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Spatially Restricted and Developmentally Dynamic Expression of *Engrailed* Genes in Multiple Cerebellar Cell Types

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Abstract

The cerebellum is a highly organized structure partitioned into lobules along the anterior–posterior (A-P) axis and into striped molecular domains along the medial–lateral (M-L) axis. The *Engrailed* (*En*) homeobox genes are required for patterning the morphological and molecular domains along both axes, as well as for the establishment of the normal afferent topography required to generate a fully functional cerebellum. As a means to understand how the *En* genes regulate multiple levels of cerebellum construction, we characterized *En1* and *En2* expression around birth and at postnatal day (P)21 during the period when the cerebellum undergoes a remarkable transformation from a smooth ovoid structure to a highly foliated structure. We show that both *En1* and *En2* are expressed in many neuronal cell types in the cerebellum, and expression persists until at least P21. *En1* and *En2* expression, however, undergoes profound changes in their cellular and spatial distributions between embryonic stages and P21, and their expression domains become largely distinct. Comparison of the distribution of *En*-expressing Purkinje cells relative to early- and late-onset Purkinje cell M-L stripe proteins revealed that although *En1*- and *En2*-expressing Purkinje cell domains do not strictly align with those of ZEBRINII at P21, a clear pattern exists that is most evident at E17.5 by an inverse correlation between the level of *En2* expression and PLC β 4 and EPHA4.

Keywords

Engrailed; Purkinje cell; Cerebellum; Compartment; Development; Stripe

Introduction

The mature cerebellum is a highly compartmentalized structure. Along the anterior–posterior (A-P) axis, it is organized into a series of ten lobules in the vermis [1], which can be grouped into four basic transverse molecular zones (anterior, central, posterior, and nodular) based on molecular marker domains [2] and along the medial–lateral (M-L) axis into three broad morphologically distinct domains, from medial to lateral: the vermis, hemispheres, and flocculi/paraflocculi. The M-L axis is further subdivided by a series of Purkinje cell parasagittal gene expression stripes that create over 100 domains defining the

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cerebellum molecular code [3]. The molecular and morphological specification of numerous subdomains can be considered a coordinate system with M-L gene expression stripes and A-P lobules providing the latitude and longitude [3]. This array could serve as a framework for organizing the expression of genes that ensure afferents form an orderly map that results in efficient information processing. Interestingly, the *Engrailed* (*En*) homeobox genes are required for patterning the cerebellum into lobules, M-L gene expression stripes, as well as spinocerebellar afferent topography [4–7]. The *En* genes thus appear to act as master regulators to organize the cerebellum for proper functioning.

The cerebellum undergoes dramatic changes in morphology and organization from its inception at E9.0 to its mature form at postnatal day (P)21, and *En1* and *En2* expression strikingly parallels the observed structural changes. The cerebellar anlage is specified around E9.0 from dorsal rhombomere 1, and expression of *En1* in this region is essential for the formation of the cerebellum [8, 9]. Expression of the *En* genes in the cerebellum is largely regionally uniform before E15.5, with *En1* expression confined to the presumptive vermis and *En2* expression extending laterally to include nearly the entire cerebellum [9]. After E15.5 when cerebellar parasagittal domains of gene expression are first apparent and foliation begins, *En1/2* expression changes dramatically, shifting from uniform expression into a series of M-L bands [10]. The patterned *En1/2* expression persists until at least the early postnatal period [10], with *En2* being detected in the adult [11]. While the spatial patterns of *En1* and *En2* expression have been described, little is known about the cell types responsible for the expression, the relationship between the embryonic M-L bands and other striped markers, or the details of *En1* and *En2* expression after cerebellum morphogenesis is complete and adult striped gene expression is established.

The emergence of embryonic Purkinje cell striped gene expression is concomitant with *En1/2* expression becoming patterned between E15.5 and E17.5. The establishment of Purkinje cell molecular signatures comes in two waves. The first wave is referred to as early-onset expression and is characterized by mostly transient expression in late embryogenesis in clusters of Purkinje cells. The distinct embryonic molecular signatures are lost during the first postnatal week due to the downregulation of some genes and de novo expression of others [12]. Both *En* genes, in addition to PCP-2/L7 [13, 14], Calbindin (CALB) [15], EPHA4 [16], and several other proteins [10] are classified as early-onset markers, and their combined banding pattern divides the perinatal cerebellum into M-L subdomains [3, 17]. The second wave is referred to as late-onset expression, including the stripe markers, Aldolase C/ZebirinII (ALDC/ZII) [18, 19] and HSP25 [20], and usually initiates with homogeneous expression in Purkinje cells during the first postnatal week followed by a downregulation in a subset of Purkinje cells by the third postnatal week when the mature stripe pattern emerges [12]. Whether the early M-L Purkinje cell subdomains maintain constant identity during development, and how that identity corresponds to the late-onset M-L molecular stripe domains, has been controversial. However, recently, two markers, PLC β 4 and Neurogranin (NG), were found to maintain patterned expression from embryonic to adult stages [21, 22]. The consistent expression pattern indicates that stable populations of cells might exist. Supporting this idea, embryonic clusters of Purkinje cells fate-mapped using an inducible Cre expressed in L7-positive Purkinje cells were found to retain their molecular identity [23]. Furthermore, a correlation between Purkinje cell birth date and their localization in M-L stripe subdomains has been reported [24]. These lines of evidence suggest that Purkinje cell molecular identities are established embryonically and mature into a molecular code consisting of parasagittal striped expression in the adult.

Purkinje cell molecular heterogeneity precedes the segregation of afferents to their terminal zones and therefore occurs independently of afferent inputs [25–27]. Thus, Purkinje cell molecular code patterning was proposed to be the precipitating event in circuit organization

in the cerebellum [25]. An understanding of the positional information encoded by Purkinje cells during development is therefore of utmost importance. Interestingly, mice lacking *En1* and/or *En2* have defects in the positioning and formation of lobules [5, 9, 28]. A series of *En1/2* temporal conditional mutants recently demonstrated that the *En* genes are necessary after E15.5 to pattern foliation [5]. Analysis of an allelic series of *En1/2* mutants, as well as mis-expression of *En2*, further identified a sensitivity of the molecular code to *En1/2* dose [6, 29]. Moreover, *En1/2* mutants exhibit defects in segregation of spinocerebellar afferent inputs into the correct M-L sub-domains during the first postnatal week [7, 30]. Since the *En* genes control patterning of both cerebellar morphology and gene expression, as well as afferent targeting, elucidating their functions will likely provide insight into how a normally functioning cerebellum is constructed. Both processes, foliation and the formation of parasagittal gene expression domains, initiate during the late embryonic period and mature during a protracted developmental period up to the end of the third postnatal week, a period of *En1/2* expression that has only been superficially analyzed.

