Cellular and Genetic Programs Underlying Cerebellum Development

Alexandra L. Joyner, Ryan Willett, and Andrew Lawton

Abstract The cerebellum is a late developing structure compared to the rest of the central nervous system (CNS) and houses more cells than the entire rest of the brain in a complex set of folds. To accommodate production of the large number of cells, the cerebellum has not only a ventricular progenitor zone that produces all the glia and inhibitory neurons but also a unique progenitor zone, the rhombic lip, dedicated to excitatory neuron production. In this chapter we discuss how the inhibitory Purkinje cells, which integrate the incoming information and moderate the output neurons of the cerebellar nuclei, play a key role during development in ensuring appropriate production of the other neurons/astrocytes of the cerebellar cortex. Key transcription factors that regulate development of the two progenitor populations and the lineage relationships of the neurons and astrocytes produced by each are described, followed by a discussion of cerebellar foliation.

Keywords Ventricular zone • Rhombic lip • Purkinje cells • Granule cells • Interneurons • Bergmann glia • Astrocytes • Cerebellar nuclei • Neural stem cells • Foliation

Introduction

The cerebellum is the region of the brain that is the latest to complete neurogenesis; in humans cerebellar development continues during the first year of life and in mouse for more than 2 weeks after birth [1–3]. It arises from the dorsal aspect of the most anterior hindbrain called rhombomere1 (Fig. 1a, b). Remarkably, the volume of the human cerebellum increases ~10× between 20 and 40 weeks of gestation, with the surface area increasing much more due to the formation of folia and lobules [6–8]. The mouse cerebellum undergoes maximum growth and foliation after birth (Fig. 1a–d). Given the late development of the CB compared to other brain regions,

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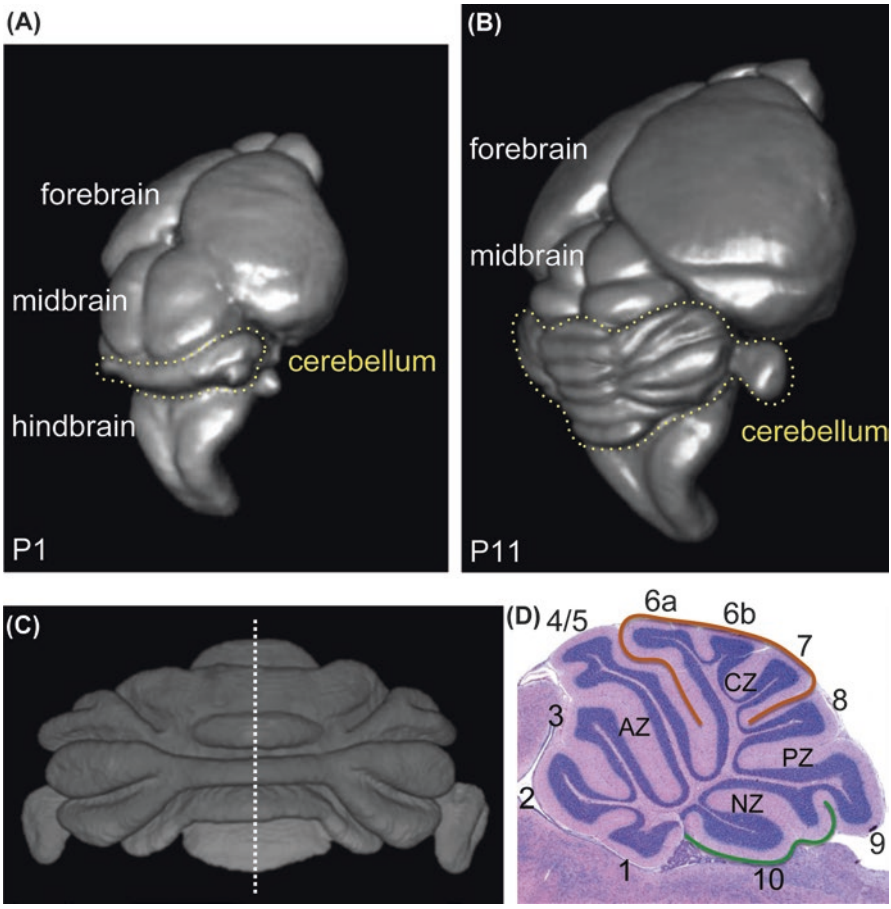

Fig. 1 The cerebellum forms in the dorsal anterior hindbrain and has its major growth and foliation after birth. (**a–c)** microMRI images illustrating mouse postnatal cerebellum development (outlined in *yellow*) (based on [4]), and (**c)** highlighting distinct foliation patterns in the medial vermis and lateral hemispheres [5]. (**d)** Hematoxylin and eosin (H&E) midline section (*dotted line* in **c**) of adult cerebellum. 1–10, lobules, *AZ* anterior zone, *CZ* central zone (outlined in *red*), *PZ* posterior zone, *NZ* nodular zone (*green*)

the cerebellum is particularly sensitive to environmental and clinical factors that impact on growth (or cause injury) around birth [9]. A better understanding of the factors that regulate progenitor cell expansion, production of neurons and glia, and their compartmentalization during foliation should pave the way for developing therapeutic approaches to stimulate endogenous progenitors to replenish cells lost due to injury.

