 1998) and rabbit anti-GABA (1:10,000, Sigma) (Villani et al., 1982). For fluorescent detection we used Alexa Fluor 568 goat anti-mouse conjugate and Alexa Fluor 647 goat anti-rabbit (1:200, Molecular Probes). All embryos and larvae for sectioning were embedded in 1.5% agar/5% sucrose and frozen in 2-methyl-butane chilled by immersion in liquid nitrogen. Sections of 10 μm thickness were obtained using a cryostat microtome. Fluorescent images of sectioned embryos were collected using a 40X oil-immersion (NA = 1.3) objective mounted on a motorized Zeiss Axiovert 200 microscope equipped with a PerkinElmer ERS spinning disk confocal system or a Zeiss LSM510 Meta laser scanning confocal microscope and imported into Volocity (Improvision). Whole mount fluorescent images were collected using a QImaging Retiga Exi color CCD camera mounted on an Olympus AX70 compound microscope and imported into Adobe Photoshop. All image manipulations were restricted to adjustment of levels, curves, saturation and hue.

Cyclopamine treatments

Embryos were incubated in Embryo Medium (EM) (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.15 mM KH₂PO₄, 0.05 mM NH₂PO₄, 0.7 mM NaHCO₃) containing 50 μM cyclopamine (CA) (Toronto Research Chemicals), diluted from a 10 μM stock dissolved in ethanol. Embryos were treated in their chorions at shield stage or following manual dechoriation with any treatments that began after 24 hpf.

Heat-shock induction

To induce expression of Dkk1, *hsDkk1GFP* embryos were collected from matings of heterozygous *hsDkk1GFP* fish and raised in EM at 28.5°C. Embryos were cooled to 24°C for one hour at 29 hpf then transferred to a microfuge tube filled with EM in a 40°C water bath for one hour. Embryos were sorted by GFP expression and only highly-expressing embryos were selected for analysis. These embryos were then placed back in EM at 28.5°C and raised until 48 hpf.

Quantification of EGFP⁺ neurons

To quantify the number of EGFP⁺ cells in a whole cerebellum *Tg(olig2:egfp)* embryos were fixed at 48 hpf. The embryos were dissected using watch maker's forceps, removing the eyes, yolk, forebrain and trunk to isolate the cerebellum. Cerebellums were mounted in 75% glycerol

on bridged coverslips. Images were collected at 2 μ M intervals through the entire depth of the cerebellum using a confocal microscope. The images were imported into Volocity and then exported to Openlab (Improvision). Each Z stack image was examined and individual EGFP⁺ cells were labeled and counted.

Results

Zebrafish cerebellar cells express *olig2*

To initiate an investigation of cerebellar patterning we examined expression of *olig2*, which encodes a bHLH transcription factor (Zhou et al., 2000; Takebayashi et al., 2000; Park et al., 2002; Lu et al., 2000), by in situ RNA hybridization. At 24 hours post fertilization (hpf), expression was evident in ventral spinal cord, as previously described (Park et al., 2002), and in ventral diencephalon but not in the cerebellum (Fig. 1A). By 36 hpf, a prominent domain of *olig2* expression appeared near the dorsal boundary between midbrain and hindbrain (data not shown). Expression was maintained at 48, 72, and 96 hpf (Fig. 1B–D) through at least 7 days post fertilization (dpf) (data not shown). Transverse and sagittal tissue sections revealed that cerebellar cells dorsal to the anterior medulla oblongata expressed *olig2* (Fig. 1H–J, N–P) in a pattern consistent with that of other cerebellar genes (Mueller et al., 2006; Mueller and Wullmann, 2003).

Previously, we described a transgenic line in which *olig2* regulatory DNA drives expression of EGFP, recapitulating endogenous *olig2* expression in spinal cord (Shin et al., 2003). The pattern of cerebellar EGFP expression was also identical to that of *olig2* RNA (Fig. 1E–G, K–M, Q–S), with the exception that EGFP was first detectable at 40 hpf, presumably reflecting a delay in EGFP folding and fluorescence following translation (data not shown). EGFP in *Tg(olig2:egfp)* embryos is cytosolic and reveals both cell bodies and processes. Notably, cerebellar EGFP⁺ cells extended long processes to the deep brainstem (Fig. 1K–M).

olig2 expression identifies a subset of cerebellar neurons

The long EGFP⁺ projections evident at 48 hpf were suggestive of axons. To determine if cerebellar *olig2*⁺ cells are neurons we labeled sections of *Tg(olig2:egfp)* 48 hpf embryos with anti-Hu antibody, which marks newly born postmitotic neurons (Marusich et al., 1994). The majority of the cerebellar *olig2*⁺ cells expressed Hu, identifying them as neuronal (Fig. 2A–C). To examine more closely the morphology of *olig2*⁺ cells we next imaged living *Tg(olig2:egfp)* larvae using confocal microscopy. High magnification dorsal views of 54 hpf animals revealed deep ventral projections, as noted above (Fig. 2D). By 7 dpf, *olig2*⁺ cells appeared to project single, extensively branched dendrites toward the exterior surface of the cerebellum (Fig. 2E).

We next tried to identify *olig2*⁺ cells using antibodies previously used as Purkinje neuron and eurydendroid cell markers. First, we labeled transverse and sagittal sections of 5 dpf *Tg(olig2:egfp)* larvae with Zebrin II antibody, which identifies Purkinje neurons in both mammals and teleosts (Brochu et al., 1990; Lannoo et al., 1991a; Lannoo et al., 1991b; Meek et al., 1992), and antibody specific to Calretinin, a calcium-binding protein expressed by a subset of eurydendroid neurons (Castro et al., 2006; Diaz-Regueira and Anadon, 2000). As expected, Zebrin II and Calretinin immunofluorescence did not colocalize, with Zebrin II⁺ Purkinje neurons dorsal to Calretinin⁺ eurydendroid neurons (Fig. 2F, G). However, neither antibody labeled *olig2*⁺ cells (Fig. 2F, G). Instead, most *olig2*⁺ cells occupied space between Zebrin II⁺ Purkinje neurons and Calretinin⁺ eurydendroid cells, although some *olig2*⁺ cells were intermixed with Zebrin II⁺ and Calretinin⁺ cells (Fig. 2F, G). Thus, Zebrin II, *olig2* and Calretinin expression mark distinct cerebellum cell populations that occupy dorsal, intermediate and ventral positions, respectively.

We reasoned that *olig2*⁺ cells might be relatively immature at 5 dpf and not yet express markers of differentiated neurons. Therefore, we labeled sections obtained from 14 dpf transgenic animals. Although *olig2*⁺ cells were closely associated with Calretinin⁺ and Zebrin II⁺ cells, they remained as distinct cell populations (Fig. 2H, I). We also used anti-Parvalbumin antibody, which has been used to mark Purkinje neurons (Porteros et al., 1998). Consistent with Zebrin II labeling, *olig2*⁺ cells did not express Parvalbumin (Fig. 2J). In goldfish, three morphologically distinct types of eurydendroid cells are distributed in the Purkinje, granule and molecular cell layers, many of which are intimately associated with Zebrin II⁺ cells and fibers (Ikenaga et al., 2005). Many *olig2*⁺ cells are similarly in close contact with Zebrin II⁺ and Parvalbumin⁺ cells (Fig. 2I, J). Because *olig2*⁺ cells extend axons to the deep brainstem they most likely are a subpopulation of eurydendroid neurons although, lacking definitive molecular marker evidence, we cannot rule out other possibilities.

The cerebellar *olig2*⁺ cell population is enlarged in the absence of Hh signaling

The distribution of Zebrin II⁺, *olig2*⁺ and Calretinin⁺ cells in the cerebellum was reminiscent of the DV organization of spinal cord neurons. In the spinal cord, specification of different cell types along the DV axis is influenced by graded Hh signaling (Jessell, 2000). Ventral spinal cord cells in mouse and zebrafish embryos that lack Hh signaling fail to express *olig2* (Lu et al., 2000; Park et al., 2002), indicating Hh promotes *olig2* expression. To test the possibility that Hh also patterns the cerebellar DV axis, we investigated *olig2* expression in embryos that were homozygous for a mutation of the *smoothened homolog (smo)* gene, which encodes a seven transmembrane protein necessary for Hh signaling (Alcedo et al., 1996; Huangfu and Anderson, 2006). As previously described (Park et al., 2002), spinal cord cells of *smo* mutant embryos did not express *olig2* (Fig. 3D). By contrast, cerebellar *olig2* expression was elevated in *smo* mutant embryos (Fig. 3E, F). From a dorsal view, the stripe of *olig2* expression was both broader in the anteroposterior axis and extended more ventrally than normal (Fig. 3B, C, E, F).

