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The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments

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Abstract

The upper rhombic lip, a prominent germinal zone of the cerebellum, was recently demonstrated to generate different neuronal cell types over time from spatial subdomains. We have characterized the differentiation of the upper rhombic lip derived granule cell population in stable GFP-transgenic zebrafish in the context of zebrafish cerebellar morphogenesis. Time-lapse analysis followed by individual granule cell tracing demonstrates that the zebrafish upper rhombic lip is spatially patterned along its mediolateral axis producing different granule cell populations simultaneously. Time-lapse recordings of parallel fiber projections and retrograde labeling reveal that spatial patterning within the rhombic lip corresponds to granule cells of two different functional compartments of the mature cerebellum: the eminentia granularis and the corpus cerebelli. These cerebellar compartments in teleosts correspond to the mammalian vestibulocerebellar and non-vestibulocerebellar system serving balance and locomotion control, respectively. Given the high conservation of cerebellar development in vertebrates, spatial partitioning of the mammalian granule cell population and their corresponding earlier-produced deep nuclei by patterning within the rhombic lip may also delineate distinct functional compartments of the cerebellum. Thus, our findings offer an explanation for how specific functional cerebellar circuitries are laid down by spatio-temporal patterning of cerebellar germinal zones during early brain development.

Keywords: Zebrafish; Cerebellum; Granule cell; Neuronal migration; Bioimaging; Cell tracing; Fate mapping

Introduction

Many developmental, histological and functional properties of the cerebellum are highly conserved among vertebrates ranging from teleost fish to mammals. This evolutionary conservation includes the position and patterning of the cerebellum under control of the organizer activity of cells at the midbrain–hindbrain boundary (MHB) (Voogd and Glickstein, 1998; Middleton and Strick, 1998; Martinez et al., 1999; Wurst and Bally-Cuif, 2001; Wang and Zoghbi, 2001), its laminar organization comprised by few neuronal cell types of distinct morphology (Nieuwenhuys, 1967; Lannoo et al., 1991; Altman and Bayer, 1997), the characteristic cerebellar feedback circuitry (Wullimann and Northcutt, 1988; Wullimann, 1998; Bengtsson and Hesslow, 2006; Ito, 2006) and, lastly, the role of the cerebellum in coordinating locomotion and contributing to motor learning (Elbert et al., 1983; Roberts et al., 1992; Fiez, 1996; Gao et al., 1996; Boyden et al., 2004; Morton and Bastian, 2004; Rodriguez et al., 2005). Thus, the cerebellum represents one of the highest conserved compartments of the vertebrate brain.

Migration of neuronal precursors is a key step in cerebellar differentiation (Goldowitz and Hamre, 1998; Sotelo, 2004). The precursors of the most common cerebellar interneurons, the granule cells, migrate over long distances. Several fate mapping studies in mouse and chick embryos have revealed their detailed migratory pathways (Miale and Sidman, 1961; Hatten and Heintz, 1995; Lin et al., 2001; Gilthorpe et al., 2002). Granule cells arise from the cerebellar part of a specific germinal zone, called the rhombic lip, which runs along the dorsal aspect of the entire hindbrain ventricle (4th ventricle) (Ben-Arie et al., 1997; Alder et al., 1996). This cerebellar rhombic lip, termed upper rhombic lip (URL), undergoes a reorientation caused by the

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rotation of the cerebellar primordium during opening of the hindbrain ventricle (Sgaier et al., 2005; Distel et al., 2006). Thus, the URL displays a mediolateral orientation, while the remaining rhombic lip of the hindbrain posterior to the cerebellum, the lower rhombic lip (LRL), displays an anterior–posterior orientation.

Recent fate mapping studies demonstrated that the upper rhombic lip successively produces different neuronal cell types during embryogenesis including neurons of various mesencephalic and hindbrain nuclei, followed by cerebellar deep nuclei and finally granule cells (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005; Wilson and Wingate, 2006). Furthermore, lineage tracing of clonally related clusters of URL cells in chick and mouse embryos have shown that the URL gives rise to parasagittal domains of granule cells (Ryder and Cepko, 1994; Mathis and Nicolas, 2003). This suggests that the URL is also spatially patterned along its mediolateral axis. Elegant evidence for such URL subdomains was provided by genetic fate mapping in mouse embryos, where the differential mediolateral extent of expression driven by the promoters from the *engrailed1* and *engrailed2* genes within the cerebellar primordium was exploited (Sgaier et al., 2005). This study showed that granule cell progenitors maintain their mediolateral coordinates after emigration from the URL until the initiation of cerebellar foliation. At this time, granule cell progenitors derived from lateral positions within the URL migrate medially to preferentially populate the posterior-most folia of the mouse cerebellum (Sgaier et al., 2005). Recent genetic experiments suggest that different cerebellar subdomains are generated by a differential sensitivity to *engrailed* gene function (Sgaier et al., 2007). Similar genetic fate mapping studies in mouse for the lower rhombic lip have shown that it is subdivided as well, along its anteroposterior axis (Farago et al., 2006). Such spatiotemporal partitioning of germinal zones provides a powerful means of specifying distinct functional compartments in the mature brain at embryonic stages (Landsberg et al., 2005; Farago et al., 2006).

The zebrafish has become an important model organism to characterize the genetic mechanisms underlying vertebrate brain development. The largely transparent zebrafish embryos are particularly suited for intravital time-lapse studies. In this fashion, molecular mechanisms of neuronal migration (Köster and Fraser, 2001; Haas and Gilmour, 2006; Kirby et al., 2006) and axon pathfinding (Dynes and Ngai, 1998; Hutson and Chien, 2002; Bak and Fraser, 2003) can be studied in the context of brain morphogenesis. Although some genetic studies on the induction of the cerebellar primordium have been performed in the zebrafish (Reifers et al., 1998; Jaszai et al., 2003; Tallafuss and Bally-Cuif, 2003; Foucher et al., 2006), cerebellar morphogenesis, circuitry formation and function remain poorly characterized. In addition, with the exception of Purkinje cells (Lannoo et al., 1991), none of the other cerebellar neuronal cell types, including the cerebellar granule cells, nor patterning within the rhombic lip have been characterized in zebrafish.

In this study we identify the granule cell population of the zebrafish cerebellum. By combining expression analysis with single cell tracing via time-lapse imaging and retrograde labeling, we characterize granule cell differentiation in the context of cerebellar morphogenesis. We also show that the zebrafish cerebellar rhombic lip is spatially patterned similar to mammals. Moreover, we determine that depending on their mediolateral positions within the URL, granule cell progenitors contribute to different functional domains of the mature zebrafish cerebellum. These findings provide an explanation for how zebrafish cerebellar circuitries of different function may be laid down by spatial patterning of germinal zones during embryonic cerebellar development.

Materials and methods

Maintenance of fish

Raising, spawning, and maintaining of zebrafish lines were performed as described previously (Kimmel et al., 1995; Westerfield, 1995). For simplicity, stable transgenic gata1:GFP embryos and adult fish of the strain 781 (Long et al., 1997) will be referred to as gata1:GFP throughout the manuscript.

RT–PCR

Cloning of a partial cDNA of the zebrafish $Gaba_A$-receptor alpha 6 subunit was performed by degenerate RT–PCR using cDNA from adult brains. The following primers were used: Gaba-up: ATGGAATTCACIATIGAYGTITTYT-TYMG and Gaba-low: GACGCATGCRWARCAIACIGCIATRAACCA to amplify an approximately 560-bp fragment, which was cloned into the pCRII-vector (Invitrogen, San Diego, CA). From this vector, a fragment containing a partial cDNA fragment of 423 bp of the zebrafish GabaRα6 subunit (accession number: EF364095) was amplified using the specific primers gabaRa6up: CAAACGTGGATAGATGACCGGCTGAA and gabaRa6lo: ACCTGTGTT-TGACTTCAACCTTTCACTAGAC followed by subcloning into the pSC-B vector (Stratagene, La Jolla, CA).

Morphological stainings

In order to visualize neuropil and the cellular organization of the cerebellum, vital embryo staining and counterstaining of tissue sections were performed by soaking overnight in 0.001% green-fluorescent Bodipy FL C_5-ceramide (D-3521, Invitrogen) or red-fluorescent Bodipy 630/650-X (D-10000, Invitrogen), respectively. For nuclear counterstaining of fixed tissue, DAPI was used at 0.1 μg/ml (Roche, Indianapolis, IN). To remove excess dye, stained specimens were rinsed in PBS/0.1% Tween-20 before images were acquired.

Expression analysis

mRNA in situ hybridization was performed as described (Köster and Fraser, 2006) with the following additions: juvenile and adult brains were dissected after sacrificing zebrafish by an overdose of MS22 (3-aminobenzoic acid ethylester, Sigma, St. Louis, MO) followed by fixation overnight in 4% paraform aldehyde/PBS/0.1% Tween-20. After hybridization, brains were embedded in 4% agarose/PBS. Vibratome sections (Microm HM 650 V, Walldorf, Germany) were cut at 100 μm thickness and mounted on SuperFrostPlus slides (Menzel, Braunschweig, Germany) prior to probe detection.