The developing cerebellum is unique among the brain regions as it has two zones that house neural stem and progenitor cells (Fig. 2a). Whereas in the rest of the central nervous system the ventricular zone (VZ) gives rise to all the neurons and

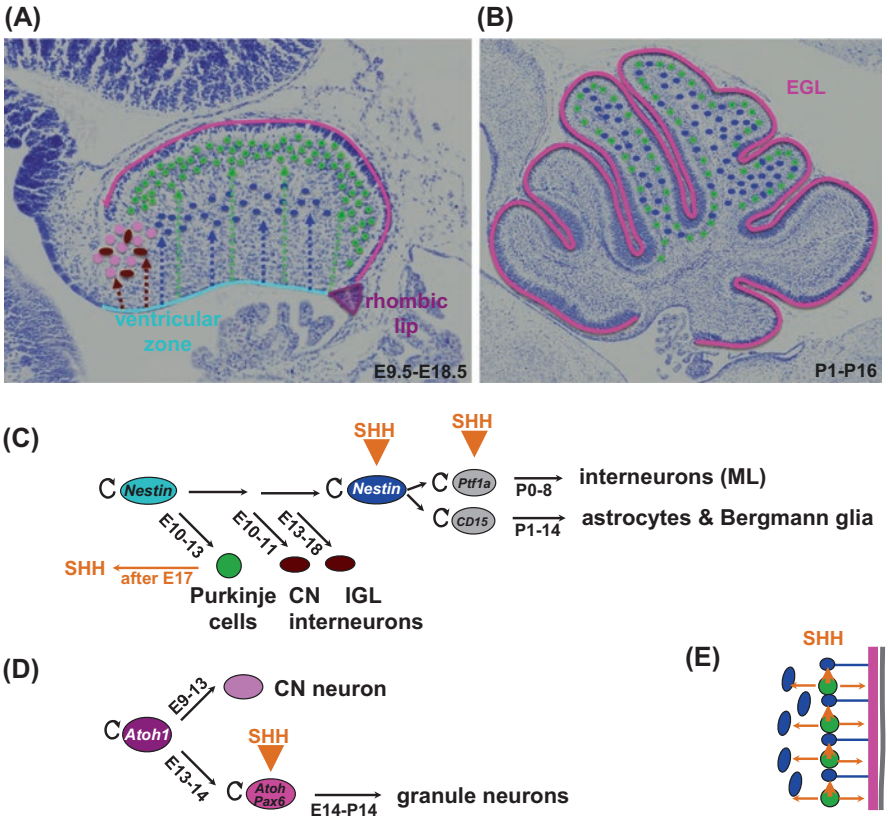

Fig. 2 Two progenitor zones produce all the neurons and the astroglia of the cerebellum at particular time points. (**a**) Midline eosin stained sagittal section of E16.5 cerebellum with ventricular zone (*turquoise*) and rhombic lip (*pink*) indicated and the cells that arise from the zones color coded as in **c** and **d**. (**b**) Midline sagittal section of P3 cerebellum showing EGL (*pink*), Purkinje cells (*green*), and Nestin-expressing progenitors. (**c**) The ventricular zone lineage is shown. (**d**) The rhombic lip lineage is shown. (**e**) SHH (*orange*) is expressed by Purkinje cells and signals to all progenitors in the postnatal cerebellum, also indicated in **c** and **d**

glia, the VZ of the cerebellum is dedicated to making only inhibitory neurons (Purkinje cells and interneurons), as well as astrocyte-like glia (astrocytes and Bergmann glia referred to as astroglia) [10]. Interestingly, most of the interneurons and astroglia are generated from intermediate progenitors that leave the VZ and proliferate after birth in the cerebellar cortex [11–14] (Fig. 2b, c). The second cerebellar progenitor zone is called the rhombic lip and generates the excitatory neurons of the cerebellum, primarily the granule cells, and projection neurons of the cerebellar nuclei [15–17] (Fig. 2a, d). Like the astroglia and interneurons, the granule cells are generated from a secondary progenitor pool made up of granule cell precursors (GCPs) that are housed in the external granule cell layer (EGL) that covers the surface of the cerebellum during development and generates granule cells that migrate

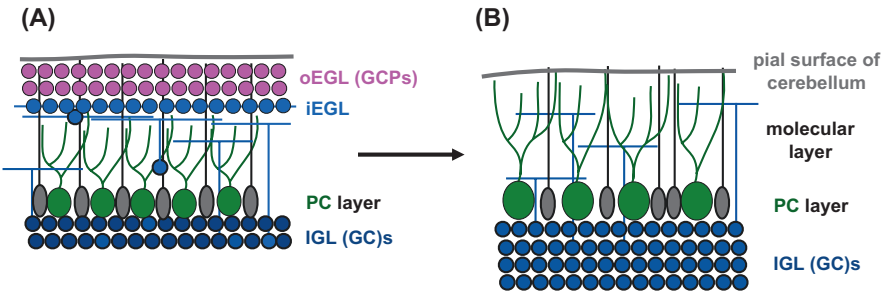

Fig. 3 Schematic drawing showing granule cell development. (**a**) During development (E15.5-P14), the cerebellum is covered with granule cells organized in a layer called the external granule cell layer (*EGL*) which is divided into an outer layer (*oEGL*) of dividing progenitors (*GCPs*) and inner layer (*iEGL*) of postmitotic granule cells (*GCs*) that extend parallel fibers (axons shown as horizontal *blue lines*). GCs migrate down the fibers (*black lines*) of Bergman glia (*grey* cell body) past the Purkinje cells (PCs, *green*) to form the inner granule cell layer (*IGL*). **(b)** Newly formed parallel fibers stack on top of older ones to form the molecular layer that also has interneurons (not shown), but the cell bodies of GCs randomly mix in the IGL. PCs express SHH, which is required for GCP proliferation

inward to form the internal granule cell layer (IGL) (Figs. 2a, b and 3). In humans, the EGL reaches a maximum volume after birth [2]. It is tempting to speculate that a dedicated transient amplifying progenitor pool evolved for the granule cells, because the granule cells comprise a majority of the neurons in the brain and thus require massive expansion of progenitor numbers during development. Curiously, the source of most oligodendrocytes for the cerebellum appears to be the VZ outside the cerebellum, likely the midbrain and/or ventral rhombomere 1 [18–20].