We conducted a detailed investigation of the cell types and M-L domains in which the *En* genes are expressed. Using *En1* and *En2* reporter lines and immunohistochemical detection of EN proteins, we identified parasagittal stripes of *En1/2* expression in Purkinje cells at E17.5 and detected weak expression at P21 in subsets of Purkinje cells. In addition, we found that populations of granule cells, interneurons, and deep cerebellar nuclei (DCN) neurons express either or both *En* genes at E17.5 and that expression persists at P21. However, there is a significant shift in the levels and domains of *En1* and *En2* expression between the two time points. Interestingly, the two genes become expressed in primarily distinct neuronal cell types at P21, with *En1* expression in a subset of DCN neurons and Purkinje cells and *En2* in unipolar brush cell interneurons and granule cells. Our expression studies have thus revealed possible unique functions for each *En* gene in postmitotic neurons.

Materials and Methods

Mouse Strains

All mouse lines were previously described and were maintained on a mixed (predominantly Swiss Webster) background: *En1^{LacZ}* [31], *Atoh1-Cre* [32], *En2^{flxLacZ}* [33], and *En2^{GFPloxIRES}* referred to as *En2^{GFP}* [5], *En2^{hd}* [4], *En1^{Cre}* [34], and *En1^{flx}* [9].

Broad and robust expression in multiple cerebellar cell types of β -galactosidase from the *En2* locus prevented visualization of the periodic pattern of *En2* expression in Purkinje cells in E17.5-P4 whole-mount cerebella. To circumvent this problem, expression of β -galactosidase driven from an *En2^{flxed-LacZ}* allele was eliminated from granule cells by expressing *Cre* from a granule cell precursor (GCP)-specific promoter *Atoh1* (*Atoh1-Cre*; *En2^{flxLacZ}*⁺ mice; Fig. 1).

Most *En1*-null mutant mice fail to form a cerebellum and die perinatally, preventing specific detection of *En2* localization in P21 *En1*-null mutant cerebella [8]. However, eliminating *En1* expression about 1 day after its initiation by combining *Cre* recombinase under the control of *En1* (*En1^{Cre}* allele) with a floxed *En1* allele (*En1^{flx}*) allows animals to mature to adulthood and to form cerebella with largely normal morphology and cytoarchitecture [6]. Thus, *En1^{Cre/flx}* mice were used for the immunohistochemical analysis of EN2 expression at P21 (Fig. 4).

Whole-Mount β -Galactosidase Visualization

Whole cerebella were dissected away from the rest of the brain, immersion fixed in 4% paraformaldehyde (PFA), and X-gal stained as previously described [35]. The staining time

for cerebella from *En1^{LacZ}* mice was 2 h at E17.5/P0 and 4 h for P4; for *En2^{fxLacZ}* mice, the staining time was 25 min at E17.5 and 35 min at P0/P4.

Immunohistochemistry and Data Acquisition

E17.5 brains were collected in cold PBS and immersion fixed overnight with 4% paraformaldehyde. P21 animals were transcardially perfused with 4% PFA, brains were dissected out and post-fixed for 1 h at 4°C in 4% PFA, and cryopreserved in 30% sucrose at 4°C overnight. Immunostaining was performed on 14-μm E17.5 or 30-μm P21 coronal cryosections. Immunostaining procedures followed standard protocols [28], except for rabbit αpan-EN and rabbit αHSP25 double labeling that was done sequentially.

For sequential immunostaining, cryosections (thawed, rinsed with PBS, and blocked in 5% BSA, 0.5% Tx100, PBS 1 h at room temperature) were incubated with rabbit αpan-EN antibody diluted 1:100 in blocking solution at 4°C overnight, followed by washes in PBS and 1-h incubation at room temperature with donkey αrabbit Cy3-conjugated secondary antibody (1:200, Jackson ImmunoResearch). This was followed by six washes in PBS (10 min) and a 1-h incubation at room temperature in blocking solution, followed by overnight incubation at 4°C with rabbit αHSP25 antibody diluted 1:500, washes with PBS, and 1-h incubation at room temperature in donkey αrabbit Cy2 (1:500, Jackson ImmunoResearch). Lack of cross-reactivity between antibodies following this protocol was clear since EN and HSP25 localize to distinct subcellular compartments. As expected, we observed nuclear labeling with the red signal representing EN localization and cytoplasmic localization of the green signal representing HSP25 immunoreactivity.

The following primary antibodies at the indicated dilutions were used: affinity-purified rabbit αEngrailed homeodomain-1 (αEnhb-1 referred to as pan-EN) 1:100 [36]; rat αGFP 1:1,000 (Nacalai Tesque, cat. no. 04404-84); rabbit αβ-gal 1:1,000 (Millipore, cat. no. A11132); rabbit and mouse αCalbindin D-28K 1:1,000 (Swant); mouse αZII 1:100 [6], generous gift of Dr. Richard Hawkes); rabbit αHSP25 1:500 (Stressgene, cat. no. SPA-801); rabbit αPAX6 1:500 (Millipore, cat. no. AB5409); rabbit αNeurogranin 1:500 (Millipore, cat. no. AB5620); rabbit αPLCβ4 1:250 or 1:500 (Santa Cruz Biotechnology, cat. no. sc-20760); goat αEPHA4 1:250 or 1:400 (R&D Systems, cat. no. AF641); mouse αLHX1/5 1:100 (DSHB, 4F2); goat αBRN2 1:500 (Santa Cruz Biotechnology, cat. no. sc-6029); rabbit αSOX2 1:500 (Millipore, cat. no. AB5603); rabbit αPAX2 1:500 (Invitrogen, cat. no. 18-0483); rabbit αTBR2 1:1,000 (Millipore, cat. no. AB9618); rabbit αTBR1 1:1,000 (Millipore, cat. no. AB9616); goat αRORα 1:250 (Santa Cruz Biotechnology, cat. no. sc-6062).

Images were acquired using Axiovision and Velocity software on a Zeiss Observer Z1 and Leica CTR 6000 microscopes. For cellular co-localization analysis MosaiX and Z-Stack modules of the Zeiss AxioVision software were used to collect single optical sections at E17.5 with a 40X objective, and a stack of 15 Z-sections with a 60X objective at P21.

Expression Pattern Analysis

Images of E17.5 coronal sections stained for GFP (*En2*) and EPHA4 or PLCβ4, and β-gal (*En1*) and PLCβ4 were analyzed using Photoshop, ImageJ, and Xcel. The dorsal surface of the cerebellum was straightened using the liquify filter in Photoshop with minimal distortion. The images were then cropped to produce a band that included the Purkinje cells along the dorsal surface. The channels were split, and for each channel, the fluorescence was converted into a graph displaying the fluorescence intensity versus distance from midline (for PLCβ4) or the far edge of the midline expression band (for EPHA4, as 0-μm point)

using the analyze gel slice function in ImageJ. The points were then extracted from the graph and a rolling average (for 50 points) was calculated and plotted in Xcel.