To quantify cerebellar *olig2*⁺ cells, we used confocal microscopy to count EGFP⁺ cells of *Tg(olig2:egfp)* embryos. At 48 hpf, an average of approximately 65 *olig2*⁺ cells occupied each half of the cerebellum of wild-type transgenic embryos (Fig. 4A). Similarly staged *smo*^{-/-};*Tg(olig2:egfp)* embryos had nearly a two-fold increase in the number of *olig2*⁺ cells (Fig. 4A). Thus in normal development, Hh limits formation of *olig2*⁺ cells in the cerebellum.

Previous studies have shown the pharmacological agent cyclopamine (CA) directly binds to Smoothened and specifically inhibits Hh signal transduction (Chen et al., 2002). Consistent with this, zebrafish embryos incubated with CA had a deficit of motor neurons, similar to *smo* mutant embryos (Chen et al., 2001; Lewis and Eisen, 2001; Park et al., 2004). To determine if CA treatment phenocopies the *smo* mutant cerebellar defect, we treated *Tg(olig2:egfp)* embryos at 6 hpf, an early gastrulation stage, and counted EGFP⁺ cells as described above. Similar to *smo* mutant embryos, CA treated embryos had an almost two-fold increase in *olig2*⁺ cell number when compared to control embryos (Fig. 4A).

To determine the time during which Hh signaling is necessary for regulating *olig2*⁺ cell formation, we utilized CA to selectively block Smoothened function at different intervals. We treated *Tg(olig2:egfp)* embryos at 24, 28, 32, 33, 34, 35, and 36 hpf, allowed them to mature to 48 hpf and counted EGFP⁺ cells. Embryos treated at 33 hpf and earlier had approximately the same number of *olig2*⁺ cerebellar cells as *smo* mutant embryos and embryos treated continuously from 6 hpf with CA. By contrast, those treated at 34 hpf and later had approximately the same number as control embryos (Fig. 4B). These data indicate Hh signaling regulates *olig2*⁺ cell development in the cerebellum before 33 hpf.

Expression patterns of Hh pathway genes do not coincide with cerebellar *olig2*⁺ cells

To investigate if Hh signaling is active in cerebellar cells we used in situ RNA hybridization to analyze expression of genes that encode components of the Hh pathway. *shh* expression was ventral to the cerebellum at 30 hpf and 48 hpf (Fig. 5A–C), spanning the critical time period for Hh regulation of *olig2*⁺ PN formation. As previously reported (Ekker et al., 1995) *tiggy winkle hedgehog (twhh)* was expressed similarly to *shh*, whereas *echidna hedgehog (ehh)* was restricted to the trunk notochord (Currie and Ingham, 1996) (data not shown). *patched 1 (ptc1)* RNA, which is expressed by cells in which the Hh pathway is active (Concordet et al., 1996; Marigo and Tabin, 1996), was prominent along the lower rhombic lip of the hindbrain but not within the cerebellum (Fig. 5D–F). *gli1*, which encodes a transcription factor that mediates Hh signaling (Karlstrom et al., 2003; Lee et al., 1997), was expressed similarly to *ptc1* (data not shown). Thus, although our functional data show that Hh signaling limits formation of cerebellar *olig2*⁺ cells, our gene expression data indicate that Hh signaling is active only in cells located at a distance from them. This raised the possibility that Hh signaling influences cerebellar *olig2*⁺ cell development indirectly, by regulating other pathways.

Spinal cord patterning and specification of neurons along the DV axis is dependant on opposing gradients of morphogens secreted from the roof plate and floor plate (Wilson and Maden, 2005). Notably, dorsal spinal cord and roof plate cells secrete Wnt1 and Wnt3a (Lee and Jessell, 1999; McMahon and Bradley, 1990; Roelink and Nusse, 1991). To investigate whether *wnt1* is a candidate to regulate cerebellar *olig2*⁺ cell development, we examined its expression using in situ RNA hybridization. At 30 hpf there were two domains of *wnt1* expression close to the cerebellum. The first domain included cells along the dorsal midbrain, extending ventrally at the midbrain-hindbrain boundary. The second domain, marked by a relatively low level of *wnt1* transcripts, included the dorsal cerebellum (Fig. 5G, H). At 48 hpf expression near the midbrain-hindbrain boundary remained strong whereas *wnt1* transcripts were no longer evident in dorsal cerebellum (Fig. 5I, J). Thus, cerebellar *wnt1* expression coincides with the critical period for Hh regulation of *olig2*⁺ neuron formation.

Wnt signaling is necessary for cerebellar *olig2*⁺ cell development

We tested whether Wnt signaling regulates formation of cerebellar neurons by investigating *olig2* expression. Lymphoid enhancer 1 (*Lef1*) mediates nuclear response to Wnt signals and functions as a transcriptional activator in the presence of β -catenin (Clevers and van de Wetering, 1997). The chromosomal deficiency *Df(LG01:lef1)^{x8}* deletes approximately 2–8 cM of Linkage Group 1, spanning the *lef1* locus (Phillips et al., 2006). In situ RNA hybridization revealed that, in contrast to *smo* mutants, *Df(LG01:lef1)^{x8}* mutant cerebellar cells failed to express *olig2* at 48 hpf whereas spinal cord expression was normal (Fig. 6D–F). Because Wnt signaling has an earlier role in forming the midbrain-hindbrain boundary (Bally-Cuif et al., 1995; Kelly and Moon, 1995) and the *Df(LG01:lef1)^{x8}* deletion removes additional genes, we used a heat-shock activated transgenic line, *hsDkk1GFP*, to conditionally express the Wnt inhibitor Dkk1. Dkk1 is a secreted ligand for LRP6, a coreceptor necessary for Wnt signal transduction (Liu et al., 2003; Semenov et al., 2001). LRP6 preferentially binds to Dkk1 in the presence of Wnt, prohibiting it from forming a complex with the Frizzled receptor and preventing pathway activation. We induced expression of Dkk1 using heat shock at 30 hpf and examined *olig2* expression at 48 hpf using in situ RNA hybridization. Similar to *Df(LG01:lef1)^{x8}* mutant embryos, transgenic heat-shocked embryos expressed *olig2* in spinal cord but not cerebellum (Fig. 6J–L). These data indicate Wnt signaling is necessary for cerebellar *olig2*⁺ neuron formation.

Our data raised the possibility that Hh signaling limits the range of Wnt activity, which is necessary for *olig2*⁺ neuron formation. Thus, we predicted that the absence of Wnt would be epistatic to loss of Hh signaling. To test this we treated *Df(LG01:lef1)^{x8}* mutant embryos with

CA at 6 hpf and examined *olig2* expression at 48 hpf. As expected, spinal cord cells did not express *olig2* in the absence of Hh (Fig. 6I). Notably, cerebellar cells also did not express *olig2* (Fig. 6G, H). Similarly, heat shock induction of Dkk1 at 30 hpf entirely blocked formation of *olig2*⁺ cells in transgenic embryos treated with CA at 6 hpf (Fig. 6M–O). Expression of *atoh1a*, which marks the rhombic lip, appeared normal in *Df(LG01:lef1)^{x8}* mutant embryos and transgenic embryos in which Dkk1 expression was induced at 30 hpf (Supplementary Fig. 1), indicating that the cerebellar primordium was intact. Thus, absence of *olig2*⁺ cells in these embryos was not due to the absence of a cerebellum. We conclude that expansion of *olig2*⁺ cell number in the absence of Hh signaling is dependent on Wnt activity, consistent with the idea that Hh limits the range of Wnt signals in the cerebellum.

Shh could limit Wnt signaling by restricting production of ligand or blocking cell response to ligand. To investigate these possibilities we first examined *wnt1* expression by in situ RNA hybridization. As shown above, at 48 hpf, *wnt1* is normally expressed at the midbrain-hindbrain boundary and seems to be restricted to a small number of cells (Fig 7A–D). By contrast, in the CA treated embryos, *wnt1* expression was expanded both posteriorly and ventrally at the midbrain-hindbrain boundary (Fig. 7E–H). Additionally, whereas overexpression of Dkk did not affect *wnt1* RNA expression (Fig. 7I, J), the *wnt1* expression domain was expanded in embryos treated with CA and overexpressing Dkk (Fig. 7K, L). These data suggest that Hh activity limits the range of Wnt signaling by limiting the number of cells that express *wnt1* RNA.

We also tested the effect of Wnt signaling on the range of Hh activity by examining *ptc1* expression. Whereas *ptc1* expression was not evident in the cerebellum of wild-type embryos (Supplementary Fig. 2A, B), *ptc1* expression appeared to be expanded into the cerebellum of *Df(LG01:lef1)^{x8}* mutant embryos (Supplementary Fig. 2C, D). In contrast, *ptc1* expression was not evident in the cerebellum of 48 hpf transgenic embryos in which Dkk was induced at 30 hpf (Supplementary Fig. 2E, F). Taken together, these data raise the possibility that, prior to 30 hpf, Wnt signaling limits the range of Hh activity in the cerebellum and is later required for formation of *olig2*⁺ neurons.