For immunohistochemical detection the following antibodies were used: polyclonal rabbit anti-GFP (TP401, 1:500, Torrey Pines Biolabs, Houston, TX), mouse anti-GFP (1:500, Molecular Probes, Eugene, OR), polyclonal rabbit anti-phosphohistone H3, PH3, (1:200, Upstate Biotechnology, Lake Placid, NY), mouse anti-human HuC/D (1:500, Molecular Probes), polyclonal rabbit anti-zebrafish Tag-1 (1:1000, received from C. Stürmer), chicken anti-mouse IgG AlexaFluor488-conjugated (1:200, Molecular Probes), goat anti-rabbit IgG Cy2- or Cy5-conjugated (1:200, Jackson ImmunoResearch West Grove, PA) and

goat anti-mouse IgG Cy3-conjugated (1:200, Jackson ImmunoResearch). Stained sections were photographed using an Axioplan2 microscope equipped with an Axiocam HRc camera. Alternatively, confocal images were acquired using an LSM510 laser-scanning microscope (all from Zeiss, Oberkochen, Germany).

Intravital imaging

Recording of still images and time-lapse microscopy of live zebrafish embryos were performed as described in detail (Köster and Fraser, 2004; Köster and Fraser, 2006). GFP-expressing cells were tracked using the NIH ImageJ software (1.34 S; http://rsb.info.nih.gov/ij/) together with the Manual Tracking plug-in.

Retrograde neuronal labeling

Transgenic gata1:GFP zebrafish larvae were anesthetized at 6 dpf with 0.01% MS22 (3-aminobenzonic acid ethylester, Sigma) in 30% Danieau/0.75 mM phenylthiourea (PTU) followed by embedding in a drop of 1.2% ultra-low gelling agarose (Sigma) with the dorsal side facing up. 6% tetramethylrhodamine-dextran, 3000 MW (Invitrogen) dissolved in PBS/0.4% Triton X-100 was microinjected into the GFP-fluorescent crista cerebellaris of the right larval hindbrain. After dye application, the agarose was removed and the larvae were maintained at 28 °C for 1 day prior to re-embedding and image recording as described previously (Köster and Fraser, 2004; Volkmann and Köster, 2007).

Results

gata1:GFP cells are of rhombic lip origin

We have recently shown that in embryos of the transgenic gata1:GFP 781-strain (Long et al., 1997) (for simplification gata1:GFP from hereon) GFP expressing neuronal precursors migrate from the dorsoposterior region of the cerebellum, likely the upper rhombic lip, toward and along the midbrain–hindbrain boundary (Köster and Fraser, 2006). The identity of this cerebellar neuronal cell type, however, remained unclear. Recent genetic fate mapping studies in the mouse embryo have defined the rhombic lip molecularly by its expression of the bHLH transcription factor Atonal1 (Math1) (Machold and Fishell, 2005; Wang et al., 2005). In contrast, cells derived from the remainder of the cerebellar ventricular zone of the cerebellum express the bHLH transcription factor Ptf1a (Hoshino et al., 2005; Wingate, 2005). This mutually exclusive expression of *atonal1* and *ptf1a* has been conserved in zebrafish (A. Babaryka & R. W. Köster, unpublished). To molecularly confirm that GFP-expressing cells in the cerebellum of the gata1:GFP zebrafish strain are derived from the rhombic lip, we characterized the mRNA expression patterns of *atonal1a* (Kim et al., 1997) and *ptf1a* (Zecchin et al., 2004) using in situ hybridization.

Shortly after the first GFP-expressing cells were detectable in the developing cerebellum, gata1:GFP embryos were fixed and processed for mRNA in situ hybridization followed by immunohistochemistry against GFP (Chapouton et al., 2006). Expression analysis by confocal microscopy revealed that GFP-expressing cells express the rhombic lip marker *atonal1a* (Figs. 1A–D). In addition, GFP-expressing cells were found to be positioned in areas spared by *ptf1a*-expression (Figs. 1E–H). This confirms that GFP-expressing cells in the developing cerebellum of gata1:GFP embryos are derived from the rhombic lip.

GFP-expressing cells were found to be distributed within the *atonal1a* expression domain in a mosaic manner and were mostly positioned at some distance to the posterior edge of the upper rhombic lip (Figs. 1A, C), where proliferation occurs. To address whether cerebellar GFP-expressing neuronal precursors still proliferate, we analyzed the co-expression of the M-phase marker phosphorylated histone 3 (PH3) and GFP by immunohistochemistry at 68 hpf, when migration of GFP-expressing neuronal precursors is predominant in the differentiating zebrafish cerebellum. Only a small number of GFP-expressing neuronal precursors co-localized with PH3-expression (Figs. 1I, J, yellow arrowhead). This is consistent with very few cell divisions of GFP-fluorescent neuronal precursors that were observed in our time-lapse studies, with dividing cells leaving the rhombic lip region soon afterwards. Instead, most of the GFP-expressing cells (Fig. 1K, yellow arrowhead) co-expressed the postmitotic neuronal marker HuC/D (Figs. 1L, M, note HuC is excluded from nucleus unlike GFP), which is expressed anterior to the rhombic lip region all the way to the MHB. This spatio-temporal relation of GFP-expression and mitotic markers suggests that GFP expression in the neuronal precursors is activated when cells are about to leave the proliferative rhombic lip to follow their migratory route.

gata1:GFP cells are glutamatergic and express granule cell marker genes

The mouse cerebellar rhombic lip sequentially generates different migratory neuronal cell types, all of which acquire a glutamatergic fate. Granule neurons are the last of the cell types to be produced (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005; Wilson and Wingate, 2006). Neuronal migration from the zebrafish rhombic lip is initiated at around 28 hpf (Köster and Fraser, 2001). In contrast, GFP-expression and onset of rhombic lip-derived migration in gata1:GFP embryos is not observed prior to 48 hpf (Köster and Fraser, 2006). This suggests that the gata1:GFP population arises late from the zebrafish rhombic lip. We therefore analyzed gata1:GFP cells for expression of genes expressed in cerebellar granule cell progenitors.

The transcription factor NeuroD and the adhesion molecule Tag1 are expressed in differentiating migratory cerebellar granule cell precursors in the mouse embryo (Miyata et al., 1999; Schüller et al., 2006; Hatten et al., 1997). When 3-day-old gata1:GFP embryos were analyzed for the expression of these genes, both of them were found to co-localize with GFP-expressing cells along their migratory pathway from the rhombic lip towards and along the MHB (Figs. 2A–A″, B–B″). Furthermore, at 4 dpf the GFP-expressing cells express the vesicular glutamate transporter 1, *vglut1*, indicative of a glutamatergic neurotransmitter identity (Figs. 2C–C″).

At 4 days post-fertilization, zebrafish larvae stand upright and begin to show directed swimming behavior coordinating balance, body posture and locomotion. Thus, beginning at