For banding pattern analysis at P21, EN-positive Purkinje cell nuclei and ZII-positive Purkinje cell somata were identified from the collapsed Z-stacks and verified in individual Z-sections when necessary, and analyzed using Photoshop and Illustrator software. Purkinje cell nuclei were identified by their position in the Purkinje cell layer (PCL), large diameter, ovoid shape, dispersed appearance, and the presence of two to four large nucleoli [37]. All Purkinje cell somata within the Z-stack identified by either ZII labeling or by the nuclear labeling were marked and counted. The resulting tracings were superimposed, allowing analysis of the numerical and positional relationship of the two markers in the PCL at P21. Two sections per lobule were analyzed from three different animals, and more than 170 Purkinje cells per section were scored.

Results

En1 and *En2* are Expressed in a Series of Parasagittal Stripes Perinatally

Expression of *En1* (closely followed by *En2*) begins at the two-somite stage throughout the mesencephalon and rhombomere 1, structures that give rise dorsally to the cerebellum and colliculi and ventrally to some of the tegmental nuclei [9, 11, 36]. During late gestation, expression of the *En* genes becomes regionalized into parasagittal stripes that likely contain clusters of Purkinje cells [9, 10]. However, the cell types responsible for the observed late embryonic *En* expression pattern have not been investigated and a large portion of cerebellar development occurs postnatally. Therefore, we conducted a detailed *En* expression analysis during late embryonic and early postnatal stages.

The three-dimensional pattern of *En1* and *En2* expression was revealed by analysis of whole-mount cerebellar preparations from reporter lines expressing β -galactosidase from either the *En1* or *En2* locus. At E17.5, *En1^{LacZ}* expression was detected in prominent superficial clusters of cells, possibly corresponding to dorsally located Purkinje cells (Fig. 1, black arrows and arrowheads). Lower and more uniform expression was also detected spanning the presumptive vermis (Fig. 1, white arrowhead), which could correspond to external granule layer (EGL) cells, inner granule cell layer (IGL), and/or immature neuronal or glial precursors. Patterned *En1* expression at E17.5 consisted of a band spanning the midline (A and B in Fig. 1, black arrowhead) and two symmetrical pairs of bilateral bands (Fig. 1, black arrows). The more central bilateral bands were located at the incipient vermis–paravermis boundary and the more lateral bands in a medial region of the future hemispheres (Fig. 1, black arrows). The medial band was wider and spanned the entire A-P axes of the cerebellum, whereas the lateral bands were more prominent in the anterior regions and tapered off posteriorly. An additional prominent cluster of *En1* expression was oriented along the M-L axis in the most anterior region of the E17.5 cerebellum, corresponding to future lobules 1–3 (asterisk). This banding pattern persisted during the first postnatal week, but the level of expression was attenuated at later stages (E, F, I, and J in Fig. 1).

Expression of *En2^{fxLacZ}* was detected in a wide domain that likely includes many cerebellar cell types at E17.5, obscuring analysis of a potential patterned expression in Purkinje cells. To circumvent this problem, we analyzed *En2*-driven β -galactosidase expression in mice where expression in granule cells was specifically eliminated (see “Materials and Methods” for details). The medial band of *En2* expression (black arrowhead) was less prominent compared to *En1*, and unlike the *En1* medial band, it did not span the entire A-P axis and shifted posteriorly from E17.5 to P4 (C, D, G, H, K, and L in Fig. 1). Additional bands and clusters of *En2* expression in more lateral portions of the vermis and hemispheres were

mostly oriented along the A-P axis and located in similar positions from E17.5 to P4 (C, D, G, H, K, and L in Fig. 1). Thus, the *En* genes are expressed in regionalized stripes perinatally during a period of intense organization and morphogenesis of the cerebellum.

Cellular Composition of the E17.5 Cerebellum

Due to the interesting three-dimensional *En* expression pattern perinatally, we sought to classify the cell types responsible for the pattern. We characterized the cell populations and their locations within the E17.5 cerebellum using protein expression markers for the various cell types (schematized in Fig. 2A). At E17.5, the cerebellum is a simple elongated structure; however, the cytoarchitecture is less well defined than the mature trilaminarily arranged adult cerebellum. Cell division and migration are still active processes, making the perinatal cerebellum an extremely dynamic structure. Two distinct progenitor zones are actively generating new cells: the EGL and the ventricular zone (VZ; Fig. 2A, green circles and red ovals, respectively). Additionally, secondary progenitors producing interneurons and glia migrate into the cerebellar cortex from the VZ and continue to expand during postnatal development (Fig. 2A, navy blue triangles). The EGL is seeded from a domain located at the interface of the EGL and VZ termed the rhombic lip (RL). The RL is the source of all PAX6-expressing GCPs [38] (Fig. 2A, gold rectangles), TBR2-expressing glutamatergic interneurons [39] (Fig. 2A, gold rectangles), and TBR1-expressing projection neurons of the DCN [40] (Fig. 2A, dark gray oval). The VZ comprised radial glial cells (RGCs), positive for the stem cell marker SOX2 [41] (Fig. 2A, red ovals), which give rise to all of the GABAergic neurons of the cerebellum, including LHX1/5 [42] and ROR α [43–45] expressing Purkinje cells (Fig. 2A, purple hexagons), and glia. Directly under the EGL, a transient band of immature Purkinje cells (Fig. 2A, purple hexagons) was apparent at E17.5, which varied in thickness depending on M-L and A-P positions. PAX2-expressing GABAergic interneuron precursors [46] (Fig. 2A, navy blue triangles) were detected comingling in the cerebellar perimeter with the Purkinje cells; however, PAX2-positive precursors extended centrally into the location of the future white matter. A second interneuron precursor population, TBR2-expressing glutamatergic interneurons, was sparsely distributed throughout the cerebellum with a huge concentration ventrally in the rhombic lip and scattered above the VZ [39]. Additionally, an apparent migratory population of BRN2-expressing neurons (Fig. 2A, aqua ovals) was detected above the VZ—the nature of these cells is not known. Lastly, the DCN neurons (Fig. 2A, large ovals), many expressing BRN2 [40, 47] (Fig. 2A, DN and IN ovals), were positioned centrally in three diffuse clusters symmetrically on either side of the midline, with the dentate nucleus (DN) most lateral followed by the interposed (IN) and then the most medial nucleus, the fastigial nucleus (FN), containing many TBR1-expressing projection neurons [40] (Fig. 2A, FN oval).