Discussion

olig2 expression marks a subset of cerebellar neurons

Although zebrafish have become an increasingly important model for investigating neural development, the composition and organization of the zebrafish cerebellum remains poorly described. Consequently, understanding of the mechanisms that pattern the zebrafish cerebellum and regulate specification of cerebellar cell fate remains incomplete. We noted that cerebellar cells of zebrafish embryos prominently express *olig2* RNA and EGFP driven by *olig2* regulatory sequences. EGFP⁺ cells of transgenic animals extend axons to the deep brainstem and form extensive dendritic arbors. *Olig2* genes, which are conserved among mammals, birds and fish, encode bHLH transcription factors necessary for motor neuron and oligodendrocyte development (Lu et al., 2002; Park et al., 2002; Zhou and Anderson, 2002) but have not yet been implicated in cerebellum development. Our data reveal *olig2* expression as a new marker of developing cerebellar cells in zebrafish and raise the possibility that *olig2* promotes specification or differentiation of some cerebellar neurons.

The long axons and highly branched dendrites of *olig2*⁺ cerebellar cells are characteristic of eurydendroid neurons. However, *olig2*⁺ neurons do not express the canonical markers of eurydendroid neurons nor of Purkinje neurons. During early larval stages cells that express Zebrin II, which marks Purkinje neurons, were mostly located just dorsal to *olig2*⁺ cells whereas Calretinin⁺ eurydendroid neurons, which occupy the granule layer of teleosts (Castro et al., 2006; Diaz-Regueira and Anadon, 2000), were located just ventral to *olig2*⁺ cells. At

later larval stages *Zebrin II*⁺, *Calretinin*⁺ and *olig2*⁺ cells remained as separate populations, although many *Zebrin II*⁺ and *olig2*⁺ cells were intermixed. Interestingly, subdivision of eurydendroid neurons into *Calretinin*⁺ *olig2*⁻ and *Calretinin*⁻ *olig2*⁺ cells might reflect morphological differences between eurydendroid neurons. Goldfish have at least three types of eurydendroid neurons that can be distinguished by the shapes of their soma and dendrite number: fusiform and polygonal cells, which are distributed throughout the molecular, Purkinje and granule layers, and monopolar cells, which have single dendrites and are mostly associated with the Purkinje layer of the ventrocaudal corpus cerebelli (Ikenaga et al., 2005). The soma of *olig2*⁺ cells are rounded, and each appears to extend a single, extensively branched dendrite into the molecular layer. This raises the possibility that *olig2* expression marks monopolar eurydendroid neurons, although definitive molecular marker evidence is still required.

Hh signaling and cerebellar patterning

Hh proteins, secreted by ventral neural tube cells and underlying notochord, function as morphogens to pattern the DV axis of the CNS (Ingham and McMahon, 2001; Jessell, 2000). This is best documented in spinal cord, where different concentrations of Shh specify distinct types of neurons that occupy different DV positions. Loss of Shh function results in loss of ventral spinal cord identity and expansion of dorsal identity (Chiang et al., 1996). Similarly, Shh is necessary for formation of dopaminergic and serotonergic neurons in the ventral midbrain and anterior hindbrain (Ye et al., 1998), indicating that its role in DV patterning extends along the entire length of the neural tube.

Hh signaling has a well established role in promoting granule neuron precursor proliferation in mice (Dahmane and Ruiz i Altaba., 1999; Corrales et al., 2004; Lewis et al., 2004) but whether it also influences formation of other cerebellar cells is less clear. Calbindin⁺ Purkinje neurons were present in mice in which *Shh* or *Gli2* were conditionally inactivated in the cerebellar primordium (Blaess et al., 2006, Lewis et al., 2004; Corrales et al., 2004). However, the distribution of Purkinje neurons was somewhat altered and they occupied slightly broader layers in mutant animals compared to wild type, reminiscent of the broadened domain of *olig2*⁺ cells we observed in zebrafish embryos deficient for Hh signaling. However, *Shh* conditional mutants had progressively fewer Purkinje neurons than wild-type mice when compared from E18.5 into adulthood (Lewis et al., 2004). The progressive decrease in cell number suggests that Purkinje neurons were lost due to lack of trophic support by the diminished granule cell population of these mice, leaving the possible role of Hh in the initial formation of Purkinje neurons an open question.

Our data raise the possibility that in zebrafish Hh signaling regulates DV pattern of the cerebellum. We found that *smo* mutant embryos had morphologically distinct cerebellums and that the *olig2* expression domain appeared to be broader in the anteroposterior axis and to extend more ventrally. Quantification of *olig2*⁺ cells using a transgenic reporter revealed a two-fold excess relative to wild type and we found no evidence of changes in the number of apoptotic cells. Pharmacological inhibition of Hh signaling using cyclopamine produced similar effects and also revealed that Hh is required as late as 33 hpf, well after formation of the cerebellar anlage, to limit the number of *olig2*⁺ cells. Although we were unable to investigate formation of *Calretinin*⁺ eurydendroid cells and Purkinje neurons, because mutant and cyclopamine-treated larvae die before expression of *Calretinin* and *Zebrin II*, the ventral expansion of *olig2* expression is strongly consistent with a role for DV pattern formation in the cerebellum by Hh signaling.

Wnt signaling is necessary for cerebellar *olig2*⁺ neuron development

Similar to ventral neural tube, patterning of dorsal neural tube also depends on secreted factors (Lee and Jessell, 1999; Liem et al., 1995). In particular, *Wnt1* and *Wnt3a* are expressed

throughout nearly the entire AP extent of the dorsal CNS (Parr et al., 1993). Mouse *Wnt1* mutants had greatly reduced or nearly absent cerebellums (Bally-Cuif et al., 1995; Thomas and Capecchi, 1990), precluding investigation of cerebellar DV patterning, but *Wnt1/Wnt3a* double mutants had a deficit of dorsal spinal cord interneurons (Ikeya et al., 1997; Muroyama et al., 2002). Zebrafish *wnt1* and *wnt3l* are expressed prominently in the midbrain at the boundary with hindbrain (Buckles et al., 2004), similar to mouse *Wnt1* and *Wnt3*. Additionally, we detected a lower level of *wnt1* transcripts in dorsal cerebellum at 30 hpf but not 48 hpf, which temporally coincides with Hh-dependent patterning of *olig2*⁺ cells. Notably, the midbrain-hindbrain boundary appeared present in embryos homozygous for a chromosomal deletion spanning *lef1*, which encodes a transcription factor that mediates Wnt signaling. This is a milder phenotype than that produced by *Wnt1* mutation in mice or simultaneous disruption of *wnt1*, *wnt3l* and *wnt10b* functions in zebrafish (Buckles et al., 2004). *olig2* expression was entirely absent from the cerebellums of *lef1* deficient embryos, suggesting that Wnt signals are required for *olig2*⁺ neuron development. To test this further without the complications of eliminating multiple genes within the chromosomal deficiency or disruption of an early developmental role of Wnt signaling in forming the cerebellar anlage, we induced expression of *Dkk1*, a Wnt inhibitor, at 30 hpf. Similar to *lef1* deficient embryos, *Dkk1* expression blocked *olig2* expression within cerebellum but not in ventral diencephalon or spinal cord. Thus, our data provide evidence that Wnt signaling is necessary for specification of *olig2*⁺ neurons in cerebellum.

The complementary effects of loss of Hh and Wnt signaling on *olig2* expression suggested that they act in opposition to regulate the number and distribution of *olig2*⁺ cells. Consistent with this, *wnt1* expression was expanded ventrally and posteriorly in CA-treated embryos. Notably, *Wnt1* expression was also expanded in the midbrain-hindbrain region of mice in which *Smo* was conditionally inactivated (Blaess et al., 2006). We also found that *ptc1* expression, which serves as an indicator of Hh signaling activity, was expanded dorsally in *lef1* deficient embryos. Finally, embryos deficient for both Hh and Wnt signaling were identical to embryos that lacked only Wnt signaling in that neither had cerebellar *olig2* expression. We propose that Hh activity limits *olig2*⁺ cell formation by restricting the ventral extent of Wnt signaling, which is necessary for *olig2*⁺ cell specification.

Pattern formation via antagonistic signaling between the Hh and Wnt pathways is an established development mechanism. For example, in the ventral spinal cord Wnt inhibitors regulate transcription of Hh-responsive genes (Lei et al., 2006) whereas in dorsal spinal cord Gli3 repressor inhibits Wnt signaling (Ulloa et al., 2007). Within the epithelium of the colon, high levels of Wnt signaling are restricted to the base of the proliferative crypt by Indian Hedgehog (*Ihh*), expressed by mature colonocytes, and *Ihh* reciprocally inhibits expression of Wnt target genes (van den Brink et al., 2004). Our data indicate that antagonistic Hh and Wnt signaling also pattern the DV axis of the zebrafish cerebellum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Richard Hawkes for Zebrin II antibody and advice and Randall Moon for use of *Tg(HSP70:dkk1)* embryos. This work was supported by a Vanderbilt University Discovery Grant, a zebrafish initiative funded by the Vanderbilt University Academic Venture Capital Fund and NIH grant NS04668 (B.A).