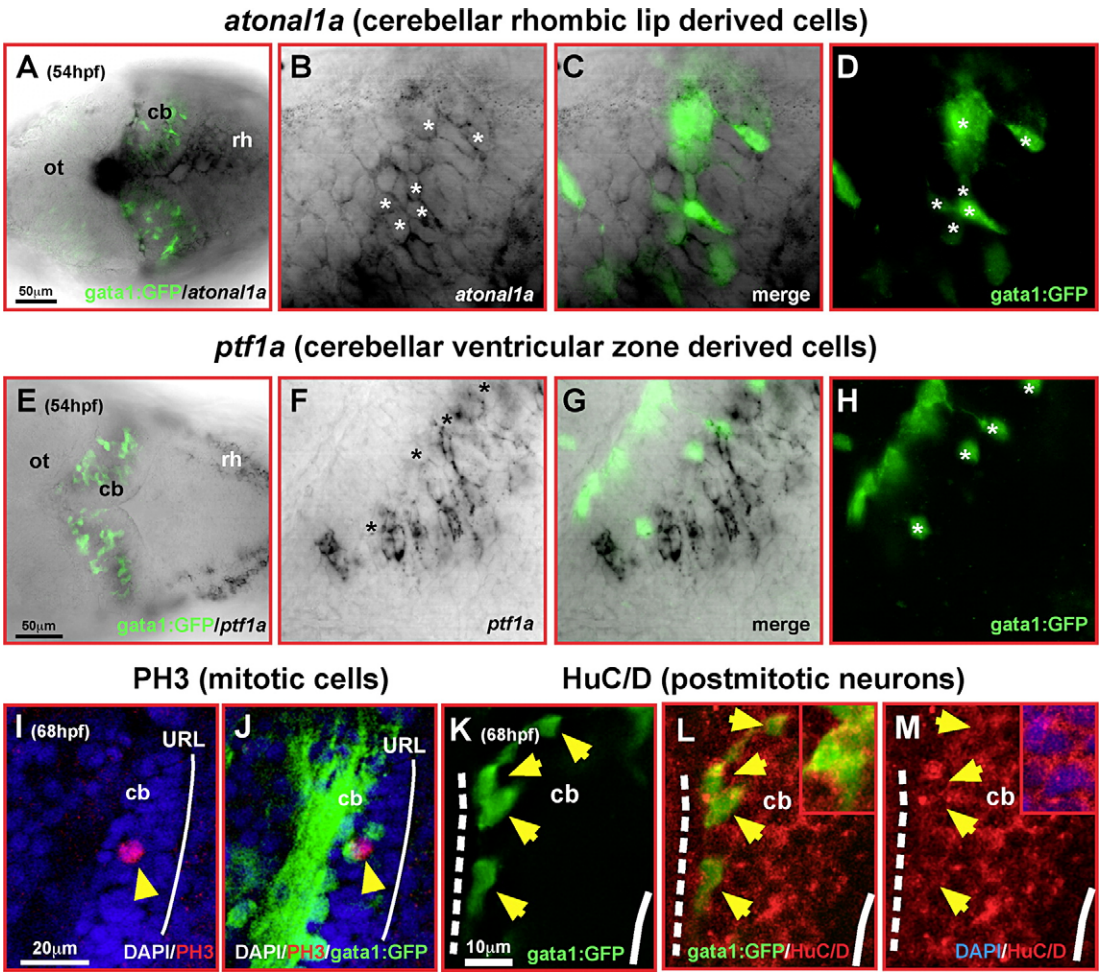

Fig. 1. Differentiating GFP-expressing cells in the cerebellum of transgenic gata1:GFP embryos are derived from the rhombic lip. Dorsal views of cerebella (A, E) or single optical section taken by confocal microscopy of one magnified cerebellar half (B–D, F–H) analyzed by immunohistochemistry against GFP-expression and mRNA in situ hybridization for zebrafish *atonal1a* (A–D) or *ptf1a*-expression (E–H), respectively. Whereas GFP-expressing cells (D, H, white asterisk) are co-expressing *atonal1a* (B), they are spared by *ptf1a*-expression and are positioned in gaps of the *ptf1a*-expression pattern (F). (I–M) Single optical section of the lateral view of the cerebellum recorded by confocal microscopy at 68 hpf. Although only few GFP-expressing cells (I, J, yellow arrowhead) co-express the mitotic M-phase marker PH3 close to the upper rhombic lip (white solid line), most GFP-expressing neuronal precursors (K–M, yellow arrowheads) co-express the neuronal postmitotic marker HuC/D (L, see also inset) close to the MHB (white dashed line; note that GFP is localized throughout the cell, while HuC is confined to the cytoplasm as shown by the DAPI nuclear counterstain in the inset of panel M). Abbr.: cb, cerebellum; MHB, midbrain–hindbrain boundary; ot, optic tectum; rh, rhombencephalon; URL, upper rhombic lip.

4 days post-fertilization zebrafish larvae likely possess functional cerebellar circuitries. During these late differentiation stages, granule cells express the transcription factor Pax6 and throughout their life the extracellular matrix molecule Reelin (Costagli et al., 2002; Schüller et al., 2006; Foucher et al., 2006). The expression of both zebrafish homologs *pax6a* (Figs. 2D–D″) and *reelin* (Figs. 2E–E″) largely co-localized with GFP-expressing cells in the cerebellum of 6 dpf gata1:GFP larvae. This developmental expression profile suggests an identity for cerebellar gata1:GFP-expressing cells as zebrafish granule cell progenitors.

GFP-expression in the gata1:GFP larval cerebellum is confined to cerebellar granule cells

Cerebellar rhombic lip-derived neuronal progenitors share many genes in their expression profile, such as *neuroD* or *reelin*, which are expressed by precursors of both granule neurons as well as deep nuclei neurons (D'Arcangelo et al., 1995; Schüller et al., 2006). In contrast, the evolutionary conserved $GABA_A$ receptor α6 subunit ($GABA_AR\alpha6$) is exclusively expressed in the cerebellum by granule cells in all vertebrates, allowing cerebellar granule cells to be identified unambiguously (Jones et al., 1996; Bahn et al., 1996; Fünfschilling and Reichardt, 2002; Aller et al., 2003).

Using primers targeted against conserved domains of goldfish $GABA_AR\alpha6$, we amplified a 423 bp cDNA fragment from zebrafish adult brain by degenerate RT–PCR. The deduced amino acid sequence of this zebrafish cDNA fragment (Fig. 3A) shows a 99.3% sequence identity to the goldfish $GABA_AR\alpha6$ homolog, also containing the characteristic α6-subunit specific Arginine-residue (Fig. 3A, red box) that determines the specific pharmacology of this $GABA_A$ receptor subunit (Lüddens et al., 1990; Bahn et al., 1996).

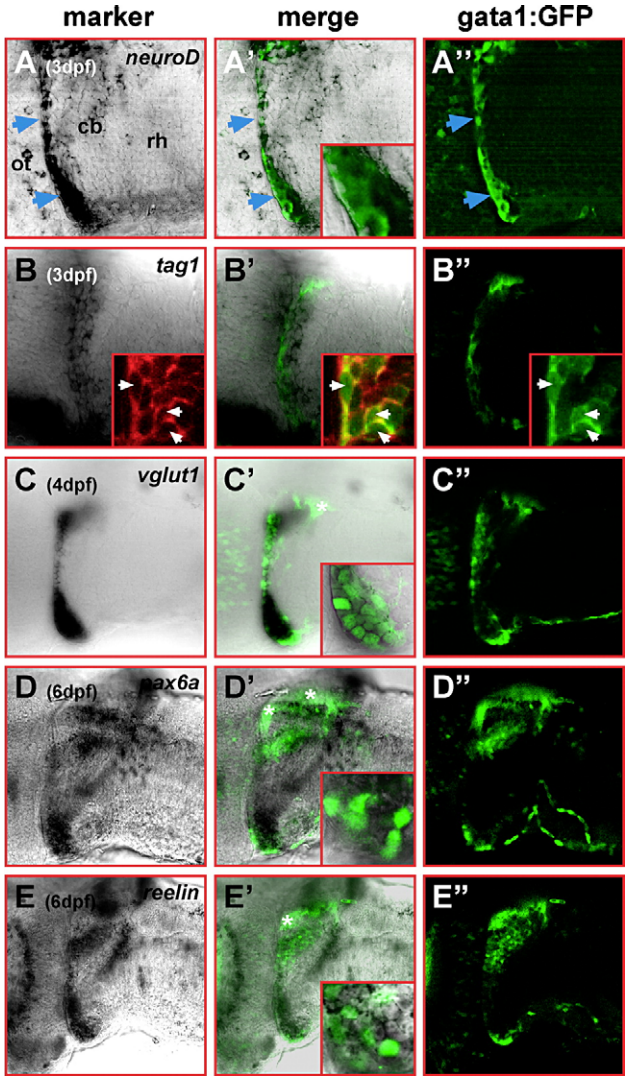

Fig. 2. Cerebellar gata1:GFP cells show a granule cell expression profile. Single optical sections of lateral views of cerebella at 3 dpf (A–A″, B–B″), 4 dpf (C–C″) and dorsolateral views at 6 dpf (D–D″, E–E″), respectively, recorded by laser scanning confocal microscopy. In the left column, the cerebellar expression pattern of (A) *neuroD*, (B) *tag1*, (C) *vglut1*, (D) *pax6a* and (E) *reelin* is displayed. In the right column, cerebellar GFP-expression of the same optical section is displayed, while the overlap of both expression patterns is shown in the column in the middle with insets demonstrating the co-expression of GFP and the respective marker gene. Insets in panels B–B″ display Tag-1 expression detected by immunohistochemistry (B) co-localizing to GFP-expressing gata1:GFP cells (B′, B″ white arrowheads). GFP-expression in the dorsal-most cerebellum (white asterisks) is confined to parallel fibers (see Fig. 6), and thus, shows no co-localization with the analyzed marker gene expression. Abbr.: cb, cerebellum; ot, optic tectum; rh, rhombencephalon.

Expression analysis of zebrafish *gaba$_A$Rα6* in 7-day-old gata1:GFP larvae showed a close overlap of both expression patterns in all cerebellar areas (Fig. 3B, a–i, note panels d–f and g–i represent magnifications of orange and blue boxed areas in picture a). Migration of granule cell progenitors in the zebrafish cerebellum along the MHB still occurs at day 7 and beyond (see Movie 2 and Movie 3 in supplementary data). This could explain why a few GFP-positive cells along the MHB may not have activated expression of *gaba$_A$Rα6* yet (Fig. 3B, g, white arrowheads). In sagittal brain sections of 1-, 2-, and 4-week-old zebrafish, expression of *gaba$_A$Rα6* was confined to the internal granule cell layer of the zebrafish corpus cerebelli (Suppl. Fig. 1A, D, G), the granule cell-containing caudal lobe (Suppl. Fig. 1E, H) and the eminentia granularis (not shown) co-localizing with gata1:GFP cells. In addition, parallel fibers from GFP-expressing gata1:GFP cells within the internal granule cell layer could be observed that project to the molecular layer (Suppl. Fig. 1C, F, I, yellow arrowhead). Expression of *gaba$_A$Rα6* in these different granule cell populations could still be found in the adult cerebellum (Suppl. Fig. 1J–M). Taken together, these data identify the GFP-expressing cells in the cerebellum of gata1:GFP embryos as cerebellar granule neurons.