En1 is Expressed in Four Purkinje Cell Subpopulations at E17.5

Purkinje cells are known to have differential protein expressions manifested in bilaterally symmetrical stripes reminiscent of the *En1* banding pattern observed in the whole-mount X-gal cerebella preparations (Fig. 1) [10, 15, 48, 49]. We therefore performed immunohistochemical labeling of coronal cerebellar sections from E17.5 *En1^{LacZ/+}* animals with antibodies against β -galactosidase (expression referred to as *En1^{LacZ}*) and the pan-Purkinje cell markers ROR α and/or LHX1/5 (purple hexagons, schematics in Fig. 2A) [42] to identify Purkinje cells co-expressing *En1*. Purkinje cells were identified by their position underneath the EGL and their expression of ROR α or LHX1/5; all cells in this domain expressed both markers (Electronic Supplementary Material (ESM) Fig. 1). In coronal sections, we identified five *En1^{LacZ}* expression bands: one band centered at the midline (Fig. 2B, Band1 (B1)) and two bilaterally symmetric pairs of bands (Fig. 2B, Band2 (B2) and Band3 (B3)). B2 abutted the expression domain of B1, but was distinct based on *En1^{LacZ}*

intensity. B3 was located in the dorsal aspect of each future hemisphere (Fig. 2B, B3). B2 and B3 cell populations contained almost exclusively Purkinje cells that were intensely positive for *En1^{LacZ}*. These two populations corresponded to the strongly X-gal-positive parasagittal stripes in A in Fig. 1 marked by black arrows. A third Purkinje cell population, distinguished by fainter labeling for *En1^{LacZ}*, was observed overlapping the dorsal domain of B1 (Fig. 2) but expanding into domain B2. This population is within the diffuse *En1* medial expression domain marked by a white arrowhead in A in Fig. 1 in which many additional cell types express *En1* (Fig. 2B and data not shown). Curiously, an additional *En1^{LacZ}* population co-expressing LHX 1/5, as well as BRN2, was identified within the ventral aspect of B1 (Fig. 2B, C box3 and 3'). However, these cells (Fig. 2B, C, box 3) are most likely not Purkinje cells due to their lack of ROR α expression and were not further characterized. Thus, by spatial distribution and molecular signatures, we identified three distinct *En1*-expressing Purkinje cell populations organized in M-L clusters at E17.5 cerebella. Additionally, many *En1^{LacZ}*-positive cells were not distributed in a recognizable pattern and did not label for LHX1/5 or ROR α , and therefore are most likely not Purkinje cells.

***En2* is Expressed in a Subset of Purkinje Cells at E17.5**

Our whole-mount X-gal (C and D in Fig. 1) analysis and previously published data [10] elucidated five bands of cells expressing a higher level of *En2* organized along the M-L axis. Upon more detailed analysis of coronal sections in *En2^{GFP/+}* tissue, two additional bands were identified, for a total of seven bands (Fig. 3A). The apparent thick diffuse band spanning the midline (black arrowhead in Fig. 1) actually consisted of a central domain of higher expressing cells (Fig. 3A, B1) that is distinct from two symmetric bands on either side of the midline (Fig. 3A, B2), followed by the two pairs of previously described M-L bands (Fig. 3A, B3 and Band4 (B4)). Compared to *En1*, the *En2* expression bands spanned greater areas and contained many more cells.

To identify the contribution of Purkinje cells to the observed pattern, we performed immunohistochemical labeling of coronal sections from *En2^{GFP/+}* animals with antibodies against GFP (expression referred to as *En2^{GFP}*) and LHX1/5 or ROR α . All bands contained *En2^{GFP}*-expressing Purkinje cells based on co-expression of GFP and LHX1/5 or ROR α (Fig. 3A, B, boxes 1 and 2). The co-localization of *En2^{GFP}* and LHX1/5 or ROR α was greatest in the area directly beneath the EGL, with the incidence of co-localization decreasing in the ventral portion of the expression band, consistent with the predominant localization of Purkinje cells in a dorsal region underlying the EGL (Fig. 2A) [42]. In addition, many *En2^{GFP}* cells did not co-label with LHX 1/5 or ROR α , and thus were most likely not Purkinje cells.

Interestingly, the Purkinje cells expressing the highest levels of *En1* and *En2* appeared to define both overlapping and distinct populations of Purkinje cells based on their relationship to additional patterned Purkinje cell markers (Fig. 5). The lateral population of *En1*-expressing Purkinje cells (Fig. 2B, B3) seemed to be located within the most lateral *En2*-expressing Purkinje cell domain B4 (Fig. 3A). However, the widest and most prominent *En2* expression band, B3 (Fig. 3A), was nearly absent of *En1* expression (Fig. 2B), while the most extensive *En1*-expressing Purkinje cell population (Fig. 2B, B1 and including B2) encompassed *En2* B1 and B2, but also included a large number of Purkinje cells only weakly expressing *En2* (Fig. 3A, area between B1 and B2).

***En1* is Expressed in a Subset of GCPs, RGCs, Interneuron Precursors, and DCN at E17.5**

We classified the *En1^{LacZ}*-positive, LHX1/5-negative cells into seven groups by immunofluorescence double-labeling analysis with the cerebellar cell type markers

described above and summarized in Fig. 2A. The identity of the strongly expressing *En1^{LacZ}* cells at the midline with radial glial morphology was confirmed to be radial glia by the co-expression of the RGC marker, SOX2 (Fig. 2B, C, box 4). A population of cells weakly expressing *En1^{LacZ}* situated in the cell-dense EGL (Fig. 2B, C, box 5) and extending posteriorly into the RL co-labeled for the GCP marker PAX6. Consistent with the observed expression from the whole mounts, the *En1^{LacZ}* GCP populations were only detected in the medial cerebellum. Scattered *En1^{LacZ}*-positive cells seen predominantly in the lateral cerebellum (Fig. 2B, C, box 6) co-labeled with a marker for interneuron precursors, PAX2. *En1^{LacZ}* was also expressed in a subset of DCN neurons, labeling a cluster of cells in the most lateral nucleus, the DN, most strongly (Fig. 2B, C, box 7). The more medial nuclei, IN and FN, identified by the expression of BRN2 (data not shown) and TBR1 (Fig. 2B, C, box 8), respectively, contained neurons expressing *En1^{LacZ}* at lower levels. Lastly, a population of strongly expressing *En1^{LacZ}* cells was situated directly above the VZ and co-expressed BRN2 (Fig. 2B, box 9). The identity of these cells is not clear.