References

- Alcedo J, Ayzenzon M, Von Ohlen T, Noll M, Hooper JE. The *Drosophila* smoothed gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* 1996;86:221–232. [PubMed: 8706127]
- Altman J, Bayer SA. Prenatal development of the cerebellar system in the rat. I. Cytogenesis and histogenesis of the deep nuclei and the cortex of the cerebellum. *J Comp Neurol* 1978;179:23–48. [PubMed: 8980716]
- Altman J, Bayer SA. Embryonic development of the rat cerebellum. III. Regional differences in the time of origin, migration, and settling of Purkinje cells. *J Comp Neurol* 1985;231:42–65. [PubMed: 3968228]
- Bally-Cuif L, Cholley B, Wassef M. Involvement of Wnt-1 in the formation of the mes/metencephalic boundary. *Mech Dev* 1995;53:23–34. [PubMed: 8555108]
- Barresi MJ, Stickney HL, Devoto SH. The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development* 2000;127:2189–2199. [PubMed: 10769242]
- Brochu G, Maler L, Hawkes R. Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. *J Comp Neurol* 1990;291:538–552. [PubMed: 2329190]
- Buckles GR, Thorpe CJ, Ramel MC, Lekven AC. Combinatorial Wnt control of zebrafish midbrain-hindbrain boundary formation. *Mech Dev* 2004;121:437–447. [PubMed: 15147762]
- Castro A, Becerra M, Manso MJ, Anadon R. Calretinin immunoreactivity in the brain of the zebrafish, *Danio rerio*: distribution and comparison with some neuropeptides and neurotransmitter-synthesizing enzymes. I. Olfactory organ and forebrain. *J Comp Neurol* 2006;494:435–459. [PubMed: 16320255]
- Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothed. *Genes Dev* 2002;16:2743–2748. [PubMed: 12414725]
- Chen W, Burgess S, Hopkins N. Analysis of the zebrafish smoothed mutant reveals conserved and divergent functions of hedgehog activity. *Development* 2001;128:2385–2396. [PubMed: 11493557]
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996;383:407–413. [PubMed: 8837770]
- Clevers H, van de Wetering M. TCF/LEF factor earn their wings. *Trends Genet* 1997;13:485–489. [PubMed: 9433138]
- Concordet JP, Lewis KE, Moore JW, Goodrich LV, Johnson RL, Scott MP, Ingham PW. Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* 1996;122:2835–2846. [PubMed: 8787757]
- Currie PD, Ingham PW. Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* 1996;382:452–455. [PubMed: 8684485]
- Dahmane N, Ruiz i Altaba A. Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* 1999;126:3089–3100. [PubMed: 10375501]
- de Zeeuw CI, Berrebi AS. Individual Purkinje cell axons terminate on both inhibitory and excitatory neurons in the cerebellar and vestibular nuclei. *Ann N Y Acad Sci* 1996;781:607–610. [PubMed: 8694453]
- Diaz-Regueira S, Anadon R. Calretinin expression in specific neuronal systems in the brain of an advanced teleost, the grey mullet (*Chelon labrosus*). *J Comp Neurol* 2000;426:81–105. [PubMed: 10980485]
- Ekker SC, Ungar AR, Greenstein P, von Kessler DP, Porter JA, Moon RT, Beachy PA. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr Biol* 1995;5:944–955. [PubMed: 7583153]
- Fiez JA. Cerebellar contributions to cognition. *Neuron* 1996;16:13–15. [PubMed: 8562076]
- Finger TE. Efferent neurons of the teleost cerebellum. *Brain Res* 1978;153:608–614. [PubMed: 81089]
- Gilthorpe JD, Papantoniou EK, Chedotal A, Lumsden A, Wingate RJ. The migration of cerebellar rhombic lip derivatives. *Development* 2002;129:4719–4728. [PubMed: 12361964]

- Hauptmann G, Gerster T. Multicolor whole-mount in situ hybridization. *Methods Mol Biol* 2000;137:139–148. [PubMed: 10948532]
- Holmes G. The Cerebellum of Man. *Brain* 1939;62:30.
- Huangfu D, Anderson KV. Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from *Drosophila* to vertebrates. *Development* 2006;133:3–14. [PubMed: 16339192]
- Ikenaga T, Yoshida M, Uematsu K. Morphology and immunohistochemistry of efferent neurons of the goldfish corpus cerebelli. *J Comp Neurol* 2005;487:300–311. [PubMed: 15892096]
- Ikenaga T, Yoshida M, Uematsu K. Cerebellar efferent neurons in teleost fish. *Cerebellum* 2006;5:268–274. [PubMed: 17134989]
- Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 1997;389:966–970. [PubMed: 9353119]
- Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001;15:3059–3087. [PubMed: 11731473]
- Ito M. Mechanisms of motor learning in the cerebellum. *Brain Res* 2000;886:237–245. [PubMed: 11119699]
- Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 2000;1:20–29. [PubMed: 11262869]
- Karlstrom RO, Tyurina OV, Kawakami A, Nishioka N, Talbot WS, Sasaki H, Schier AF. Genetic analysis of zebrafish *gli1* and *gli2* reveals divergent requirements for gli genes in vertebrate development. *Development* 2003;130:1549–1564. [PubMed: 12620981]
- Kelly GM, Moon RT. Involvement of *wnt1* and *pax2* in the formation of the midbrain-hindbrain boundary in the zebrafish gastrula. *Dev Genet* 1995;17:129–140. [PubMed: 7586754]
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn* 1995;203:253–310. [PubMed: 8589427]
- Krauss S, Concordet JP, Ingham PW. A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 1993;75:1431–1444. [PubMed: 8269519]
- Lannoo MJ, Brochu G, Maler L, Hawkes R. Zebrin II immunoreactivity in the rat and in the weakly electric teleost *Eigenmannia* (gymnotiformes) reveals three modes of Purkinje cell development. *J Comp Neurol* 1991a;310:215–233. [PubMed: 1955583]
- Lannoo MJ, Ross L, Maler L, Hawkes R. Development of the cerebellum and its extracerebellar Purkinje cell projection in teleost fishes as determined by zebrin II immunocytochemistry. *Prog Neurobiol* 1991b;37:329–363. [PubMed: 1758964]
- Lee J, Platt KA, Censullo P, Ruiz i Altaba A. *Gli1* is a target of Sonic hedgehog that induces ventral neural tube development. *Development* 1997;124:2537–2552. [PubMed: 9216996]
- Lee KJ, Jessell TM. The specification of dorsal cell fates in the vertebrate central nervous system. *Annu Rev Neurosci* 1999;22:261–294. [PubMed: 10202540]
- Lei Q, Jeong Y, Misra K, Li S, Zelman AK, Epstein DJ, Matise MP. Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. *Dev Cell* 2006;11:325–337. [PubMed: 16950124]
- Lewis KE, Eisen JS. Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* 2001;128:3485–3495. [PubMed: 11566854]
- Liem KF Jr, Tremml G, Roelink H, Jessell TM. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* 1995;82:969–979. [PubMed: 7553857]
- Lin JC, Cepko CL. Biphasic dispersion of clones containing Purkinje cells and glia in the developing chick cerebellum. *Dev Biol* 1999;211:177–197. [PubMed: 10395781]
- Liu G, Bafico A, Harris VK, Aaronson SA. A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. *Mol Cell Biol* 2003;23:5825–5835. [PubMed: 12897152]
- Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* 2002;109:75–86. [PubMed: 11955448]

- Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* 2000;25:317–329. [PubMed: 10719888]
- Marigo V, Tabin CJ. Regulation of patched by sonic hedgehog in the developing neural tube. *Proc Natl Acad Sci U S A* 1996;93:9346–9351. [PubMed: 8790332]
- Marusich MF, Furneaux HM, Henion PD, Weston JA. Hu neuronal proteins are expressed in proliferating neurogenic cells. *J Neurobiol* 1994;25:143–155. [PubMed: 7517436]
- Mathis L, Bonnerot C, Puelles L, Nicolas JF. Retrospective clonal analysis of the cerebellum using genetic lacZ/lacZ mouse mosaics. *Development* 1997;124:4089–4104. [PubMed: 9374405]
- McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 1990;62:1073–1085. [PubMed: 2205396]
- Meek J, Hafmans TG, Maler L, Hawkes R. Distribution of zebrin II in the gigantocerebellum of the mormyrid fish *Gnathonemus petersii* compared with other teleosts. *J Comp Neurol* 1992;316:17–31. [PubMed: 1573049]
- Molven A, Njolstad PR, Fjose A. Genomic structure and restricted neural expression of the zebrafish wnt-1 (int-1) gene. *Embo J* 1991;10:799–807. [PubMed: 2009859]
- Morton SM, Bastian AJ. Cerebellar control of balance and locomotion. *Neuroscientist* 2004;10:247–259. [PubMed: 15155063]
- Mueller T, Vernier P, Wullimann MF. A phylotypic stage in vertebrate brain development: GABA cell patterns in zebrafish compared with mouse. *J Comp Neurol* 2006;494:620–634. [PubMed: 16374795]
- Mueller T, Wullimann MF. Anatomy of neurogenesis in the early zebrafish brain. *Brain Res Dev Brain Res* 2003;140:137–155.
- Muroyama Y, Fujihara M, Ikeya M, Kondoh H, Takada S. Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev* 2002;16:548–553. [PubMed: 11877374]
- Park HC, Mehta A, Richardson JS, Appel B. *olig2* is required for zebrafish primary motor neuron and oligodendrocyte development. *Dev Biol* 2002;248:356–368. [PubMed: 12167410]
- Park HC, Shin J, Appel B. Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. *Development* 2004;131:5959–5969. [PubMed: 15539490]
- Parr BA, Shea MJ, Vassileva G, McMahon AP. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* 1993;119:247–261. [PubMed: 8275860]
- Phillips BT, Kwon HJ, Melton C, Houghtaling P, Fritz A, Riley BB. Zebrafish *msxB*, *msxC* and *msxE* function together to refine the neural-nonneural border and regulate cranial placodes and neural crest development. *Dev Biol* 2006;294:376–390. [PubMed: 16631154]
- Porteros A, Arevalo R, Brinon JG, Crespo C, Aijon J, Alonso JR. Parvalbumin immunoreactivity during the development of the cerebellum of the rainbow trout. *Brain Res Dev Brain Res* 1998;109:221–227.
- Raymond JL, Lisberger SG, Mauk MD. The cerebellum: a neuronal learning machine? *Science* 1996;272:1126–1131. [PubMed: 8638157]
- Roelink H, Nusse R. Expression of two members of the Wnt family during mouse development--restricted temporal and spatial patterns in the developing neural tube. *Genes Dev* 1991;5:381–388. [PubMed: 2001840]
- Schutter DJ, van Honk J. The cerebellum on the rise in human emotion. *Cerebellum* 2005;4:290–294. [PubMed: 16321885]
- Schwaller B, Buchwald P, Blumcke I, Celio MR, Hunziker W. Characterization of a polyclonal antiserum against the purified human recombinant calcium binding protein calretinin. *Cell Calcium* 1993;14:639–648. [PubMed: 8242719]
- Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr Biol* 2001;11:951–961. [PubMed: 11448771]
- Shin J, Park HC, Topczewska JM, Mawdsley DJ, Appel B. Neural cell fate analysis in zebrafish using *olig2* BAC transgenics. *Methods Cell Sci* 2003;25:7–14. [PubMed: 14739582]
- Stoick-Cooper CL, Weidinger G, Riehle KJ, Hubbert C, Major MB, Fausto N, Moon RT. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* 2007;134:479–489. [PubMed: 17185322]

- Thach WT, Goodkin HP, Keating JG. The cerebellum and the adaptive coordination of movement. *Annu Rev Neurosci* 1992;15:403–442. [PubMed: 1575449]
- Thomas KR, Capecchi MR. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 1990;346:847–850. [PubMed: 2202907]
- Traiffort E, Charytoniuk DA, Faure H, Ruat M. Regional distribution of Sonic Hedgehog, patched, and smoothed mRNA in the adult rat brain. *J Neurochem* 1998;70:1327–1330. [PubMed: 9489757]
- Ulloa F, Itasaki N, Briscoe J. Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signaling. *Curr Biol* 2007;17:545–550. [PubMed: 17331723]
- van den Brink GR, Bleuming SA, Hardwick JC, Schepman BL, Offerhaus GJ, Keller JJ, Nielsen C, Gaffield W, van Deventer SJ, Roberts DJ, Peppelenbosch MP. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet* 2004;36:277–282. [PubMed: 14770182]
- Varga ZM, Amores A, Lewis KE, Yan YL, Postlethwait JH, Eisen JS, Westerfield M. Zebrafish smoothed functions in ventral neural tube specification and axon tract formation. *Development* 2001;128:3497–3509. [PubMed: 11566855]
- Wang VY, Zoghbi HY. Genetic regulation of cerebellar development. *Nat Rev Neurosci* 2001;2:484–491. [PubMed: 11433373]
- Wilson L, Maden M. The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Dev Biol* 2005;282:1–13. [PubMed: 15936325]
- Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* 1998;93:755–766. [PubMed: 9630220]
- Zhou Q, Anderson DJ. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 2002;109:61–73. [PubMed: 11955447]

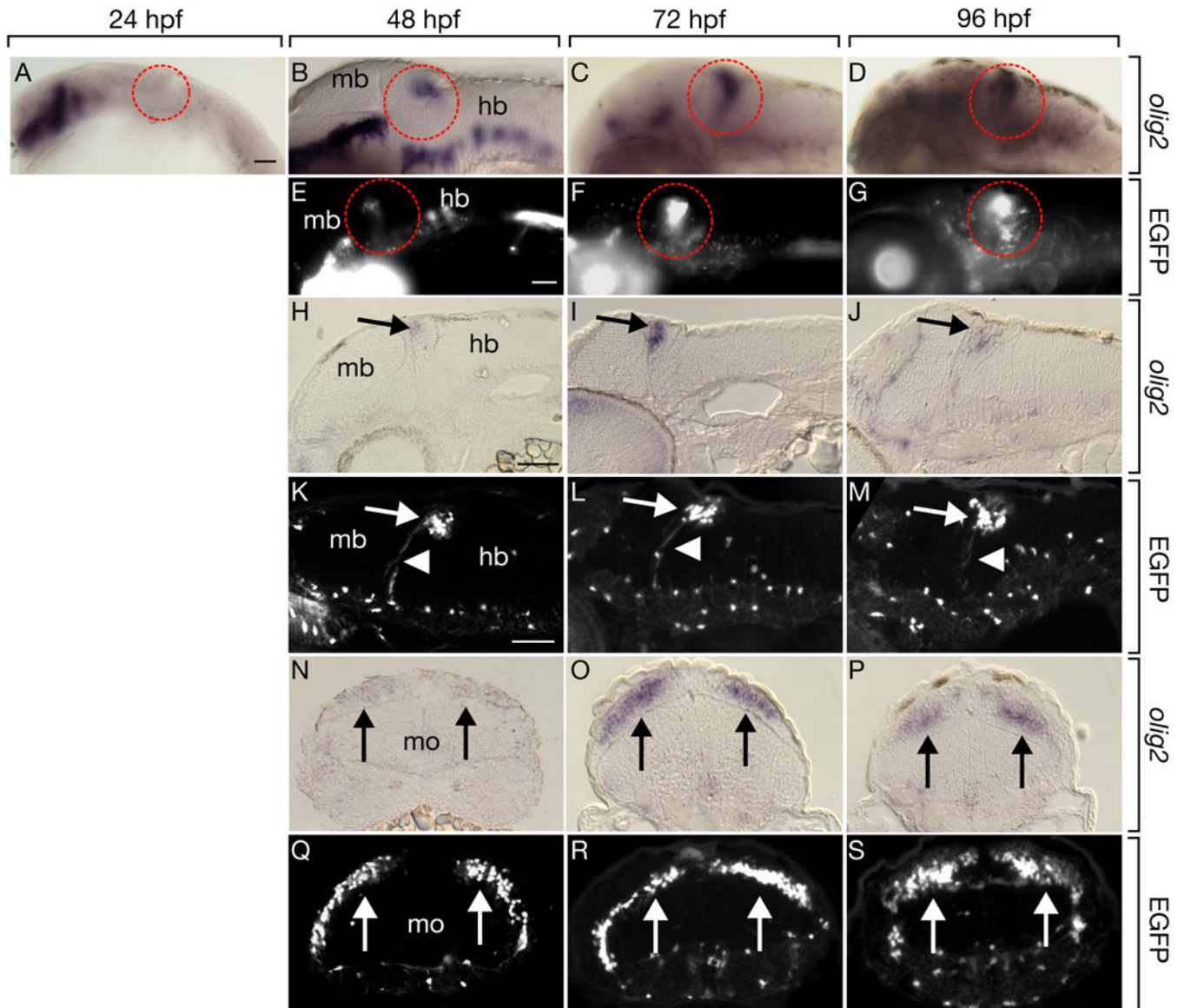


Fig. 1. Transgenic reporter gene expression recapitulates endogenous *olig2* RNA expression in zebrafish cerebellum. Lateral whole mount views and sagittal sections are shown with dorsal up and anterior to the left. Transverse sections are oriented dorsal up. Midbrain (mb), hindbrain (hb) and medulla oblongata (mo) are marked. (A–D) Developmental time course of *olig2* expression detected by in situ RNA hybridization in whole embryos. Dashed circles mark where cerebellum is located. (E–G) EGFP expression driven by the *Tg(olig2:egfp)* transgene in live embryos. Expression within cerebellum is circled. (H–J) Sagittal sections through cerebellum with *olig2* RNA expression marked by arrows. (K–M) Arrows mark EGFP⁺ cells in cerebellum of sagittally sectioned *Tg(olig2:egfp)* embryos and arrowheads show axonal extensions from EGFP⁺ cells. Transverse sections through cerebellum showing *olig2* RNA (N–P) and EGFP (Q–S) expression marked by arrows. Scale bars represent 20 μ m in all panels.