In this chapter, we use mouse as a model system to describe development and foliation of the cerebellum (Fig. 1) and the generation of the various neurons and astroglia of the cerebellum since precise knowledge of the VZ and RL lineages has been obtained with genetic fate mapping studies. Cumulative fate mapping with a site-specific recombinase such as Cre labels all cells that ever expressed Cre, and if the gene is specific to one progenitor pool, then all the cell types generated from the pool can be determined [21] (Fig. 4). The temporal sequence of cell-type generation is determined by genetic inducible fate mapping (GIFM). This method only labels cells expressing Cre during a particular ~24 h period [22]. Furthermore, using GIFM, the initial marked population can be precisely determined, as well as the descendants of the population at any later developmental stage or in the adult. Using promoters specific to each stem/progenitor population, detailed knowledge of the cerebellar lineages has thus been uncovered.

In this chapter we define the lineage relationships of each stem/progenitor pool, the temporal pattern of cell-type generation, and some of the proteins that regulate progenitor cell number expansion and differentiation. We include a discussion of how the numbers of each neuron/astroglial type in the cortex might be scaled to attain the correct relative proportions of different cell types and the possible contributions of the progenitor pools for replenishment of cells after an injury at

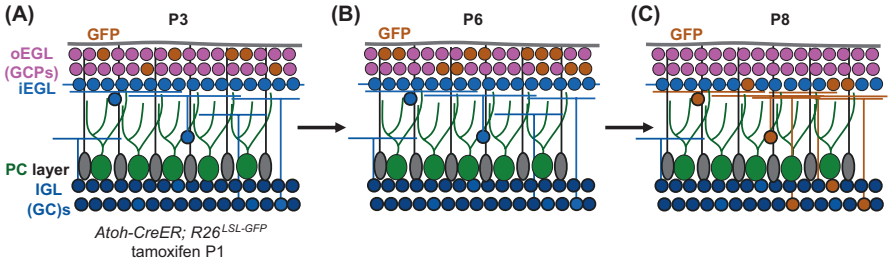

Fig. 4 Schematic illustrating genetic inducible fate mapping (GIFM). An *Atoh1-CreER* transgene is expressed only in granule cell precursors (*pink*, *GCPs*) in the outer external granule cell layer (*oEGL*). A reporter allele, $R26^{LSL\text{-}Gfp}$, expresses GFP in cells that have active Cre. Tamoxifen is injected into *Atoh1-CreER,* $R26^{LSL\text{-}Gfp}$ mice at P1, and it binds CreER and allows it to move from the cytoplasm to the nucleus and induce recombination of loxP sites in the $R26^{LSL\text{-}Gfp}$ allele (*LSL* = loxP-stop of transcription sequence-loxP), which allows GFP expression. A small number of GCPs are initially labeled with GFP (*brown*) (**a**) and then expand in number (**b**) and then differentiate (**c**). All cells in a clone differentiate at the same time; a clone is shown in **c**. Colors and labels are as described in Fig. 3

birth. This is especially relevant to premature births, since the cerebellum is particularly vulnerable to clinical and environmental factors around birth because much of its growth occurs in the third trimester and continues after birth. We end with a description of how the complex three-dimensional folded structure of the cerebellum develops in mouse and discuss how particular efferent neural circuits are enriched in specific subsets of lobules and the possible implications of this spatial division of functions for evolution of new cerebellar functions.

Early Patterning of the Neural Tube and Specification of the Cerebellar Territory

The cerebellar anlage is specified in the dorsal aspect of the anterior hindbrain called rhombomere 1 around embryonic day 9 (E9) in mouse [23–26]. Chick transplantation studies around two decades ago demonstrated that the boundary between the midbrain and hindbrain (referred to as the isthmus) is an organizing center that initiates development of r1 and the midbrain (reviewed in [27–29]). Dorsally an epithelial structure (isthmus) can be seen at E18.5 in mouse that links the cerebellum to the tectum (Fig. 5). The key isthmic organizer gene is *Fgf8* (fibroblast growth factor 8), as it is expressed in the isthmus (E8.5–12.5), is required to induce formation of the anlage of the midbrain and r1 [30], is sufficient to induce and pattern the midbrain and rhombomere 1 [31, 32], and is necessary up until E12 for cerebellum development [30, 33]. The secreted factor WNT1 is also expressed near the isthmus and is required for development of the midbrain and cerebellum [34, 35]. The molecular interactions of FGF8 with the transcription factor OTX2, required in the midbrain,