***En2* is Expressed in a Subset of GCPs, RGCs, Interneuron Precursors, and DCN at E17.5**

Since Purkinje cells did not account for all of the *En2^{GFP}*-expressing cells, we sought to further classify the cell populations expressing *En2*. Many LHX1/5-negative cells within the seven bands had neuronal morphology, and their location was consistent with the known location of interneuron precursors at E17.5. Indeed, a large number of PAX2-positive interneuron precursors were intermixed with Purkinje cells in all seven *En2^{GFP}* expression bands (Fig. 3A, B, box 4' and 5). Additionally, an interneuron precursor population that was *En2^{GFP}*-positive was observed directly above the VZ and appeared to be migrating laterally (Fig. 3A, B, box 6). This apparent migratory population was heterogeneous and also included PAX2-negative, BRN2-expressing cells (Fig. 3A, B, box 7), which appeared to be distinct from the interneurons. In support of this, the BRN2-expressing cells in this region were always more lateral and some cells in the region expressed *En1^{LacZ}*, whereas the PAX2-positive, *En2^{GFP}*-expressing cells were more medial and did not appear to express *En1^{LacZ}*. Additionally, a dorsal population of *En2^{GFP}*-expressing cells was found to co-label with a marker of unipolar brush cell interneurons, TBR2 (Fig. 3A, B, box 8). A central population of *En2^{GFP}*-positive cells off the midline expressed the DCN marker BRN2 (Fig. 3A, B, box 9). We also detected weak expression of *En2^{GFP}* in the RL (Fig. 3, box 11) and the EGL (Fig. 3A, B, box 10) in both lateral and medial regions, with the strongest EGL expression in the central region of the presumptive vermis. The central EGL with the strongest *En2^{GFP}* expression included the *En1^{LacZ}*-positive EGL domain, but was not limited to this population. Lastly, we identified a population of SOX2-expressing RGCs at the midline that expressed *En2^{GFP}* (Fig. 3A, B, box 3), a similar population to the *En1^{LacZ}*-expressing RGCs. Similar to Purkinje cells, the other neuronal cell types of the cerebellum appeared to contain both overlapping and distinct populations of *En1*- and *En2*-expressing cells.

***En1* and *En2* are Expressed in Diverse and Largely Non-overlapping Neuronal Populations at P21**

The apparent reduction in *En1/2* expression levels by P4 (Fig. 1) led us to investigate cerebellar *En1* and *En2* expression at a later time point, P21, when the mature cytoarchitecture has been established. Utilizing reporter strains and antibody labeling in mutants lacking *En1* or *En2* (Fig. 4), we identified expression of not only *EN2* as was previously described [11] but also low levels of *EN1* in the cerebellum at P21. Interestingly, expression of each gene was largely restricted to distinct cell types. A low level of *En1^{LacZ}* expression (*LacZ*⁺ cells) was detected predominantly in the PCL and the molecular layer (ML), corresponding to the location of Purkinje cell bodies and dendrites, whereas *En2^{GFP}* expression (*GFP*⁺ cells) was largely confined to the IGL and the ML, corresponding to the

location of granule cells and their axons (Fig. 4A, B). Additionally, *En1*, but not *En2*, was detected in the DCN (Fig. 4A, dotted line). *En1^{LacZ}* and *En2^{GFP}* were also detected in cells in the ML, likely corresponding to interneurons, as has been recently reported [5].

Due to the low levels of expression from the reporter lines at this stage, the novelty of these findings, and the knowledge that protein expression can be profoundly regulated by translational and posttranslational modifications, we investigated the distribution of EN1 and EN2 proteins at P21 with the pan-EN antibody [36]. Immuno-fluorescence analysis of wild-type cerebella at P21 detected the expression of EN1/2 proteins in most granule cells, most DCN cells, and in ML and IGL interneurons (Figs. 4, 7, and 9 and ESM Fig. 2), as well as relatively weaker expression in subsets of Purkinje cells arranged in a periodic pattern along the M-L axis (Figs. 4, 7, 8, and 9). The pan-EN antibody staining thus confirmed the expression seen with the reporter strains. Postnatal EN1/2 expression in Purkinje cells appeared to peak during the third postnatal week and then decline (data not shown). Consistent with previous reports, EN1/2 expression in granule cells was continuously detected at all postnatal ages analyzed and was comparable in labeling intensity to the expression in the inferior colliculus and midbrain nuclei (data not shown). Interestingly, the relative levels of EN1/2 in the IGL varied several folds across cerebellar subregions, with the highest levels of expression in the IGL at P21 detected in the nodular zone (ESM Fig. 3). The majority of basket and stellate interneurons in the ML at P21 expressed EN1/2, as shown by co-labeling with antibodies to Parvalbumin (basket, stellate, and Purkinje cell marker), but not Calbindin (Purkinje cell specific marker; ESM Fig. 2). EN1/2 immunoreactivity at P21 was detected in all DCN subdivisions (ESM Fig. 2).

Since our antibody recognizes both EN proteins, we analyzed wild-type tissue for a composite pattern and tissue from either *En2* mutants (*En2^{hd/hd}*) [4] or *En1* conditional mutants (*En1^{cre/lox}*) [6, 9] to reveal the specific expression pattern of each gene. In *En2^{hd/hd}* mutants, EN immunore-activity in Purkinje cells and DCN was similar to wild types; however, labeling in granule cells was only detected in the most medial vermis (Fig. 4C and data not shown). In *En1^{cre/lox}* mutants, EN immunoreactivity in granule cells was similar to wild types; however, it was largely diminished in Purkinje cells and the DCN (Fig. 4C and data not shown). In both mutants, interneuron labeling was reduced compared to wild types, but still present. Thus, the majority of EN protein in granule cells at P21 is contributed by *En2* expression, whereas EN expression in Purkinje cells and the DCN is predominantly contributed by *En1*. These results are consistent with in situ hybridization results for *En1* and *En2* published by the Allen Brain Institute (Allen Institute for Brain Science ©2009; <http://mouse.brain-map.org>).

***En1*- and *En2*-Expressing Purkinje Cells Do Not Strictly Follow Boundaries of Known Early-Onset Stripe Markers at E17.5**

High expression of the *En* genes in subsets of Purkinje cells both at E17.5, a time when immature Purkinje cell gene expression clusters are present, and at P21, when mature stripe marker expression is achieved, led us to compare the *En1* and *En2* M-L expression domains relative to known Purkinje cell stripe markers. Consistent with the idea that the *En* genes could regulate the establishment of molecular stripe subdomains in Purkinje cells, stripe patterns of early, late, and continuous markers are disrupted in *En1/2* mutants [6, 7, 10]. The earliest markers to exhibit a periodic pattern of expression in Purkinje cells are *En1*, *En2*, and NG, which become patterned around E15.0, shortly after all the Purkinje cells have become postmitotic [10, 21]. PLCβ4, CALB, and EPHA4 subsequently become patterned by E17.5 [15, 22, 50]. Expression of all known markers expressed early, except NG and PLCβ4, becomes absent or uniform in Purkinje cells during the first postnatal week due to either downregulation of expression or upregulation in previously negative domains [3]. NG

expression is down-regulated in Purkinje cells around P20; however, expression of PLC β 4 remains patterned into adulthood [22].