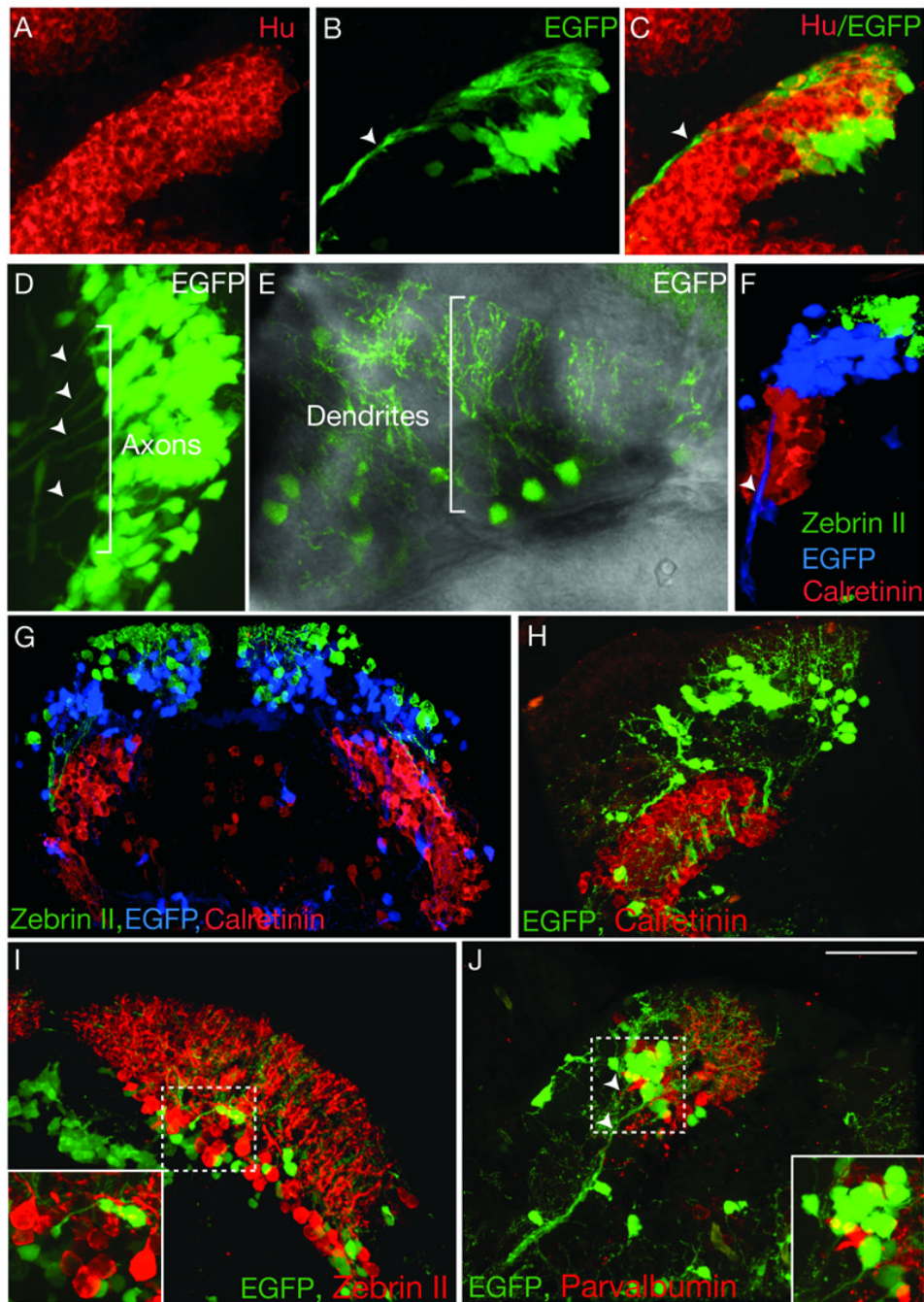


Fig. 2. Identification of cerebellar cell types reveals distinct populations of *olig2*⁺ neurons organized along the DV axis. (A–C) Sagittal section through cerebellum of 48 hpf *Tg(olig2:egfp)* transgenic embryo showing EGFP⁺ cells expressed Hu (red). Arrowheads mark long axonal projections. (D) Dorsal view with anterior to left of one half of the cerebellum in living *Tg(olig2:egfp)* embryo at 56 hpf. Bracket and arrowheads mark EGFP⁺ axons extending from EGFP⁺ cells ventrally along midbrain-hindbrain boundary. (E) Dorsal view of cerebellum in living *Tg(olig2:egfp)* larva at 7 dpf, anterior is along the bottom. Bracket indicates extensive dendritic branches originating from EGFP⁺ cells. (F) 3-D reconstruction of 5 dpf sagittal section of a *Tg(olig2:egfp)* larva illustrating DV organization of Zebrin II⁺ (green), EGFP⁺ (blue) and

Calretinin⁺ (red) cells. Arrowhead marks ventrally extending EGFP⁺ axons. (G) Transverse section a 5 dpf *Tg(olig2:egfp)* larva showing Zebrin II, EGFP and Calretinin labeling of distinct populations and DV arrangement. (H–J) Sections of 14 dpf *Tg(olig2:egfp)* transgenic larvae labeled with Calretinin, Parvalbumin and Zebrin II antibodies. (H) Transverse hemisection shows EGFP⁺ cells dorsal to Calretinin⁺ eurydendroid cells. (I) Transverse hemisection showing EGFP⁺ cells intermixed with Zebrin II⁺ Purkinje neurons. Inset shows enlargement of area framed by dashed lines. (J) Sagittal section showing distinct populations of Parvalbumin⁺ Purkinje neurons and EGFP⁺ neurons. Arrowheads mark EGFP⁺ axons extending toward deep brainstem. Inset shows enlargement of area framed by dashed lines. Scale bar = 20 μm for all panels except G, for which it represents 40 μm.

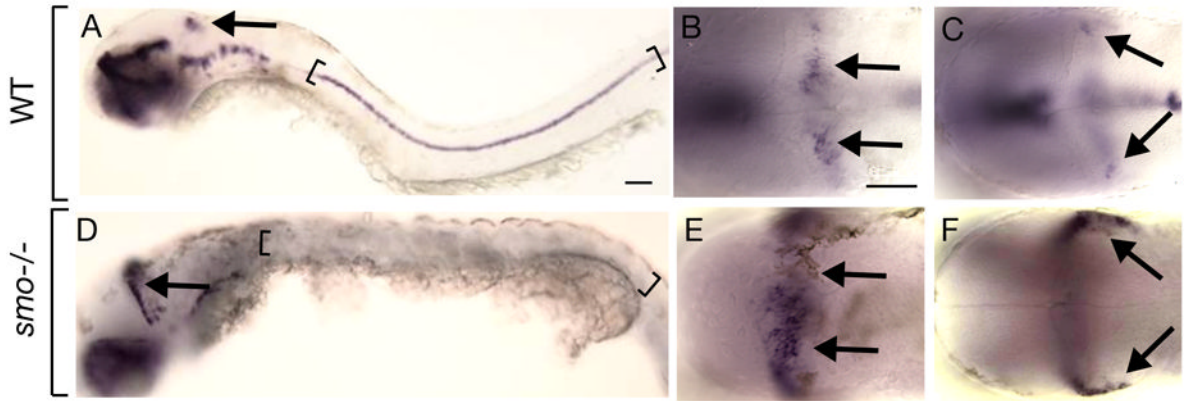


Fig. 3.

Hh signaling restricts cerebellar *olig2* expression. Lateral (A, D) and dorsal (B, C, E, F) views of 48 hpf embryos with anterior to the left. Arrows indicate cerebellar *olig2* expression. Brackets mark ventral spinal cord. (A) Wild-type embryo illustrating *olig2* expression within cerebellum and spinal cord. Dorsal (B) and ventral (C) focal planes of wild-type cerebellum.