The cerebellar rhombic lip is spatially patterned producing different granule cell clusters

Time-lapse studies of neuronal migration in zebrafish embryos of the stable transgenic gata1:GFP strain have suggested that the rhombic lip of the zebrafish cerebellum is patterned along its mediolateral axis (Köster and Fraser, 2006). As observations from these studies suggested that several neuronal clusters appeared to originate from different regions of the rhombic lip, we aimed to corroborate this finding by tracing individually migrating granule precursor cells in time-lapse recordings at higher magnification ($n=3$ movies).

Starting at about 48 hpf GFP-expressing granule precursor cells appear along the entire cerebellar rhombic lip (Fig. 4A) and set out to migrate soon afterwards anteriorly towards the MHB (Figs. 4A–C). About 10 hours later, the initial formation of GFP-expressing granule cell clusters can be observed at the MHB (Fig. 4C). These clusters are positioned dorsomedially (Figs. 4F, J, orange dashed circle) and ventrolaterally (Figs. 4F, J, blue dashed circle), remain stationary but grow further in size due to continued neuronal migration (see Movie 1 in supplementary material and additional movies in supplementary material of Köster and Fraser, 2006). Furthermore, continued time-lapse imaging up to 7 dpf demonstrated that GFP-expressing granule cells stayed within their clusters (see Movie 2 and Movie 3 in supplementary material covering a developmental period from 100–113 hpf and 144–162 hpf, respectively). Neither exchange of granule cells between these brightly labeled clusters nor emigration out of these clusters in different directions was apparent. This indicates that granule precursor cells cease migration upon reaching the forming clusters and become stationary.

When individual GFP-fluorescent cells were manually traced, it became evident that migrating granule precursor cells contributing to the dorsomedial cluster (marked orange) originate in the medial third of the cerebellar rhombic lip adjacent to the dorsal midline. In contrast, migrating cells contributing to the ventrolateral cluster (marked blue) originate in the lateral two thirds of the URL (Figs. 4A–C, F, J). Interestingly, we identified a third almost stationary GFP-expressing population that remained positioned along the URL (Figs. 4D–F, J, marked yellow). Due to the large number of GFP-expressing cells situated within the URL at earlier time-

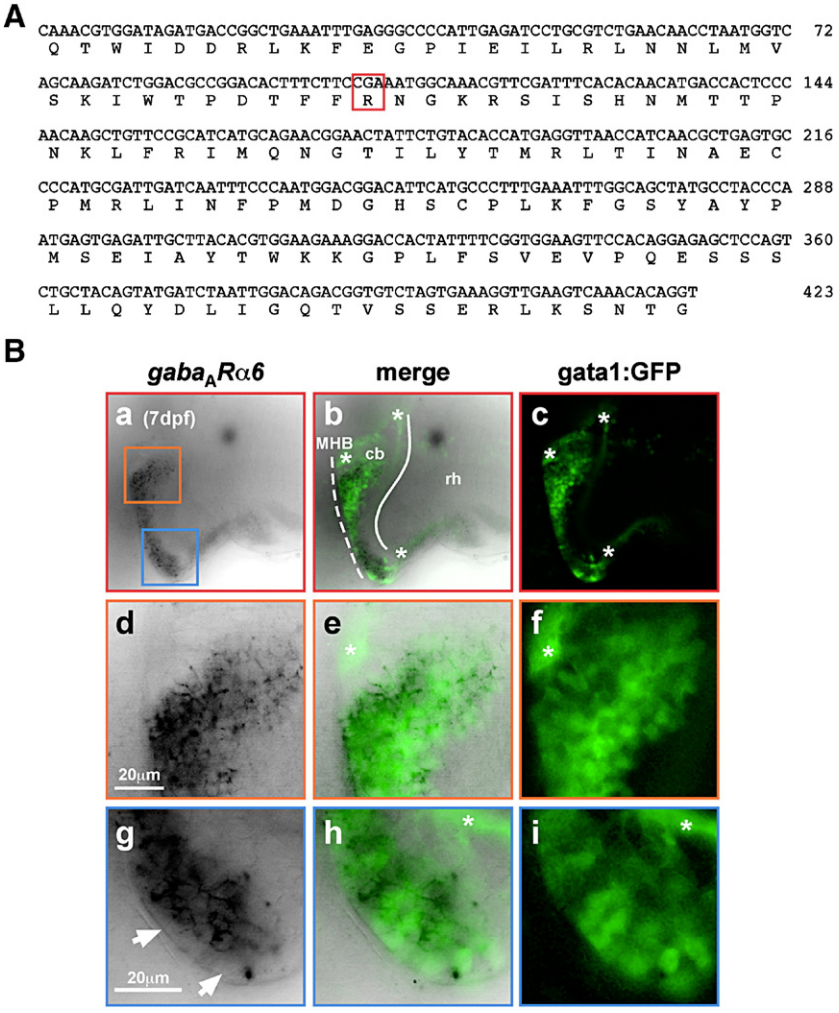

Fig. 3. gata1:GFP cells in the cerebellum are granule neurons. (A) Nucleotide and deduced amino acid sequence of isolated zebrafish *gaba$_A$Rα6*-cDNA fragment. The characteristic subunit-specific Arginine-residue conserved throughout vertebrates is demarcated by a red box. (B) mRNA in situ hybridization to analyze *gaba$_A$Rα6*-expression in zebrafish larvae at 7 dpf. In single optical sections recorded by confocal microscopy, 7 dpf larvae of the gata1:GFP strain display a close overlap of *gaba$_A$Rα6*- and GFP-expression in the cerebellum [a–c, dorsolateral view to simultaneously show GFP-expressing cells in the dorsal (orange box) and the ventrolateral (blue box) cerebellum, respectively]. Magnifications of the developing dorsal cerebellum (d–f) and the ventrolateral cerebellum (g–i) demonstrate an almost complete overlap of *gaba$_A$Rα6*- and GFP-expression. Few GFP-positive cells along the MHB (g, white arrowhead), which do not co-express *gaba$_A$Rα6*-mRNA, may represent not yet terminally differentiated granule cells arriving after migration along the MHB. GFP-expression in zebrafish granule cells is also confined to parallel fibers (marked by white asterisk in panels b, c, e, f, h, i; see also Fig. 6), and thus, shows no co-localization with *gaba$_A$Rα6*-mRNA expression. Abbr.: cb, cerebellum; MHB, midbrain–hindbrain boundary; rh, rhombencephalon.

points, these cells could only be discerned clearly at about 68 hpf (Fig. 4D), leaving their time-point of activation of GFP-expression unclear.

To further support our manual tracing results, we independently traced GFP-expressing cells via software-assisted visualization (ImageJ 1.34 S, Manual Tracking plug-in) of migratory routes. This software-assisted tracing confirmed our results demonstrating the different migratory routes and final positions of URL-derived cells (Figs. 4G, H, J, orange, blue) depending on their origin within the URL. In addition, the existence of a third stationary population remaining at the dorsoposterior edge of the differentiating cerebellum was confirmed (Figs. 4I, J, yellow). From dorsal projections of the time-lapse data, it may appear as if granule precursor cells from the medial and lateral subdomains of the rhombic lip simply migrate parallel to one another. Lateral projection of the end-point of the time-lapse data though reveals that granule precursor cells from the medial subdomain of the rhombic lip remain in dorsal positions in the cerebellum close to the MHB (Fig. 4J, orange dashed circle). In contrast, granule precursor cells from the lateral subdomain of the rhombic lip not only migrate in an anterior direction but also follow a ventrally oriented migratory route (Fig. 4J, blue dashed circle). Depth-coding of the time-lapse data demonstrates that the dorsomedial and the ventrolateral granule cell clusters are located about 30 to 50 μm apart (Fig. 4K) along the dorsoventral axis of the cerebellum at 3 dpf. This distance increases until 7 dpf to 50–100 μm (Fig. 4L) due to continued growth of the cerebellum. From these cell-tracing experiments it is evident that granule cells follow divergent migratory routes (dorsal versus lateral-ventral) and settle in different parts of the cerebellum depending on their place of origin.