The five bands of *En1*^{LacZ}-expressing Purkinje cells in the dorsal domain of the cerebellar cortex (A in Fig. 5, yellow bars) were directly compared to the expression of the early stripe markers—EPHA4 (C in Fig. 5), PLC β 4 (D in Fig. 5), CALB (E in Fig. 5), and NG (F in Fig. 5)—by double immunofluorescence analysis. At the molecular level, *En1*^{LacZ} B1 could be broken down into at least four distinct domains (schematized in Fig. 6) of alternating CALB immunoreactivities (E' in Fig. 5 and C in Fig. 6), starting with a central region being only CALB-positive (domain 1) followed by a domain negative for CALB, but expressing EPHA4, PLC β 4, and NG (domain 2), then another CALB-positive region also expressing PLC β 4 (domain 3) and lastly a CALB-negative and EPHA4-positive domain (domain 4; C'–F' in Fig. 5 and A–D in Fig. 6). B2 contained the brightest *En1*^{LacZ}-expressing Purkinje cells and also labeled for NG and CALB (D' and E' in Fig. 5 and B and C in Fig. 6). The most lateral band, B3, had low levels of expression of EPHA4 and CALB (C' and E' in Fig. 5 and A and C in Fig. 6). Thus, *En1*^{LacZ}-expressing Purkinje cell domains do not strictly align with any of the stripe markers analyzed. However, a clear pattern seemed to exist that could be characterized as an inverse correlation between *En1* expression and PLC β 4, as seen by the line graph in H in Fig. 5.

The central *En2*^{GFP} band, B1 (B in Fig. 5), contained a group of dorsally located Purkinje cells at the midline that co-expressed CALB, and likely *En1*. B2 co-expressed NG (D'' in Fig. 5 and B' in Fig. 6) and CALB (E' in Fig. 5 and C' in Fig. 6). The wide B3 expression domain contained Purkinje cells that co-expressed all four of the stripe markers analyzed (C'–F' in Fig. 5), albeit with varying expression levels, suggesting that smaller subgroups of cells have distinct expression signatures. The most medial dorsal aspect of the band strongly expressed PLC β 4 (F'' in Fig. 5) and CALB (E'' in Fig. 5), whereas the most lateral dorsal domain expressed CALB and NG (D'' in Fig. 5) more strongly. The mid region of the band displayed low levels of all three of these markers and was negative for EPHA4 (C'' in Fig. 5 and A' in Fig. 6), and the lateral third of the band expressed EPHA4 at high levels. Lastly, the most lateral band, B4, had a similar expression signature as the lateral *En1*^{LacZ} band (B2) with low levels of expression of EPHA4 and CALB (Fig. 5). While the *En2* expression bands seem to be heterogeneous with respect to the other stripe markers examined, it is notable that an inverse correlation seemed to exist between the Purkinje cells with the highest expression of *En2* and the highest expression of PLC β 4 and EPHA4 (G and I in Fig. 5). In conclusion, although the pattern of *En1* and *En2* expression at E17.5 in Purkinje cells does not precisely correlate with the expression pattern of any of the early-onset markers tested, a pattern does seem to be present. These results thus lend new insight into the complexity of the combinatorial heterogeneity among subpopulations of embryonic Purkinje cells

EN Expression in Purkinje Cells is Patterned at P21

Given the inverse relationship between high levels of EN1/2 and the early and late marker PLC β 4, we next asked whether any of the boundaries of EN1 or EN2 expression in Purkinje cells correlate with the molecular stripe subdomains defined by ZII and HSP25 expression. These two markers are among the most extensively studied adult Purkinje cell molecular code markers, and their differential expression along the A-P axis can be used to demarcate the cerebellum into four zones. ZII is a marker of M-L subdomains in the anterior and the posterior zones, whereas HSP25 defines M-L subdomains in the central and the nodular zones [2, 3, 20]. Furthermore, PLC β 4 is expressed in all ZII-negative regions [6, 51].

Examination of EN1/2 and marker protein in individual Purkinje cells revealed that EN1/2 are expressed within ZII-positive and ZII-negative territories, as well as HSP25-positive and

-negative territories (Figs. 7 and 9). Furthermore, ZII and HSP25 – positive cells did not uniformly express EN1/2. However, EN1/2-positive (EN⁺) and -negative (EN⁻) Purkinje cell clusters at P21 exhibited a reproducible gross periodic pattern along the M-L axis at different A-P levels. In order to quantitatively analyze the distribution of EN⁺ Purkinje cells relative to the ZII stripe domains in the anterior and posterior zones, EN⁺, ZII⁺, and ZII⁻ Purkinje cells were traced from Z-stack images of coronal sections through lobules III and IX stained with pan-EN and ZII antibodies, as well as Hoechst dye, to reveal nuclei of all Purkinje cells (Fig. 8A, EN⁺ domains outlined by red lines and ZII⁺ domains represented by green lines; see “Materials and Methods” for details). In lobule III, the minority of Purkinje cells expressed either EN or ZII (Fig. 8B, 17% EN⁺ and 10% ZII⁺ out of a total of 693 Purkinje cells scored). The distribution of the two markers showed only a partial coincidence of the two proteins since about 30% of all ZII⁺ Purkinje cells were EN⁺ and approximately 13% of all ZII⁻ Purkinje cells were EN⁺ (Fig. 8C). In lobule IX, the majority of Purkinje cells expressed EN and ZII (Fig. 8B, 65% EN⁺ and 73% ZII⁺ out of 893 Purkinje cells scored). A bias, albeit not statistically significant, was detected toward co-segregation of the two markers in lobule IX (Fig. 8C, 74% of all ZII⁺ Purkinje cells expressed EN, whereas 50% of all ZII⁻ cells expressed EN). In conclusion, patterned expression on EN1/2 expression in Purkinje cells at P21 shows only a weak correlation with the ZII stripe subdomains (Fig. 9).

Discussion

The cerebellum is the most highly compartmentalized structure in the adult mouse brain. The *en* gene is a key regulator of body compartmentalization in *Drosophila*, raising the question of whether En1/2 plays a similar role in establishing compartments in the mammalian cerebellum. In this study, we characterized the spatial localization of mouse *En1* and *En2* expression at the perinatal period and at P21 and identified the specific cell types responsible for *En1* and *En2* expression at E17.5 and P21, time points during which the cerebellum undergoes a transformation from a smooth ovoid to a highly foliated structure. First, we show that both *En1* and *En2* are expressed in most neuronal cell types in the cerebellum and also in a subpopulation of RGCs at E17.5. Second, we show that expression of both *En* genes persists until at least P21; however, the cellular and spatial distributions of *En1* and *En2* expression undergo profound changes. Third, we compared the distribution of EN⁺ Purkinje cell domains relative to early- and late-onset Purkinje cell M-L stripe genes and found that a simple correlation does not exist. No one marker shares a perfectly overlapping or distinct expression pattern with either *En1* or *En2*. However, *En*-expressing Purkinje cells at E17.5 and P21 localize to distinct M-L compartments that tend not to express high levels of PLCβ4 and EPHA4 at E17.5 and that show a weak correlation with the ZII-positive domains at P21. Our results suggest that *En1* and *En2* expression in multiple cerebellar cell types could contribute to the cerebellum patterning defects seen in *En1/2* mutants. We are currently examining the possible roles *En1* and *En2* play in different cell types by generating cell type-specific conditional mutants and analyzing changes on foliation and striped gene expression.