(D–F) Similar views of *olig2* expression in *smo* mutant embryo. Note expansion of cerebellar expression and absence of spinal cord expression. Scale bars represent 20 μ m for all panels.

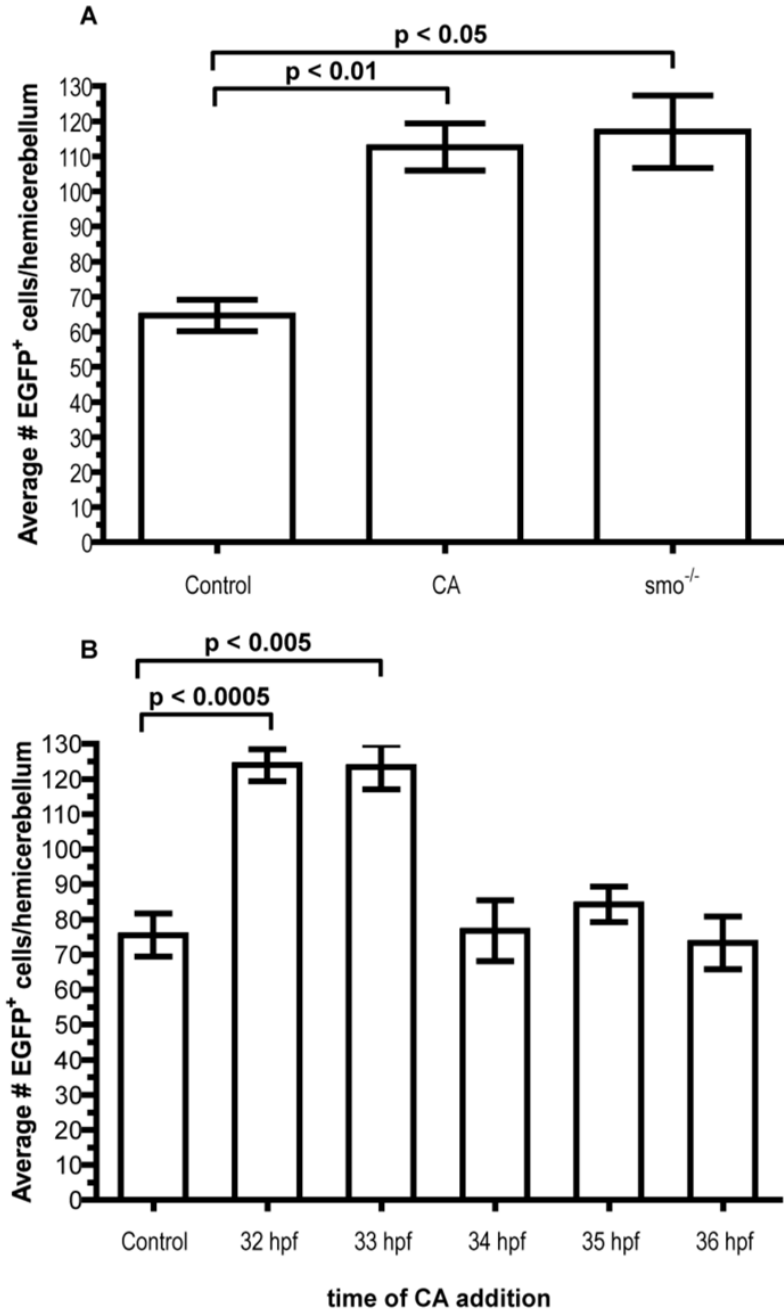


Fig. 4.

Temporal regulation of *olig2*⁺ cell number by Hh signaling. (A) Graph depicting average number of EGFP⁺ cerebellar cells. n = 14 hemicerebellums for each condition. Statistical significance calculated in accordance to Student's t-test. (B) Time course assay of EGFP⁺ cells in *Tg(olig2:egfp)* embryos treated with CA. 32 and 33 hpf treatments produced significant increases compared to control. n = 10 hemicerebellums for each time point except 35 hpf, where n = 11.

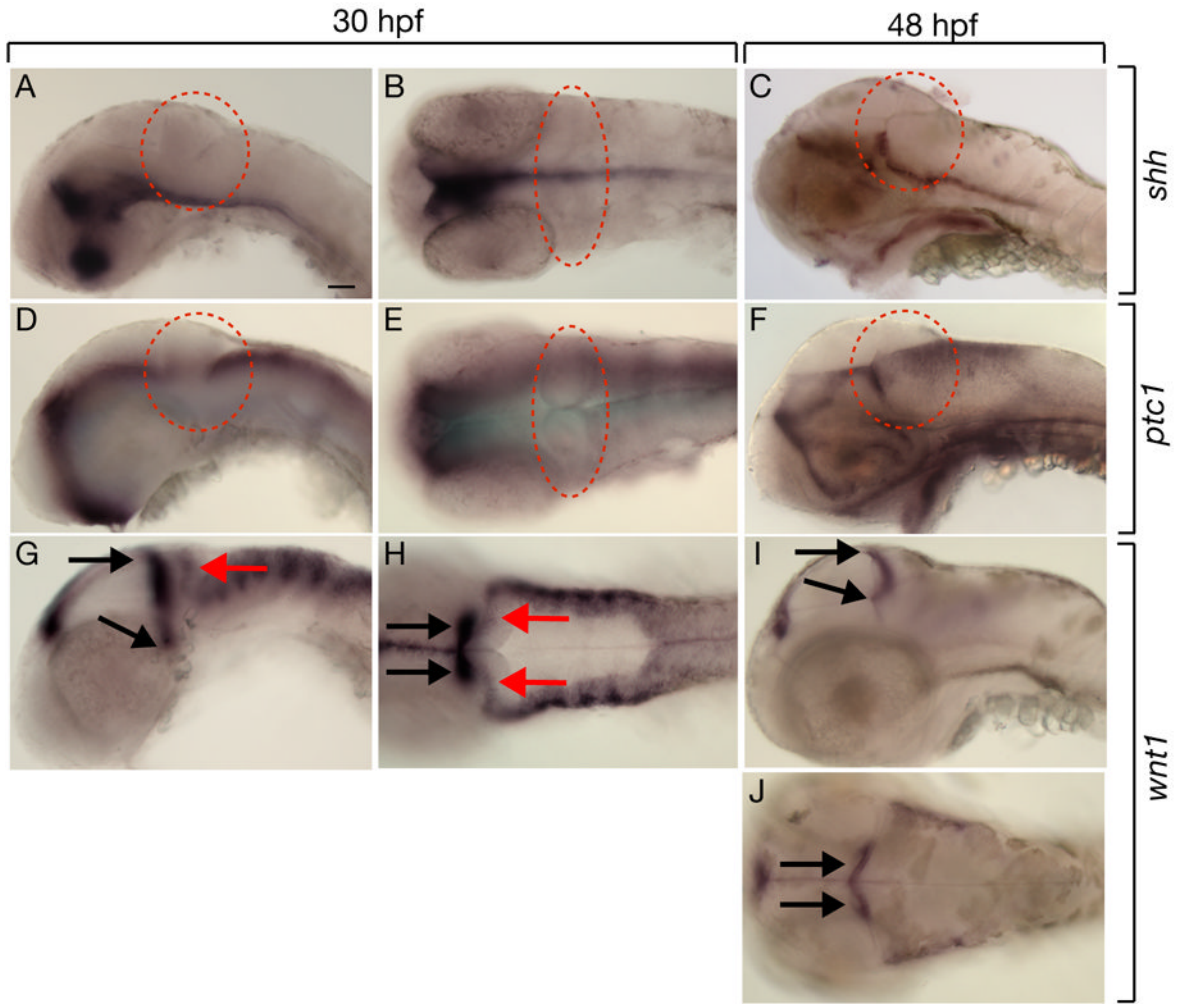


Fig. 5. Zebrafish cerebellum expresses *wnt1* but not Hh pathway genes. All panels are lateral or dorsal views with anterior to the left. Dashed circles mark cerebellums. (A–C) Expression of *shh* was ventral to cerebellum. (D–F) Expression of *ptc1* at 30 hpf was absent from cerebellum but by 48 hpf was present in the lower rhombic lip. (G, H) Expression of *wnt1* at 30 hpf, red arrows mark transient *wnt1* expression within the cerebellum and black arrows mark expression at the midbrain-hindbrain boundary. (I, J) By 48 hpf, *wnt1* expression was no longer present within the cerebellum but persisted at the midbrain-hindbrain boundary. Scale bars represent 20 μ m in all panels.