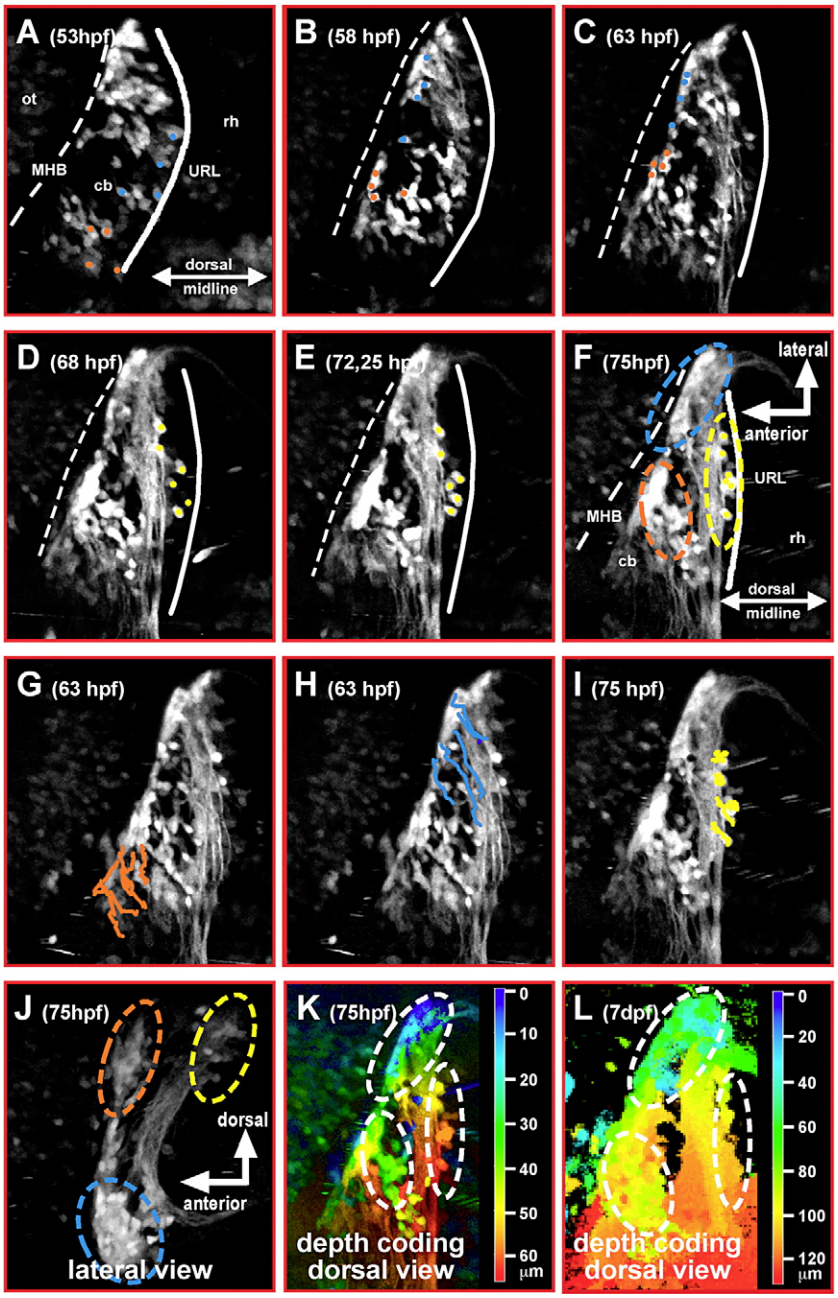

Fig. 4. Tracing of migrating GFP-expressing granule precursor cells in gata1:GFP transgenic embryos reveals a spatial pattern of the cerebellar rhombic lip. (A–F) Dorsal view of one cerebellar half; maximum brightness projections from individual time-points of a time-lapse microscopy study of a transgenic gata1:GFP embryo are shown. Manually traced granule precursor cells contributing to the dorsomedial cluster have been marked by an orange dot, cells contributing to the ventrolateral cluster are marked by a blue dot (A–C). A smaller stationary population of GFP-expressing granule precursor cells (yellow dots) remains at the dorsoposterior edge of the differentiating cerebellum (D–F). (G–I) Independently, migratory routes were visualized by ImageJ supported cell tracing. Routes have been overlaid onto pictures of individual time-points (G, H, 63 hpf; I, 75 hpf) at which tracing has been finished. (J) Lateral view of the cerebellum, maximum intensity projection (stack of 21 images each 3 µm apart) showing that the medial and lateral granule cell clusters marked in dorsal view projections (F) are positioned apart from one another along the dorsoventral axis of the cerebellum. While granule precursor cells migrating towards the medial cluster (orange) remain in dorsal positions, migration of granule precursors cells heading towards the lateral cluster involves a strong ventral component. Depth coding of granule cell positions indicates that the medial and lateral granule cell clusters are about 30 to 50 µm apart along the dorsoventral axis of the cerebellum at 3 dpf (K), increasing to values of 50 to 100 µm at 7 dpf (L) due to continued growth of the cerebellum. Abbr.: cb, cerebellum; MHB, midbrain–hindbrain boundary; ot, optic tectum; rh, rhombencephalon; URL, upper rhombic lip.

gata1:GFP cells in the cerebellum represent all the cerebellar granule cell populations in teleosts

The co-expression of *gaba$_A$Rα6* in GFP-expressing cells in the cerebellum of gata1:GFP larvae suggested that the spatial patterning of the zebrafish cerebellar rhombic lip during early embryonic stages corresponds to the different granule cell populations present in the mature teleost cerebellum. We therefore addressed the final localization of the forming granule cell clusters in larval and juvenile gata1:GFP zebrafish brains.

First, we aimed to reveal when layer formation in the cerebellum occurs. We reasoned that layer formation would represent a developmental period when granule progenitor cells settle in their final location and can be attributed to distinct granule cell populations. Towards this end, we performed in vivo Bodipy Ceramide stainings to label the neuropil of the developing molecular layer. Such membrane staining demonstrated that at 60 hpf the zebrafish cerebellum is not yet subdivided in different recognizable cellular domains (Fig. 5A). At 3 dpf the first patches of neuropil on the dorsal side of both cerebellar halves appear (Fig. 5B, blue arrowheads), indicating that cerebellar layer formation has been initiated. This is further supported by our observations that around 3 dpf migration of neuronal precursors diminishes and that zebrafish Purkinje cells initiate the expression of the terminal differentiation marker ZebrinII (not shown). Finally, at 6 dpf intense Bodipy Ceramide staining demarcates a prominent and continuous molecular layer covering the entire zebrafish cerebellum (Fig. 5C, blue asterisk). Thus, cerebellar lamination must be well under way after 1 week of zebrafish embryonic development.

To address the final localization of the GFP-expressing granule cells, gata1:GFP larvae and juvenile fish were sectioned, processed for immunohistochemistry against GFP

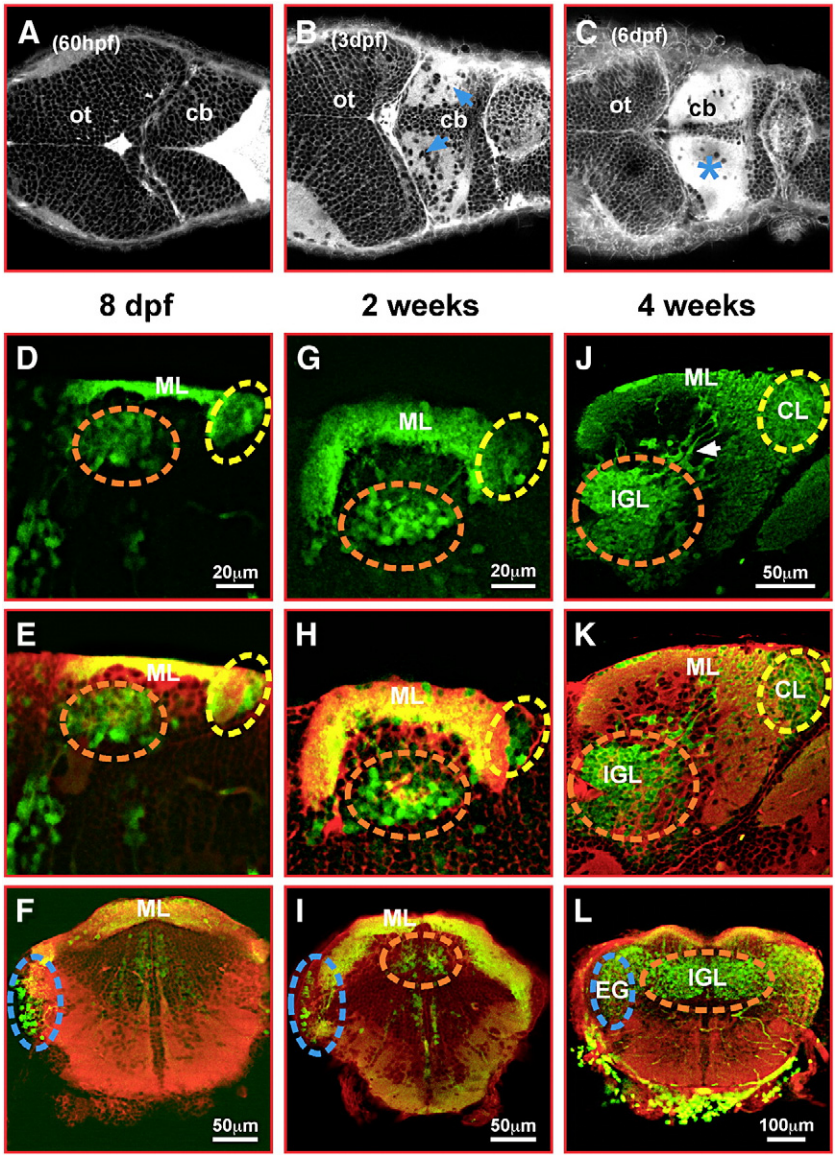

Fig. 5. gata1:GFP granule cells contribute to the corpus cerebelli, the eminentia granularis and the cerebellar caudal lobe. (A–C) Dorsal views of the embryonic and larval zebrafish cerebellum stained with Bodipy FL C_5-ceramide. Staining of cellular membranes, intense in neuropil (blue arrows and asterisk), was recorded by optical sectioning using laser scanning microscopy. (D, E, G, H, J, K) sagittal sections and (F, I, L) transverse sections through the cerebellum of 8 dpf (D–F), 2 weeks (G–I) and 4 weeks (J–L) old gata1:GFP larval and juvenile fish. GFP-expression (D, G, J) was visualized by immunohistochemistry followed by red fluorescent counterstaining with Bodipy 630/650-X (E, F, H, I, K, L) and image recording using laser scanning confocal microscopy. A dashed circle marks the forming IGL of the corpus cerebelli (orange), the eminentia granularis (blue) and the caudal lobe (yellow), respectively. Note GFP-expressing granule cells still appearing to migrate into the IGL of the corpus cerebelli at 4 weeks (J, white arrow). Abbr.: cb, cerebellum; CL, caudal lobe; EG, eminentia granularis; IGL, internal granule cell layer; ML, molecular layer; ot, optic tectum.