Engrailed Genes are Expressed in Multiple Cerebellar Cell Types in Developmentally Dynamic Patterns

We uncovered that *En2* (GFP-expressing cells in *En2^{GFP/+}* mice) is expressed primarily in two of the three germinal zones of the E17.5 cerebellum, the RL and the EGL. *En1* expression (*LacZ*-expressing cells in *En2^{lacZ/+}* mice) overlaps spatially with *En2* in these cell populations, but is more restricted, being confined to the medial aspects of both the RL and EGL. In the VZ at E17.5, *En2* expression is restricted to a cluster of RGCs at the midline that appears to also express *En1*. Expression of *En1* and *En2* in three cerebellar

germinal zones at E17.5 suggests a possible late (or continuous) role for these genes in the proliferation of progenitors and/or specification of their progeny.

En2 is broadly expressed throughout the cerebellar cortex at E17.5 and is present in all of the main neuronal cell types (excluding DCN neurons), including Purkinje cells (identified by LHX1/5 and/or ROR α immunoreactivity), GCPs (identified by PAX6), GABAergic interneurons (PAX2-positive, which include Golgi, basket and stellate cell precursors), and unipolar brush cell precursors (TBR2 double labeling). *En2*-expressing cells are more abundant in the medial regions (corresponding to the future vermis and paravermis), tapering off toward the lateral parts of the cerebellar cortex (corresponding to the future hemispheres and the floccular region). On the contrary, *En1* expression is restricted to the medial cerebellum, although in sub-populations of the same neuronal cell types, with the exception being DCN neurons that mainly express *En1*. Interestingly, the relative levels of *En1*, *En2*, and *En1+2* differ within the expression domains, further subdividing M-L subregions into smaller molecularly distinct subdomains. This raises an intriguing possibility that a complicated molecular code involving relative levels of *En* expression in M-L subdomains could contribute to the specification of Purkinje cell subdomains. This possibility is consistent with previous analysis of an allelic series of *En* mutants [6, 9].

DCN neurons and Purkinje cells continue to express *En1* at P21, whereas *En2* is mainly expressed in granule cells; both *En1* and *En2* continue to be expressed in mature cerebellar interneurons. Thus, *En* genes are expressed in postmitotic neurons in partially overlapping yet distinct expression patterns, suggesting diverse roles in differentiation, maturation, and maintenance of both glutamatergic and GABAergic cerebellar cell types.

Potential roles for the *En* genes in cerebellar neuro-genesis and neuronal differentiation based on their gene expression are consistent with previously published mutant analyses in which cerebellar size and the number of neurons is reduced in a gene copy number-dependent manner [5, 9, 52, 53]. Moreover, *Drosophila en* plays a role in midline serotonergic neuron differentiation [54], and in mammals, *En1* expression in GABAergic spinal cord interneurons is necessary to specify their targeting to somatic motor neurons [55]. Interestingly, *En1* and *En2* expression in midbrain dopaminergic neurons appears not to be required for their specification, but rather for their continuous survival and axon path finding and targeting [56, 57]. However, it is not clear whether the *En* genes play similar roles in the differentiation of neurons in the cerebellum. Our detailed description of *En* expression in proliferating and postmitotic neurons of the perinatal and P21 cerebellum provides a foundation for future examination of cellular mechanisms and molecular targets of *En* function during cerebellar development.

***En1* and *En2* are Expressed in Largely Distinct Cell Types at P21**

Despite the fact that expression of both *En1* and *En2* is attenuated during the first postnatal week (Fig. 1), we found that both genes are expressed at P21, the age at which cerebellar development is largely complete. Interestingly, at this age, the two major cerebellar neuronal cell types, Purkinje and granule cells, express *En1* and *En2*, respectively, and almost distinctly. This was unexpected since based on the high sequence similarity, overlapping embryonic expression patterns, and developmental rescue when the *En1* coding sequences are replaced by *En2* sequences, early studies postulated largely overlapping functions for the two mammalian *Engrailed* genes. Recent studies, however, have demonstrated that EN1 and EN2 protein functions are distinct as EN2 supports foliation more effectively than EN1 [9], whereas EN1 and EN2 differentially regulate ZII and HSP25 expression [6]. The distinct cellular expression of EN1 and EN2 at P21 raises the possibility of additional non-overlapping functions in the postnatal cerebellum.

Homeobox transcription factors are thought of as master regulators of developmental processes. Our expression profile in the cerebellum suggests possible, yet unexplored, roles in circuit refinement, maintenance, or plasticity in mature neurons. What roles could a homeobox transcription factor play in postmitotic neurons? In *Drosophila*, several molecules involved in signaling, axon elongation, and adhesion are direct transcriptional targets of *en* [58–60]. These classes of proteins mediate axon growth and targeting in mammals as well; however, whether they are direct transcriptional targets of mammalian *En* genes has not yet been determined. The cellular mechanisms by which *En* genes act in axon targeting in the cerebellum also remain to be determined despite *En1/2* recently being shown to be required for the normal topography of spinocerebellar afferents [7]. We now show that multiple cell types could be involved in this process. EN proteins might also function in a paracrine way since EN1 can be secreted and internalized by neurons and the protein can promote neurite outgrowth in vitro, as well as growth cone turning [61, 62].

Engrailed Parasagittal Stripe Expression in Purkinje Cells Does Not Precisely Correlate with Early- or Late-Onset M-L Molecular Compartments

Around E15.5, periodic M-L clusters of high *En* expression become apparent, constituting one of the earliest markers of cerebellar M-L compartmentalization [10]. Two major classes of cerebellar afferents, climbing and mossy fibers, arrive in the cerebellar cortex during late embryogenesis [63–65], co-incident with the emergence of the periodic pattern of *En* expression in Purkinje cells. Both climbing and mossy fibers undergo a protracted period of elaboration and refinement of synaptic contacts with their cerebellar targets, resulting in major shifts in their targeting territories during the first three postnatal weeks [7, 66]. Mature patterns of cerebellar molecular expression bands in Purkinje cells emerge by P21, concomitantly with the mature target specificity of cerebellar afferents. Interestingly, *En* genes were shown to participate in both processes—*En1/2* mutants exhibit defects in both the determination of molecular subdomains in Purkinje cells in the embryonic and early postnatal stages, as well as the adult, and in afferent targeting by P6 [6, 7]. Based on these results, it has been proposed that *En1/2* could directly regulate the expression of classical M-L molecular markers, such as EPHA4 or ZII.