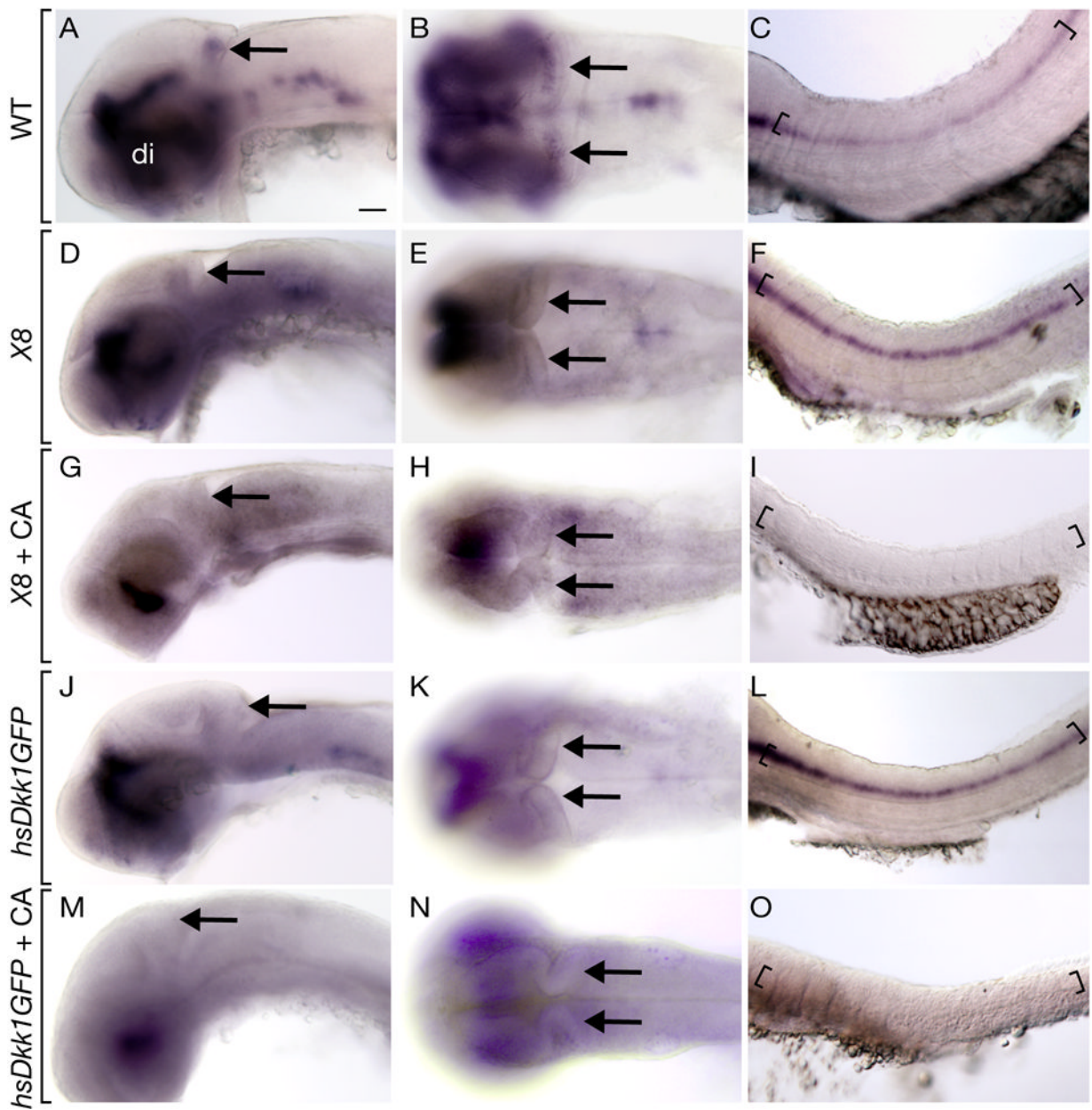


Fig. 6. Wnt signaling is necessary for cerebellar *olig2* expression. All embryos shown are 48 hpf. Anterior is to the left in all panels, the first column depicts a lateral whole mount view, the second column a dorsal view and the third a lateral view of the spinal cord. Black arrows mark cerebellum, brackets mark ventral spinal cord and ventral diencephalon is labeled (di). (A–C) Wild-type *olig2* expression. (D–F) *Df(LG01:lef1)^{x8}* (*X8*) mutant embryos expressed *olig2* in spinal cord but not cerebellum. (G–I) *Df(LG01:lef1)^{x8}* mutant embryos treated with CA at 6 hpf expressed *olig2* in ventral diencephalon but not cerebellum or spinal cord. (J–L) *hsDkk1GFP* embryos heat shocked at 30 hpf expressed *olig2* in ventral diencephalon and spinal cord but not cerebellum. (M–O) *hsDkk1GFP* embryos treated with CA at 6 hpf expressed *olig2* in ventral diencephalon but not spinal cord or cerebellum. Scale bars represent 20 μ m in all panels.

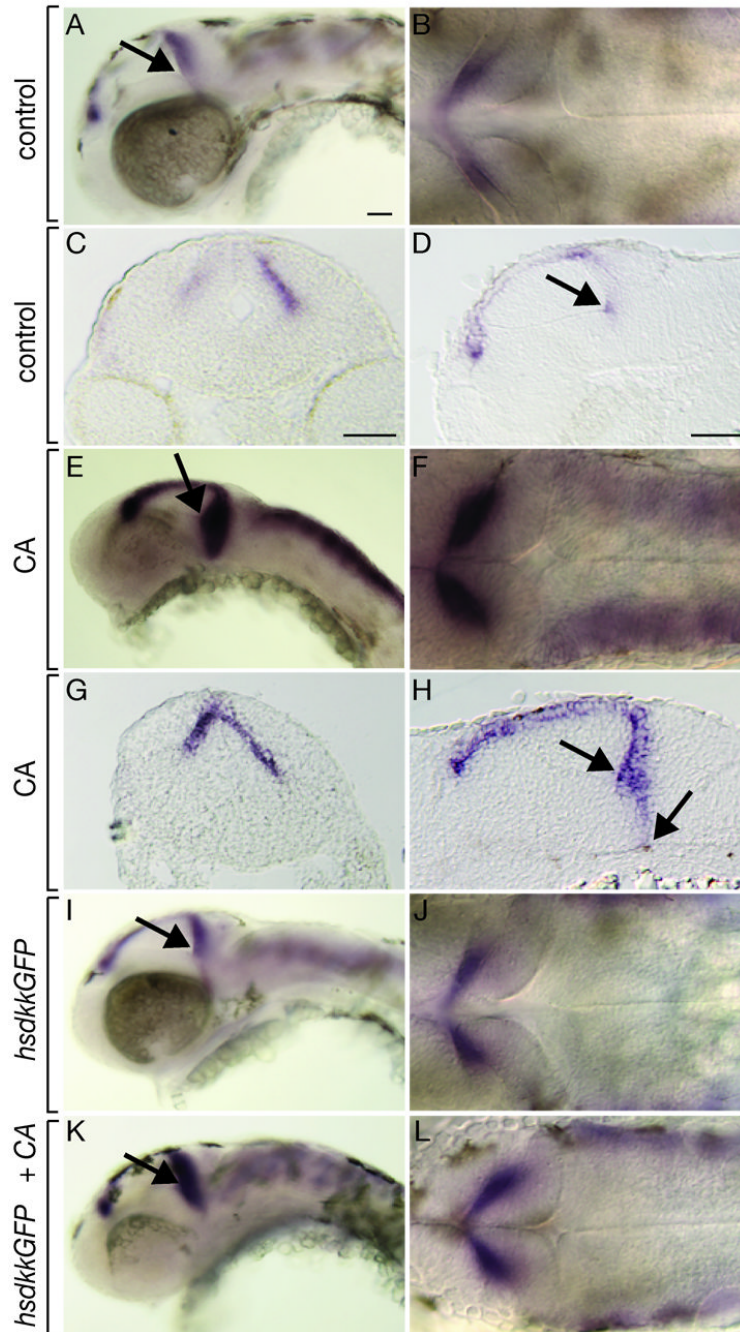


Fig. 7. Inhibition of Hh signaling causes expansion of the *wnt1* expression domain. All embryos shown are 48 hpf. Black arrows in whole mount views depict the morphological line of the midbrain adjoining the midbrain-hindbrain boundary. Lateral whole mount (A), dorsal whole mount (B), transverse section (C) and sagittal section (D) showing *wnt1* expression in control embryos. The black arrows mark the base of the 3rd ventricle, with which the ventral extent of *wnt1* expression coincides in the midbrain-hindbrain region. (E–H) Similar views of *wnt1* expression in embryos treated with CA beginning at 6 hpf. *wnt1* expression in the midbrain-hindbrain region is expanded both ventrally and posteriorly. (I, J) Expression of *wnt1* in embryos that overexpressed Dkk1 at 30 hpf was similar to controls. (K, L) *wnt1* expression was expanded

in embryos treated with CA at 6 hpf and heat shocked to induce Dkk1 expression at 30 hpf. Scale bars represent 20 μm in all panels.