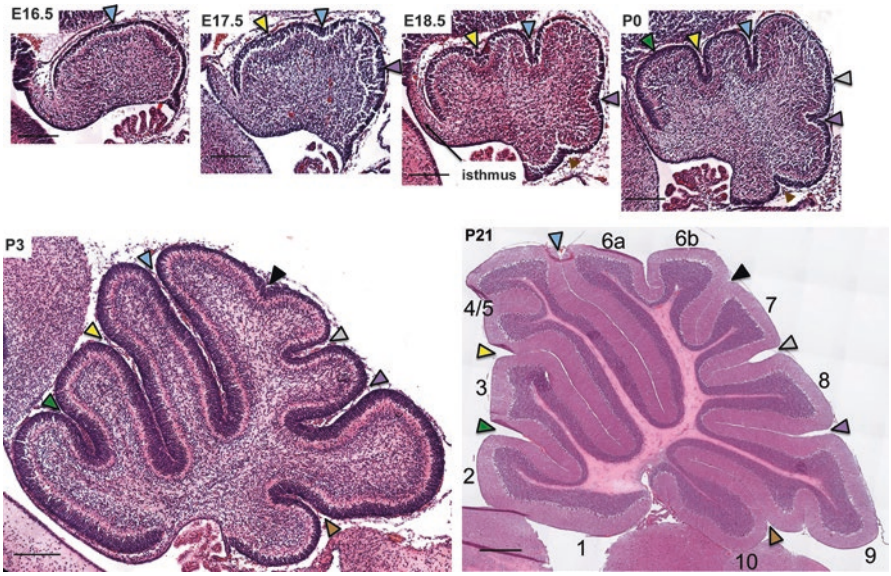

Fig. 5 Stereotypical formation of fissures during mouse cerebellum development. Midsagittal H&E sections of the cerebellum at the indicated stages. The same fissures are indicated by colored *arrowheads*. The lobules are numbers at P21. *Line* indicates 200 μm for E18.5-P3 and 500 μm for P21

and GBX2, required in the hindbrain, have been reviewed extensively, and we refer you to a detailed recent review by Martinez [28]. The dorsal-ventral axis of r1 and the midbrain is determined primarily by the morphogen sonic hedgehog (SHH), expressed by the ventral midline or floor plate [36–38]. The engrailed homeobox transcription factors (EN1/EN2) are key target patterning genes of both FGF8 and WNT signaling, with *En1* being required for the initial formation (specification) of most of the midbrain and r1 and the two genes then involved in regulating growth and foliation of the cerebellum [39, 40]. Double-mutant experiments, including conditional removal of the genes in particular lineages, have revealed overlapping and unique roles of *En1* and *En2* after the cerebellar territory is established [41–43].

Ventricular Zone Lineage

Cumulative genetic fate mapping using a line of mice in which *Cre* was inserted by gene targeting into the *Ptf1a* gene (knockin) (*Ptf1a*^{Cre}), demonstrated that only inhibitory neurons are generated from the VZ, as well as astrocytes and Bergmann glia [10] (Fig. 2c). Traditional ^{3}H-thymidine or BrdU birth dating experiments and GIFM using *Ascl1*^{CreER} revealed that Purkinje cells and interneurons of the cerebellar nuclei (CN) are the first neurons to be born during E10–13, with the interneurons being born over a shorter period [1, 12] (Fig. 2d). Interneurons are then born in an

inside (IGL) to outside (outer molecular layer) spatial progression [12, 14]. During the production of Purkinje cells, the GLI3 repressor side of the SHH pathway may play a role in proper production of ventricular zone-derived cells [37, 44]. Astrocytes and oligodendrocytes are primarily born after birth. A chick-quail chimera analysis traced the main source of oligodendrocytes to the VZ of the midbrain [19]. An earlier study in mouse also using transplantation provided evidence that the source for oligodendrocytes in the mouse cerebellum is also outside the structure and showed that oligodendrocyte precursors populate the cerebellum around E15.5 and then expand in number [20]. A recent fate mapping study argues mouse oligodendrocytes are derived from the hindbrain [18]. Curiously, a small population of Bergmann glia is born at around E13.5 [12], but most are born after birth during the major growth phase of the cerebellar cortex [11, 12, 45]. In addition, the interneurons that settle in the IGL and CN are the main interneurons derived directly from the VZ.

Interestingly, Purkinje cells have distinct settling patterns under the surface of the cerebellar cortex, depending on the day they are born, with successive waves of Purkinje cells forming different wide stripes along the anterior-posterior axis [12, 46]. Purkinje cells throughout the cerebellum initially settle into an aggregate of cells called the Purkinje plate at E14.5 before migrating outward to settle into a multilayered Purkinje cell layer (PCL) by E18.5 under the cerebellar surface. As expansion of the cerebellum continues through the postnatal growth phase, Purkinje cells resolve into a monolayer by approximately postnatal day 5 (P5) [12, 47]. Purkinje cells in the lobules of the central zone (CZ in Fig. 1d) are the last to form a monolayer, correlating with delayed generation of granule cells in these lobules [48].

The Purkinje cells initially exhibit simple morphology of a leading apical neurite and trailing axon left behind as they migrate to the PCL from the Purkinje plate (fusiform). At around P0 they undergo a sequence of cell shape changes; first their apical neurite collapses and the cells take on a stellate morphology with numerous short perisomatic neurites (~P6), and then they evolve a distinct bipolar morphology with a highly elaborated dendritic configuration that is flattened in an ramified espaliered fashion within the sagittal plane (P8 onward, [49]). The Purkinje cells of the central zone are the last to differentiate.

A medial-lateral corticonuclear topographic projection map of Purkinje cell axons to the cerebellar nuclei can be seen as early as E15.5 in mice [50], and electrophysiological recordings can be made early postnatally. While the vast majority of Purkinje cell axons project into the cerebellar nuclei, Purkinje cells of the flocculus, paraflocculus, and the nodulus of the vermis (lobule 10) instead route into the vestibular nucleus of the hindbrain.