and subsequently counterstained with Bodipy 630/650-X to outline the cellular organization of the cerebellum. At 8 dpf sagittal sectioning of the cerebellum through the position of the embryonic dorsomedial granule cell cluster (marked orange in Fig. 4) indicated a GFP-expressing granule cell population at the medial base of the cerebellum (Figs. 5D, E, orange dashed circle). Cerebellar sections after 2 and 4 weeks of zebrafish development identified this population as the internal granule cell layer of the forming corpus cerebelli (Figs. 5G, H, J, K, orange dashed circle). Thus, the embryonic dorsomedial gata1:GFP cluster, which arises from the medial rhombic lip, likely represents the later granule cell population of the corpus cerebelli.

Transverse sections of the cerebellum at 8 dpf targeting the ventrolateral gata1:GFP cell cluster (marked blue in Fig. 4) revealed in addition to the medial corpus a granule cell population at the lateral ventral edge of the larval cerebellum (Fig. 5F, blue dashed circle). Sectioning after 2 and 4 weeks of development identified this population as the granule cells of the eminentia granularis in the mature zebrafish cerebellum (Figs. 5I, L, blue dashed circle). Thus, granule cell precursors arising in the lateral rhombic lip (Fig. 4) are likely to represent the later granule cell population of the eminentia granularis.

Finally, sagittal sections at 8 dpf through the position of the embryonic dorsoposterior gata1:GFP cell cluster (marked yellow in Fig. 4) revealed a third granule cell population at the dorsoposterior edge of the larval cerebellum (Fig. 5D, E, yellow dashed circle). Sectioning after 2 and 4 weeks of larval development identified this structure as the granule cell population of the caudal lobe of the mature cerebellum (Figs. 5G, H, J, K, yellow dashed circle). The granule precursor cells found to remain almost stationary within the dorsoposterior rhombic lip in our time-lapse recordings (Fig. 4, yellow marked cells, see Movie 1) are thus likely to correspond to the later granule cell population of the zebrafish cerebellar caudal lobe.

gata1:GFP granule cells project commissural axons into the crista cerebellaris

Teleost cerebellar granule cells from the eminentia granularis and the caudal lobe but not the corpus cerebelli project axons, called parallel fibers, posteriorly into the crista cerebellaris (Caird, 1978; Montgomery, 1981; Puzdrowski, 1989). Consistent with being granule neurons, GFP-expressing cells in the cerebellum of transgenic gata1:GFP embryos display prominent axonal projections posteriorly into dorsoanterior regions of the hindbrain at 8 dpf (Fig. 6A, blue arrowheads). Furthermore, these projections were found to be positioned within the crista cerebellaris when cerebella of 4 weeks old juvenile gata1:GFP fish were sagittally sectioned and stained for GFP-expression by immunohistochemistry (Figs. 6B–B″, blue arrowhead).

To further characterize these GFP-labeled granule neuron axons, their projections were followed by intravital time-lapse analysis ($n=3$ movies) using confocal scanning microscopy (see Movie 1 in supplementary material). The first parallel fibers were recognizable at about 58 hpf, 10 hours after the onset of granule cell migration, predominantly projecting from the ventrolateral granule cell cluster (Fig. 6D, blue dashed circle) along the posterior region of the developing cerebellum (Fig. 6D, blue arrows). These parallel fibers extended towards the cerebellar dorsal midline where they crossed to continue their projection into the contralateral half of the cerebellum (Figs. 6E–F, blue arrows). Occasionally, contacts between axon terminals from both cerebellar halves meeting close to the dorsal midline were observed (Fig. 6E, F, yellow circle), followed by a quick avoidance and continuation of commissural axon extension (Figs. 6D–H, blue and red arrows). After formation of the dorsal commissure, parallel fibers turned at the lateral edge of the cerebellum at about 65 hpf finally extending into the dorsoposterior crista cerebellaris (Figs. 6I–K, green arrow).

Retrograde labeling in the crista cerebellaris discriminates the granule cells of the vestibulocerebellar system in zebrafish

In our initial time-lapse and cell-tracing analysis, diverse populations of granule precursor cells emanating from different regions along the cerebellar rhombic lip and settling in different clusters had been distinguished (Fig. 4 and Fig. 5). Only parallel fibers of the vestibulocerebellar system (composed of eminentia granularis and caudal lobe), but not of the corpus cerebelli, project into the crista cerebellaris (Figs. 7D, H) (Puzdrowski, 1989; Wullimann, 1998). This offers a means of discriminating between granule cells of the vestibulocerebellar and non-vestibulocerebellar system by retrograde labeling.

Rhodamine-dextran was focally injected into one half of the forming crista cerebellaris of 6 dpf gata1:GFP transgenic larvae (Fig. 7, white asterisk) (Volkmann and Köster, 2007). This dye is actively transported in a retrograde manner only within injured axons. One day after rhodamine-dextran application, maximum intensity projections of recorded image stacks revealed many labeled cells in areas of the cerebellum where the gata1:GFP expressing granule cells of the ventrolateral (blue dashed circle) and the dorsoposterior cluster (yellow dashed circle) are positioned (Figs. 7A–C). Further analysis of single optical sections indeed revealed individual GFP-expressing granule cells of these two populations marked by rhodamine-dextran (Figs. 7E–G). Independent retrograde labeling experiments using DiI-crystals confirmed that these two granule cell populations project axons into the crista cerebellaris (not shown). Granule cells of the forming eminentia were labeled in both cerebellar halves, indicating that the granule cell projections from the eminentia granularis into the crista cerebellaris are of dual composition containing ipsilateral and commissural contralateral axons. This is consistent with parallel fibers of the bifurcating granule cell axons projecting into both halves of the vertebrate cerebellum (Altman and Bayer, 1997). In contrast, projections of the caudal lobe granule cell population into the crista cerebellaris occur predominantly ipsilaterally (Fig. 7E) and only a few contralateral projections were observed (Fig. 7F). Currently, we have no explanation for this discrepancy in the projection of granule cells of the