Postnatal Cerebellar Cortex Progenitor Populations and Lineages

Ventricular zone-derived progenitors are present in the postnatal cerebellar cortex and proliferate and give rise to interneurons in the molecular layer for about a week after birth in mouse (Fig. 2b, c). These progenitors also give rise to astrocytes and

additional Bergmann glia for over a week after birth [11–14]. Elegant genetic fate mapping studies combined with marker analysis were used to address the location and lineage relationships of stem/progenitors in the cerebellar cortex [11, 45]. Using several *CreER* lines (*GLI1*^{CreER}, *Tnc*^{CreER}, *Ptf1a*^{CreER} knockin alleles) to mark Nestin-expressing stem/progenitor cells, and proteins that mark interneurons (PAX2) or astrocytes (GFAP), it was found that *Tnc*- and *Cd133*-expressing multipotent progenitors give rise to both a unipotent *Ptf1a*-expressing progenitor that expands the interneuron population during the first week after birth and to a *Tnc*- and *Cd15*-expressing progenitor dedicated to the astroglial lineage that likely gives rise to both astrocytes and Bergmann glia [11]. PAX2^{+} immature interneurons are generated in an inside-to-outside manner (basket and then stellate interneurons) in the molecular layer and then mature during the first few weeks after birth. A recent study addressed the location of the multipotent and unipotent progenitors using a *Glast*^{CreER} allele [45]. Tamoxifen was administered to the surface of the cerebellum to label only astroglial cells in the Purkinje cell layer that had a radial process extending to the surface. Interestingly, they demonstrated that *Glast*^{CreER} cells in the Purkinje cell layer generate new Bergmann glia and astrocytes, whereas progenitors in the white matter generate astrocytes and interneurons based on a clonal analysis. Finally, Nestin-expressing progenitors situated along the inner edge of the EGL have been proposed to produce GCPs [51], but it seems possible they normally give rise to interneurons in the white matter.

In vitro stem cell assays support the in vivo genetic fate mapping demonstration of multipotent stem/progenitor cells in the early postnatal cerebellar cortex. Stem cells isolated from the P3–7 cerebellum by FACS based on expression of CD133^{+} and the absence of lineage markers (PSA-NCAM, TAPA-1, and O4) or cells with a low level of *Tnc*$^{YFP\text{-}CreER}$ that also express *Cd133* and *Gli1* can form multipotent clonal neurospheres in culture that can differentiate into the expected interneurons and astrocytes, as well as granule cells and oligodendrocytes [11, 52]. Moreover, when transplanted into a P3 cerebellum, the cells form rare Purkinje cells, as well as many interneurons, astrocytes, and oligodendrocytes [52]. Since almost all CD133^{+} cells express *Tnc*$^{YFP\text{-}CreER}$ (and thus SOX2 and Nestin) [11], these studies indicate that cerebellar stem cells have a greater differentiation capacity (plasticity) in vitro than is seen during normal development. In another study, cells taken from the cerebellum of E14.5, P0, or adult mice and depleted of GCPs (ATOH1^{-}) also formed multipotent neurospheres with a similar differentiation capacity to CD133^{+} stem cells in culture and after transplantation [53]. Thus, rare stem cells remain in the adult cerebellum that can form most neuron types and glia when presented with the appropriate environment. These results raise the possibility that rare quiescent stem cells in the early postnatal or adult could be mobilized to replace neurons or glia after an injury if the necessary inducing factors can be identified.

Purkinje cells play a key role in growth of the cerebellum, as they express the mitogen sonic hedgehog (SHH), [13, 54, 55] which signals to both GCPs and white matter stem/progenitor cells [11] (Fig. 2c–e). SHH signaling in GCPs is required for their proliferation and viability after E16 [54, 56, 57]. Furthermore, deletion of *Shh* in Purkinje cells or ablation of HH signaling in white matter stem cells reduces expan-

sion of the pool of *Tnc*^{CreER}-labeled white matter stem/progenitor cells and production of interneurons and astroglia [11]. In addition, application of SHH to cerebellar slice cultures stimulates interneuron production [58]. Purkinje cells can coordinate growth of all cell types produced in the cerebellar cortex except, possibly oligodendrocytes, via SHH secretion (reviewed in [59]). How SHH is delivered from Purkinje cells to the outer EGL and white matter progenitors and whether there are other sources of HH ligands that regulate cerebellar neurogenesis remain open questions.

The bHLH transcription factor PTF1a is key to VZ cells, as in its absence all cerebellar inhibitory neurons are lost and astrocytes are depleted [10, 60, 61]. Interestingly some VZ-derived mutant cells are transformed into rhombic lip-derivative neurons and cell types normally generated from the VZ ventral to the cerebellum. Furthermore, PTF1a is sufficient to largely specify a generic inhibitory cell phenotype, as ectopic expression of PTF1a in several excitatory neuron progenitors in the nervous system induces a network of inhibitory neuron gene expression and repression of excitatory neuron genes [10, 62]. The related bHLH protein-encoding gene *Ascl1* plays a more limited role in generation of cerebellar interneurons [12]. Curiously, climbing fiber neurons also require *Ptf1a* for their survival, migration, and differentiation from the more posterior hindbrain, and in the absence of *Ptf1a*, some precursors take on a mossy fiber fate [63]. Genes that regulate Bergmann glia generation and function are absolutely critical for cerebellar growth, foliation, and formation of a normal cortical architecture [64].

Rhombic Lip Lineage

The rhombic lip (RL), formed by E9.5 at the posterior rim of the cerebellar anlage where the pial surface contacts the ventricular zone, is the source of all glutamatergic neural subtypes of the cerebellum (Fig. 2a,c). Cells arising from the RL spread anteriorly across the surface of the cerebellar anlage and sequentially produce two cell populations: postmitotic cerebellar nuclei (CN) and then proliferating granule cell precursors. Lineage tracing and birth dating studies have shown that the earliest population of cells emerging during E9.5–E12.5 migrate to and accumulate into two clusters of cells bilaterally symmetrically displaced from the midline, known as the nuclear transitory zone [16, 17]. Immature CN cells migrating from the rhombic lip to the nuclear transitory zone are ATOH1^{+}/PAX6^{+}, and as they migrate into the nuclear transitory zone, the proteins are downregulated and CN progenitors sequentially express TBR2, TBR1, and reelin [65].

Cells leaving the rhombic lip from E13.5 onward become cerebellar granule cell precursors (GCP) [16]. These cells remain at the cerebellar surface for the duration of embryonic development and form a dense proliferative layer called the external granule layer (EGL). As development advances, growth of the cerebellar anlage and concomitant EGL expansion subsume the nuclear transitory zone into an interior position where they are reorganized into two bilateral groups of three distinct nuclei in mouse: (from medial to lateral) the fastigial, interpositus, and dentate nucleus. The

three individual nuclei are clearly distinct by birth in mouse, and TBR1 or BRN2 expression marks the fastigial nuclei or the interpositus and dentate nuclei, respectively [65]. The human CN shows a massive expansion of the dentate nucleus compared to mouse, likely due to the vast expansion of hemisphere lobules. Additionally, two separate nuclei are found in human in the place of the mouse interpositus: the human globose and emboliform nuclei. The Purkinje cell axons converging on the CN become myelinated during postnatal gliogenesis. In the mature cerebellum, the CN reside in the confluence of white matter just dorsal to the cerebellar peduncles.

Initiation of SHH expression in Purkinje cells at E17.5 profoundly enhances GCP proliferation and commences the main period of granule cell neurogenesis that drives the major portion of cerebellar growth (Fig. 1a, b). At this time the EGL takes on a bilayer structure; the outer EGL (oEGL) contains the actively proliferating GCPs, and the inner EGL (iEGL) is populated by postmitotic and differentiating GCPs (Fig. 3). The GCPs of the iEGL migrate medial-laterally for approximately a day before they descend along Bergmann glia fibers to create the inner granule layer (IGL). As they descend, the incipient granule cells (GCs) leave a trailing apical neurite in the molecular layer, which bifurcates into a parallel fiber that extends medial-laterally and synapses onto Purkinje cells.

The bHLH protein ATOH1 is required for generation of GCPs and for most cerebellar nuclei projection neurons [17, 66]. One function of ATOH1 is to induce *Gli2* expression, and thus to enhance SHH signaling in GCPs [67], and likely regulate many other genes required for granule cell proliferation (e.g., *MycN* and cyclin D1) and differentiation [68]. There appears to be an antagonistic relationship between the rhombic lip protein, ATOH1, and the VZ transcription factor PTF1a, as mis-expression of each protein in the complementary progenitor zone leads to inhibition of the other gene [69]. Mossy fiber neurons also require *Atoh1* for their development [17].

Granule Cell Precursor Cell Behaviors

The role of granule cells in cerebellar development and function and the identification of GCPs in the etiology of the tumor medulloblastoma [70] have attracted interest in their proliferative behaviors. Of particular interest is how the expansion of the GCP population drives postnatal cerebellar growth and morphology (foliation). Analysis of GCP clones revealed that granule cell precursors primarily undergo symmetrical divisions to expand the number of the cells in a clone during postnatal development [71–73]. Shortly before the clones differentiate, the GCPs within a clone undergo an added burst of proliferation before they differentiate over a small temporal window. A single GCP at the E17.5 stage produces an average of 250 granule cells, requiring at least eight cell divisions. Despite the degree of synchrony in proliferative behaviors and differentiation timing of these GCP clones, individual cells within the clones over time exhibit poor synchrony of cell cycle phases [72].

Clonal studies have also provided insight into how the complex form of the cerebellum is shaped. During postnatal development, the cerebellum expands to a far

greater extent along the anterior-posterior axis than in the medial-lateral axis, due in part to orientation bias of the GCPs to divide in the anterior-posterior axis along with tangential migration within the EGL [72]. Conversely, as GCPs differentiate into nascent GCs and descend into the IGL, they favor a medial-lateral spread within the growing lobule. Lineage birth dating studies capable of labeling these parallel fibers have shown that parallel fibers are laid down in an inside-to-outside fashion with the earliest born granule cells innervating the deep molecular layer and the late born GCs innervating the outmost extent of the molecular layer [48, 73] (Figs. 3 and 4). In contrast to the ordered laminar arrangement of parallel fibers, the cell bodies of new GCs settle at random depths in the underlying IGL. The base of each fissure that separates the cerebellum into lobules acts as a boundary to the movement of GCPs [72]. Thus, after fissure formation GCPs are maintained in the nascent lobule. This intriguing finding suggests that lobules may not simply be anatomical units, but could also have functional uniqueness and act as separate developmental units.

Regional differences appear in the cerebellum with respect to granule cell proliferation and differentiation. Granule cell production in the anterior (lobules 1–5) and posterior (lobules 8–10) cerebellum predominates over the central region (lobules 6/7) in the perinatal period, but this delayed growth in the central zone is compensated for by the perdurance of a thicker EGL in lobules 6/7 around P14, whereas the EGL is exhausted in all other cerebellar regions [48]. Taking these observations together with a delay in onset of SHH expression in lobules 6/7 and that the intercrural and declival fissures are the latest vermis fissures to form, a picture emerges that the central zone encompassing lobules 6/7 has a general developmental delay that continues for days after cessation of development in the rest of the cerebellum. It will be interesting to determine the dynamics of regional growth in the vermis of the human cerebellum, as well as in the hemispheres.