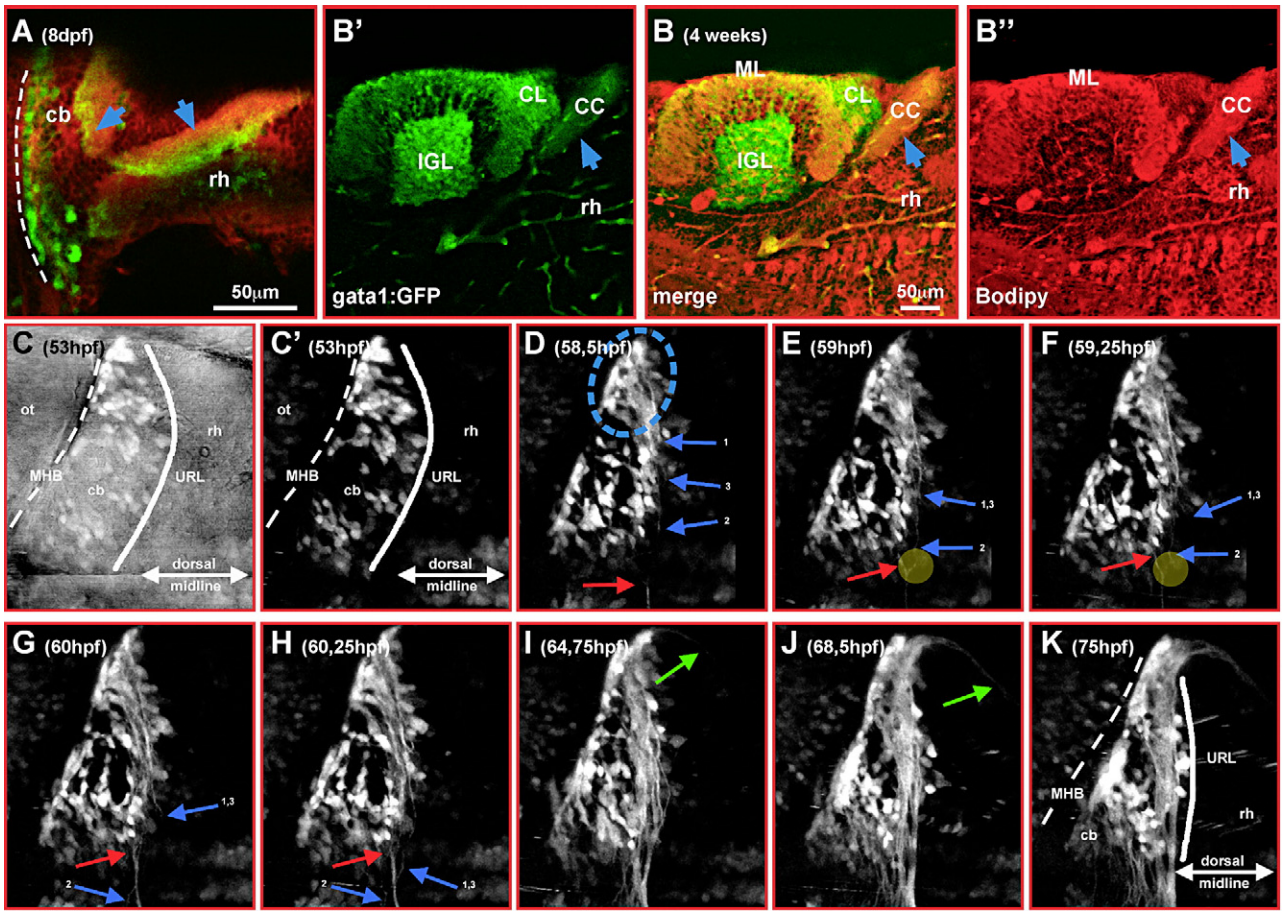

Fig. 6. gata1:GFP granule cells project commissural axons into the crista cerebellaris. (A) Optical section of the cerebellum of 8 dpf gata1:GFP transgenic larvae. Note the dorsoposterior projection out of the cerebellum (blue arrow). Sagittal sectioning of 4-week-old gata1:GFP cerebella, immunohistochemistry against GFP-expression (B′) and counterstaining with Bodipy 630/650-X (B″) reveal positioning of these projections within the crista cerebellaris (B–B″, blue arrow). (C–K) 3-D time-lapse analysis (dorsal view) to characterize axonal projections into the crista cerebellaris (60 µm stacks of 21 individual images spaced 3 µm were recorded every 12 min). Brightest point projections of images of individual time-points are displayed. Individual axons are marked with arrows; note the contact and avoidance of growing axons from opposite cerebellar halves close to the dorsal midline (E, F, yellow circle; see slow motion sequence at the end of Movie 1). Around 65 hpf first projections into the forming crista cerebellaris can be observed (see green arrows in Movie 1 in supplementary material). Abbr.: cb, cerebellum; CC, crista cerebellaris; CL, caudal lobe; IGL, internal granule cell layer; MHB, midbrain–hindbrain boundary; ML, molecular layer; ot, optic tectum; rh, rhombencephalon; URL, upper rhombic lip.

zebrafish vestibular cerebellum. Further electrophysiological and behavioral experiments are required to unravel the functional consequence of this projection pattern of the zebrafish vestibular system.

Nevertheless, these retrograde labeling results support our findings from the GFP-expression analysis in the larval and juvenile cerebellum of gata1:GFP zebrafish. Taken together, our studies identify the ventrolateral gata1:GFP cell cluster in the embryonic cerebellum as the granule cell population of the future eminentia granularis, while the embryonic dorsoposterior cell cluster represents the granule cells of the future caudal lobe (Figs. 7D, H). Both granule cell populations together form the vestibulocerebellar system of the zebrafish cerebellum. In contrast, retrograde labeling either with rhodamine-dextran (Figs. 7A–C, orange dashed circle) or by using DiI revealed that granule cells of the dorsomedial cluster never project axons into the crista cerebellaris. This identifies the embryonic dorsomedial gata1:GFP cell cluster as the future granule cell population of the corpus cerebelli (Figs. 7D, H), representing the non-vestibulocerebellar system in zebrafish. Based on our tracing of individual granule cell progenitors, the embryonic zebrafish rhombic lip is spatially patterned, with the lateral rhombic lip generating granule cells of the vestibulocerebellum, while granule cells of the zebrafish non-vestibulocerebellar system originate from the medial rhombic lip.

We have shown that migration from the zebrafish URL initiates at about 28 hpf (Köster and Fraser, 2001), whereas zebrafish granule cell progenitors begin to emigrate from the URL at about 48 hpf. This suggests that in common with mammals, the zebrafish URL generates different cerebellar neuronal cell types over time (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005; Wilson and Wingate, 2006). In addition, our data revealed that the zebrafish cerebellar rhombic lip is spatially patterned along its mediolateral axis such that different subpopulations of cerebellar granule cells are generated with different positions, connectivities and functions. Thus, rather than being a simple proliferative zone, the zebrafish

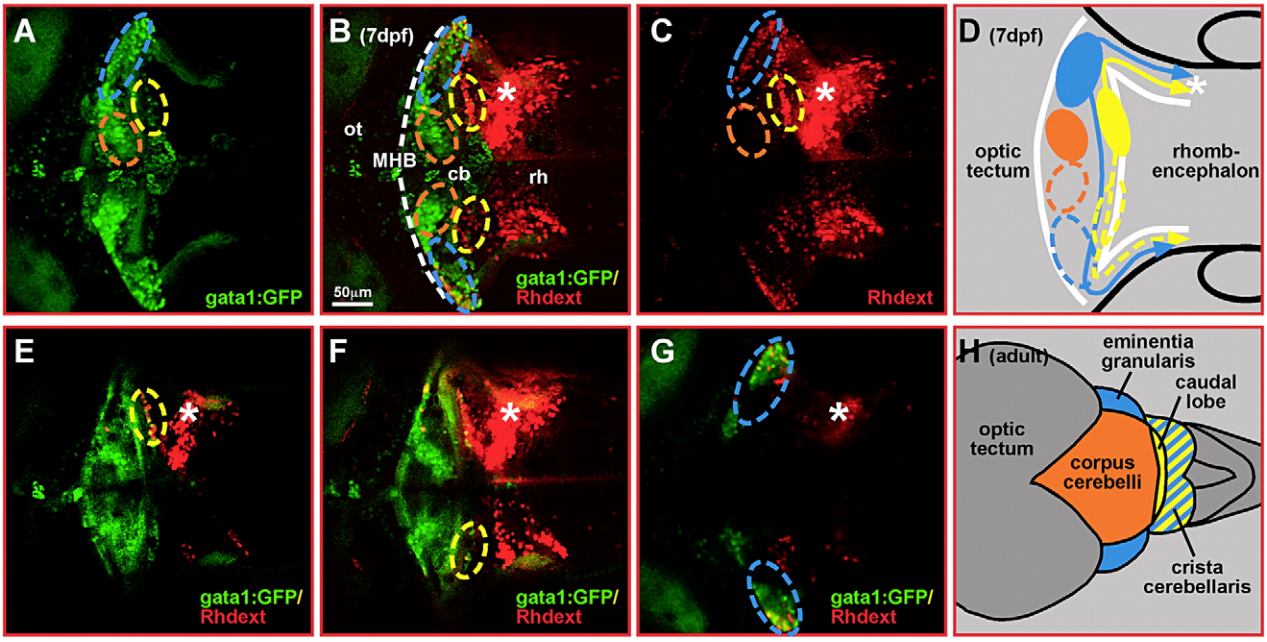

Fig. 7. Rhodamine-dextran retrograde labeling of crista cerebellaris projections discriminates between granule cells of the vestibulo- and non-vestibulocerebellar system. Rhodamine-dextran was microinjected into one half of the GFP-fluorescent crista cerebellaris (white asterisk) in transgenic gata1:GFP embryos at 6 dpf. (A–C) Maximum intensity projections of 3-D image stack (148 µm, 75 images at 2 µm distance) recorded by laser scanning confocal microscopy at 7 dpf reveal many rhodamine-dextran labeled cells in areas of the ventrolateral (blue dashed circle) and dorsoposterior (yellow dashed circle) gata1:GFP granule cell clusters. (E–G) Single optical sections indeed revealed co-labeling of GFP-cells with rhodamine-dextran in these granule cell populations. In contrast, rhodamine-dextran labeled cells were never found in the area or to co-localize with cells of the dorsomedial granule cell cluster (orange dashed circle). (D, H) Schematic drawing of granule cell projections deduced from rhodamine-dextran retrograde labeling; note that granule cells of the dorsomedial cluster (marked orange) do not project into the crista. These studies identify the embryonic ventrolateral (D, blue dashed circle) and dorsoposterior (D, yellow dashed circle) gata1:GFP granule cell clusters as granule cells of the adult vestibulocerebellar system in zebrafish (H, schematic drawing of dorsal view) formed by the eminentia granularis (blue) and the caudal lobe (yellow), respectively. Abbr.: cb, cerebellum; MHB, midbrain–hindbrain boundary; ot, optic tectum; rh, rhombencephalon.

cerebellar rhombic lip is a complex germinal structure with distinct characteristics in time and space.