Development of Cerebellar Afferents

Climbing fibers from the inferior olive innervate the cerebellar anlage as a fasciculated axon bundle beginning at E15.5–E16.5 in mice, and by late E16.5 the first synapses with Purkinje cells are observed [74–78]. By birth, these axons defasciculate and innervate the developing Purkinje cell multilayer, with each Purkinje cell receiving multiple climbing fiber inputs [79]. The supernumerary climbing fiber inputs are eliminated in an activity-dependent fashion between the second and third postnatal week so that each adult Purkinje cell is innervated by a single climbing fiber axon [80–82]. Mossy fibers arrive in the cerebellar anlage between E13.5 and E15.5 [83] and form transient contacts with Purkinje cells by birth. Within the first postnatal week, the mossy fibers establish cell-cell contacts with synaptic ultrastructural features, but in the second postnatal week, they withdraw to refine their synaptic connections with their proper GC and Golgi cell targets in the internal granule layer [84].

Development of Cerebellar Foliation and Relationship to Afferent and Efferent Circuitry

During development the cerebellum increases in size and undergoes a dramatic progressive foliation transforming the smooth outer surface into a highly foliated collection of lobules separated by fissures (Figs. 1 and 5) [85]. In the human cerebellum, there are additional shallower folia along the surface of the lobules, and they form at an early stage of the fetal foliation process. Foliation creates dramatically more surface area in the cerebellum along the anterior-posterior axis, maximizing the number of cells in the layered cortex and thus the quantity of functional circuits that the cerebellum can host in the spatial constraints of the posterior skull. Foliation also correlates with spatial separation of distinct functional regions within the cerebellum. Afferents to the cerebellum from the spinal cord and brain target particular locations within the medial-lateral axis of the cerebellum. Furthermore they project to particular lobules [86]. For example, the spinocerebellar circuit projects only to the anterior and posterior zones of the vermis. Within the lobules, some circuits target regions that correspond to the longitudinal cerebellar zones (stripes) defined by different gene expression [86, 87]. There is also a spatial relationship between Purkinje cells and the cerebellar nuclei they project to, generally medial to lateral, but it seems likely there is also an anterior-posterior code. Thus, efferent functions have spatial domains.

The murine foliation pattern is highly consistent across individuals and has minor strain-specific variation [88]. The pattern of foliation varies depending on the medial-lateral position in the cerebellum (Fig. 1c), and fissures form in a specific sequence (Fig. 5). In mouse, as in all mammals, the medial cerebellum, or vermis, has ten primary lobules [86, 87]. The lateral hemispheres have their own distinct pattern as do the most lateral paraflocculi and flocculi.

Foliation begins during the last embryonic days, around E16 to E17, and the last fissure begins to form by ~P5 (Fig. 5). The first indication of foliation at E16.5–17.5 is a regional inward thickening of the EGL, which will correspond to the base of the newly forming fissure. Following this thickening of the EGL, the outer surface of the cerebellum indents (Figs. 5 and 6). Around this time GCPs within the base of the forming fissure become elongated, and the local Bergmann glia direct their fibers to the center of the indention (Fig. 6b) [89]. The intervening regions between the fissure bases expand outward. By following the foliation process through to completion, it can be seen that the fissure bases hold their relative spatial positions and thus are called anchoring centers, as the lobules expand to their final size [4, 89].

As discussed previously, the proliferation of GCPs in the EGL and the resulting growth of the cerebellum are dependent on SHH supplied by the underlying Purkinje cells [54, 56, 57]. Reducing the level of SHH signaling reduces the overall growth of the cerebellum and concomitantly reduces the degree of foliation. The EGL becomes thinner and the first appearance of anchoring centers is delayed. Additionally, foliation is precociously halted. However, the fissures that do form correspond to the earliest fissures suggesting that while SHH provides the growth that is necessary for foliation to proceed, it does not control the pattern of foliation.

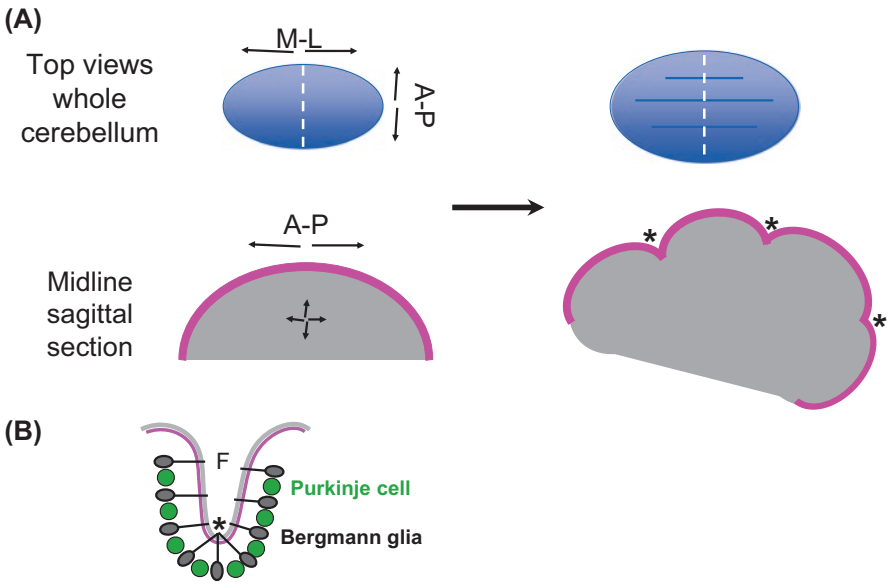

Fig. 6 Model of cerebellum foliation based on differential expansion of layers. (**a**) As the outer layer, the EGL (*pink*) expands more rapidly than the inner mass of the cortex (*grey*), the EGL buckles creating anchoring centers (*). White *dotted lines* in top views indicate where sagittal sections are positioned. Fissure placement is proposed to be directed by the differential expansion of the layers and the shape (*ovoid*) of the initial cerebellar anlagen. (**b**) The Bergmann glial fibers (*black*) connect the outer surface (*thick grey line*) to the inner buckling Purkinje cell (*green*) layer that contains the cell bodies of the Bergmann glia (*dark grey*), and a fissure (*F*) forms above each anchoring center as the cerebellum continues to expand. *A-P* anterior-posterior, *M-L* medial-lateral

When the level of SHH is increased beyond wild-type levels, the cerebellum is larger and has an extra fissure [57]. Intriguingly, this extra fissure is placed in a conserved position similar to where the rat has an additional fissure. Consistent with the requirement for HH signaling in GCP proliferation, induction of activating HH signaling mutations specifically in the GCP lineage results in the SHH subgroup of medulloblastoma [70, 90, 91].

The proliferation of GCPs is temporally and spatially regulated within the cerebellum. Maximum proliferation in the lobules of the central zone (6–7) is delayed and maintained longer relative to the other cerebellar zones. This difference is attenuated in the cerebellum of $En1^{+/-};En2^{-/-}$ mutants that have an abnormal foliation pattern such that proliferation in the anterior, posterior, and nodular zones is more similar to the central zone [48]. Because granule cells do not disperse across fissure boundaries, this creates self-contained lobules; this allows any lobule-specific granule cell behavior to fine-tune the shape of the lobules [72].

Blocking the generation of Bergmann glial cells has revealed that there are at least two separable stages of anchoring center formation: an inward thickening of the EGL and formation of an indentation on the outer cerebellar surface. The cere-

bellum is covered by the pial surface as well as the end feet of the Bergmann glial processes. In the absence of Bergmann glia, the EGL thickens, but the outer edge of the cerebellum fails to subsequently bend inward. Consequently, fissures fail to appear at the cerebellar surface. Nevertheless, many granule cells are displaced deep into the cerebellum and form a fissure-like mass, possibly at the positions of the initial EGL thickening. As a result, the layers of the cerebellum are not well defined, and the foliation pattern is severely disrupted when Bergmann glial development is disrupted [64, 92, 93].

In addition to acting as a physical bridge between the outer surface of the cerebellum and the Purkinje cell layer, Bergmann glial fibers provide the scaffolding for the radial migration of newly born granule cells from the EGL to the inner granule layer (Fig. 3). Disrupting the development, or orientation, of Bergmann glial fibers thus leads to aberrant GC migration and the ectopic accumulation of GCs in the molecular layer. In some cases this disruption is severe and can distort foliation [94]. Thus, the Bergmann glia play a key role in cerebellar foliation and formation of a normal cytoarchitecture.

Alterations in the timing of anchoring center appearance change the resulting foliation pattern. In *En2* null mutants, the appearance of the anchoring centers for the secondary and prepyramidal fissures surrounding lobule 8 is reversed in developmental time. This results in a lengthening of the prepyramidal fissure and a shortening of the secondary fissure and a corresponding foliation pattern change in the intervening lobule 8 [39, 42]. Interestingly, the initial changes in the EGL and Bergmann glia that signal the formation of an anchoring center appear normally even when the entire anchoring center either forms prematurely or is delayed [89].

Little is known about the tissue-scale mechanical or physical forces that underlie cerebellar foliation. However, the cerebral cortex is also a folded tissue in primates, and many models have been proposed to describe the formation of sulci and gyri during cerebral gyrification. Many of these models are based on a system of differential or constrained growth of a tissue bilayer. Differential growth rates between connected layers can lead to tissue buckling and subsequent surface folding. These models take into consideration that the pattern of foliation can be shaped by adjusting the starting size of the tissue, the difference in the growth rates of the layers, and the mechanical properties of the layers [95–100]. It is interesting to speculate whether similar forces could be responsible for the initial appearance of anchoring centers in the cerebellum (Fig. 6a). Like the cerebral cortex, the cerebellar cortex can be considered as divided into multiple layers. One model of cerebellar folding used a tri-layer model of differential growth [101]. In this model the EGL and the IGL were considered separated by a “soft” Purkinje cell layer. This three-layer system when modeled to have a higher outer growth rate allowed for surface wrinkling even if the outer and inner layers had similar measures of stiffness. These models suggest that the comparative rates of growth of the outer and inner cerebellum are important to foliation, as is the initial shape of the cerebellar anlage.

It is exciting to speculate about the evolution and functionality of the compartmentalized lobule structure and the spatial segregation of afferent project fields to particular lobules and zones. The cerebellum is involved in diverse roles including

cognition and social behaviors. The cerebellar hemispheres have undergone tremendous expansion in humans, and they house the majority of long-range circuits that involve the neocortex. It is possible that as the neocortex expanded and became folded into gyri and sulci, there was similar spatial segregation of neuronal circuits into particular neocortex folds. This would be one way for developmental programs to be divided into subunits that could have separate regulatory rules. For example, different numbers of neurons could be generated in each subunit, as well as different types of neurons and different proportions of inhibitory and excitatory neurons and astrocytes. A fold with a particular function in the neocortex could then connect with a specific fold in the cerebellum, completing the interacting circuit. Nevertheless, redundancy and duplication of function have been built into the cerebellum that minimize the consequences of local damage in adults. We propose that developmental regulatory mechanisms are in place to buffer the developmental processes from small injuries that occur. A question for the future is whether stem or progenitor cells in the developing or adult cerebellum can replace damaged neurons long after they are born and the progenitors no longer normally generate the cell type.

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