Discussion

The identification of individual neuronal cell types and the analysis of their specific developmental differentiation program is a key step towards understanding the morphogenesis of the vertebrate nervous system. In particular, comparison of neuronal differentiation in different vertebrate species allows one to distinguish evolutionary conserved and divergent processes of brain development and to exploit the specific advantages of each individual model organism for an overall comprehensive insight into the mechanisms of neuronal circuitry formation. Zebrafish have become one of the favorite model organisms to tackle the characterization and conservation of basic genetic and cell biological mechanisms underlying central nervous system development in vertebrates. Despite numerous studies addressing cellular and molecular mechanisms of fore-, mid- and hindbrain development, the cerebellum has been largely neglected. For example, zebrafish cerebellar neuronal cell types are poorly characterized if at all; molecular markers are barely established and the time-course of cerebellar differentiation is almost unknown. This is particularly surprising as the cerebellum represents one of the most conserved brain compartments in vertebrates promising that cerebellar research in zebrafish will be directly meaningful for higher vertebrates and mammals including humans.

We have narrowed this knowledge gap by characterizing the differentiation program of zebrafish cerebellar granule cells, the most numerous neuronal cell population of the cerebellum. Combining expression analysis with in vivo time-lapse imaging, we have shown that zebrafish granule cell precursors start to differentiate at about 48 hpf. At this developmental stage, granule cell precursors leave the germinal zone, the cerebellar rhombic lip; become postmitotic; and initiate migration over long distances towards and along the MHB (Köster and Fraser, 2006). Consistent with the onset of granule cell cluster formation at about 60 hpf and their growth due to the continued arrival of additional granule cell precursors, subsequent initiation of layer formation in the zebrafish cerebellum begins at 72 hpf. While the peak of migration occurs from 48 hpf until about 84 hpf, granule cells continue to be added over the following days (see Movie 2 and Movie 3 in supplementary data); and it appears that even in juvenile zebrafish, granule cell migration still contributes at a low rate into the internal granule cell layer (IGL) of the corpus cerebelli (Fig. 5J, white arrow; note few individual GFP-expressing cells appearing to move from the dorsoposterior region of the cerebellum into the IGL of the corpus cerebelli). Prominent expression of the terminal differentiation marker *gaba$_A$Rα6* in cerebellar granule cells can already be found at 4 dpf in the

zebrafish cerebellum. Thus, cerebellar differentiation in zebrafish occurs within 2 days. This is much faster when compared to mice, in which granule precursor cells become induced at about embryonic day 13 with migration from the external to the internal granule cell layer peaking well after birth and first *gaba$_A$Rα6* expression being observed about 2 weeks after birth (Hatten et al., 1997; Machold and Fishell, 2005; Wang et al., 2005). Thus, cerebellar development in zebrafish is temporally far more condensed than in other vertebrate model organisms, making zebrafish especially suited for intravital time-lapse studies, in which relative short periods of image recording cover almost the entire cerebellar differentiation program. Our characterization of cerebellar morphogenesis and granule cell development in zebrafish, the establishment of molecular markers and the identification of a transgenic line displaying GFP-expression throughout the granule cell population will largely facilitate such in vivo imaging studies.

We have used an intravital time-lapse approach to follow granule cell migration emanating from the cerebellar rhombic lip. This specific proliferation zone in the dorsoposterior cerebellum was initially thought to exclusively give rise to cerebellar granule cells (Alder et al., 1996), making this germinal zone unique in providing a source for isolating and culturing a single neuronal cell type. Recently though, it has become evident by genetic fate mapping and time-lapse analysis that the cerebellar rhombic lip produces different neuronal populations in a temporal sequence, with granule cells being generated last (Lin et al., 2001; Köster and Fraser, 2001; Machold and Fishell, 2005; Wang et al., 2005; Wilson and Wingate, 2006). Additionally, spatial patterning within the mouse cerebellar rhombic lip was demonstrated by genetic fate mapping approaches (Mathis and Nicolas, 2003; Sgaier et al., 2005, 2007). Our in vivo time-lapse studies and individual cell-tracing analysis suggest that such a spatial patterning also occurs in the zebrafish cerebellar rhombic lip. Beyond this, we found that the mediolateral pattern of the embryonic cerebellar rhombic lip corresponds to different functional domains in the mature cerebellum. While granule progenitor cells of the zebrafish corpus cerebelli arise close to the dorsal midline, granule cell precursors populating the future eminentia granularis originate in lateral positions of the rhombic lip. Thus, the mediolateral positioning of granule cells in the mature cerebellum is already reflected in the embryonic germinal zone prior to neuronal migration. Continued time-lapse imaging showed that this spatial organization of the cerebellar rhombic lip is maintained over several days well beyond the peak of granule cell migration (see Movie 2 and Movie 3 in supplementary data).

Our observations cannot resolve whether fate specification of granule precursor cells towards a corpus cerebelli or an eminentia granularis identity occurs within the rhombic lip. Alternatively, granule precursor cells may learn during migration after having left the rhombic lip which granule cell cluster to join. Based on our data we favor that granule cell specification occurs already inside the rhombic lip. Migratory routes of granule cells emanating from the rhombic lip and heading towards the different clusters are very directional, do not cross or intermingle even close to the rhombic lip, which we would expect if granule precursor cells learn about their destination only after having left the rhombic lip. This directional migratory behavior can again be found at 4 dpf and 6 dpf, respectively. Here, granule precursor cells in more lateral positions do not leave the rhombic lip in an anterior direction but initially migrate laterally within the rhombic lip until they migrate ventrally to join the ventrolateral granule cell cluster (see cells marked with blue dot in Movie 2 and Movie 3 in supplementary data). Granule cells migrating within the rhombic lip towards the dorsal midline were never observed. Heterotopic transplantations of granule cell precursors to different positions inside the rhombic lip could help to answer this open question of when granule precursor cell specification and determination to a vestibular or non-vestibular fate occurs.

Granule cells of the eminentia granularis and the corpus cerebelli clearly represent two different types of granule cells with different functions in teleosts. Granule cells of the eminentia and the caudal lobe (also termed auricles) receive neuronal input mainly from the vestibular system and the lateral line system (Puzdrowski, 1989; Wullimann, 1998). These cerebellar domains in zebrafish resemble most closely the vestibulocerebellum in mammals mainly serving balance control of the organism. In contrast, granule cells of the zebrafish corpus cerebelli receive input from spinocerebellar and tectocerebellar fibers transmitting proprioceptive and other sensory inputs (Nieuwenhuys, 1967; Wullimann and Northcutt, 1988; Wullimann, 1998). The zebrafish corpus cerebelli is mostly homologous to the non-vestibular cerebellar system, the diencephalo-, mesencephalo- and spinocerebellum in mammals serving coordination of locomotion and higher motor functions. Our in vivo cell-tracing studies combined with retrograde labeling reveal that in zebrafish, granule cells of the vestibular and the non-vestibular cerebellar system arise in spatially distinct regions along the mediolateral axis of the cerebellar rhombic lip. Thus, over the last years, it has become evident that the cerebellar rhombic lip is much more than just a granule cell producer being very dynamic in generating divergent cell types through temporally and spatially changing differentiation programs. Knowing the precise origin of different granule precursor cells within the cerebellar rhombic lip offers a powerful means to more specifically target distinct granule cell populations of the mature cerebellum. This will allow genetic manipulations, for instance, via electroporation or viral infection, to specifically target granule cells of the vestibular or non-vestibular cerebellar system in zebrafish, thereby improving the specificity of cerebellar research.

Given the high evolutionary conservation of the cerebellum, our findings of spatial patterning within the cerebellar rhombic lip representing functional domains of the mature cerebellum could well hold true for higher vertebrates. Here, spatial subdivisions of the rhombic lip could give rise to granule cells of the later vermis, paravermis and hemispheres, respectively. These cerebellar subdivisions also receive different neuronal input and serve different functions in controlling body posture and locomotion (vermis), coordination of extremity movements

(paravermis) and higher motor skills needed for example for speech control (hemispheres).

Prior to granule cell progenitors, the rhombic lip in mice has been shown to produce neurons of the later deep nuclei, the only output structures of the cerebellum (Machold and Fishell, 2005; Wang et al., 2005; Wilson and Wingate, 2006). Interestingly, the different cerebellar subdivisions in mice release their neuronal output via different deep nuclei, which are positioned at the base of the mammalian cerebellum in a mediolateral organization. For example, the medially positioned cerebellar vermis connects to the fastigial nucleus at the medial base of the cerebellum, the paravermis to the nucleus interpositus, whereas the lateral hemispheres project to the dentate nucleus, the most laterally positioned deep nucleus at the cerebellar base. Thus, the spatial pattern of the cerebellar rhombic lip could not only hold true for granule cells but also for the earlier generated deep nuclei neurons. This offers the exciting possibility that the spatial pattern of the embryonic rhombic lip already manifests the different positional and functional subdivisions of the mature cerebellum. Thus, individual rhombic lip precursors could be dedicated due to their spatial position to produce deep nuclei neurons and their corresponding cerebellar interneurons (granule cells) of the same cerebellar circuitry over time. This offers a means of how cerebellar subdivisions and their highly regular circuitries are being laid down by temporal and spatial patterning during early embryonic development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.024.

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