We show using whole-mount preparations that *En1* and *En2* are expressed in prominent M-L bands at P0 and P4, in addition to E17.5 [9]. Detailed analysis in coronal sections confirmed *En1* and *En2* expression in periodically arranged subsets of dorsally located Purkinje cells, underlying the EGL, and identified by LHX1/5 and ROR α immunoreactivity. *En1* and *En2* banding patterns in Purkinje cells only partially overlap. *En1* is expressed in a medial stripe spanning the midline and in two additional pairs of clusters symmetrically located on both sides of the midline, positioned at the incipient vermis–paravermis boundary, and in the incipient medial hemisphere region. *En2* expression in dorsal Purkinje cells forms one midline stripe and three, rather than two, bilaterally located pairs of clusters that generally span larger areas and encompass more cells, but appear to include many of the *En1* expression domains. Interestingly, the *En1* and *En2* stripe domains did not precisely co-label with the early-onset M-L molecular markers examined (EPHA4, PLC β 4, CALB, and NG). However, *En1* and *En2* Purkinje cells tended not to express PLC β 4, and the *En2* domains tended to be complementary to high-level EPHA4 expression. These data suggest that a complicated molecular code of embryonic Purkinje cells exists, consisting of smaller clusters of molecularly distinct Purkinje cells within the larger expression bands previously identified.

Furthermore, the pattern of *En1* and *En2* in Purkinje cells is developmentally dynamic, undergoing a profound shift from the perinatal to late postnatal period. During the first postnatal week, the level of *En1* and *En2* expression appears to go down, being lost in the majority of Purkinje cells (Fig. 1). At P21, periodically arranged clusters of Purkinje cells

express a low level of EN1. The EN1 domains, however, do not correlate precisely with the ZII expression domains, which are complementary to PLC β 4, although EN1 expression does show a weak preference for the ZII⁺ domains. Curiously, EN1 expression in Purkinje cells appears to be downregulated during the fourth postnatal week (data not shown). Since there is no major lateral migration of Purkinje cells during the postnatal period, the changes in EN1/2 expression domains in Purkinje cells must be attributed to temporal changes in *En1/2* gene expression during the first postnatal month, resulting in two distinct waves of expression in partially distinct Purkinje cell populations at E17.5 compared to P21. This is unlike NG and PLC β 4 that have been suggested to be maintained in the same cells throughout the embryonic and postnatal periods. Nevertheless, as would be predicted for a stable population of molecularly defined Purkinje cells, the general complementary expression of EN1 and PLC β 4 in the embryo matches the tendency for EN1 to be expressed in ZII-positive Purkinje cells in the adult.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expression of the *Engrailed* genes during perinatal cerebellar development. Both visualization of *En1^{LacZ}* and *Math1-Cre; En2^{floxLacZ}* reveal that both *En1* (A, B, E, G, I, J) and *En2* (C, D, G, H, K, L) are expressed by parasagittal clusters of ventricular zone-derived cell types along the A-P axis at the time points indicated. Cells of the EGL also express *En1* and *En2* with *En1* expression only in the medial cerebellum and *En2* throughout the entire EGL. The expression is dynamic and, as the cerebellum develops, anterior stripes are lost

Fig. 2.

En1 is expressed in bands of Purkinje cells and in subsets of interneurons, radial glia, granule cell precursors, and DCN neurons at E17.5. **A** Schematic of cell types and location within a representative coronal section from an E17.5 cerebellum. **B** Mid-coronal section from an *En1^{LacZ/+}* cerebellum showing overall pattern of *En1*-expressing cells. Green lines indicate the observed expression bands referred to in text and boxed regions (**B**) (boxes 1–9) indicate position of high magnification images for cell marker analysis (**C**) (boxes 1–9)

Fig. 3.

En2 is expressed in a patterned array of Purkinje cells and in subsets of other cerebellar cell types at E17.5. **A** Mid-coronal section from an *En2*^{GFP/+} cerebellum showing overall pattern of *En2*-expressing cells. *Green lines* indicate the observed expression bands referred to in text and *boxed regions (A)* (boxes 1–11) indicate position of high magnification images for cell marker analysis in **(B)** (boxes 1–11)

Fig. 4. *En1* and *En2* expression in P21 cerebellum. **A** Coronal section through cerebellum of *En1^{LacZ/+}; En2^{GFP/+}*. *En1^{LacZ}* (red in the composite and the middle panel) localized mainly by Purkinje cells (band of expression corresponding to Purkinje cell somata and dendrites in the PCL and ML) and DCN (highlighted by dotted line in the middle panel). *En2^{GFP}* (green in the composite and the right panel) localized mainly to the IGL, suggestive of predominant expression in granule cells. **B** Higher magnification image of boxed region in (A) showing *En1^{LacZ}* expression (red in the composite and the middle panel) in subsets of the Purkinje cells and *En2^{GFP}* (green in composite and right panel) expression in small densely packed cells in the IGL, consistent with expression in granule cells. **C** Engrailed proteins in P21 cerebella localize to cell nuclei as detected with pan-EN antibody. In WT cerebella (left panel), EN localized to small and large cells in the IGL, large cells in the PCL, and cells in the ML, consistent with expression in granule cells, Purkinje cells, as well as IGL and ML interneurons. In *En2*-null (*En2^{hd/hd}*, middle panel), EN immunoreactivity is gone from the majority of IGL granule cells, but remains in Purkinje cells and some interneurons. In *En1* conditional mutant (*En1^{Cre/lox}*, right panel), EN immunoreactivity disappears from Purkinje cells, but remains in most granule cells and interneurons. Scale bars are 500 μ m in (A) and 20 μ m in (B) and (C). ML molecular layer, PCL Purkinje cell layer, IGL inner granule cell layer

Fig. 5.

Comparison of *En1* or *En2* expression in Purkinje cells with early-onset stripe markers. Representative coronal sections from *En1^{LacZ/+}* and *En2^{GFP/+}* cerebella showing pattern of *En1* (A) and *En2* (B) expression in E17.5 brains. Brain sections were double-labeled for either *En1^{LacZ}* (C'–F') or *En2^{GFP}* (C''–F'') in green and the early-onset stripe markers in red EPHA4 (C' and C''), Neurogranin (NG) (D', D''), CALB (E', E''), and PLCβ4 (F', F''), with individual stripe marker expression in (C–F) for clarity. White arrowheads (A, C'–F') indicate position of *En1*-expressing Purkinje cells in B2 and B3. Line graphs show the fluorescence intensity (percentage of maximum) relative to the distance (μm) from the edge of the midline expression band (0-μm point) for *En2^{GFP}* and EPHA4 (G) or midline (0-μm point) for *En1^{LacZ}* and PLCβ4 (H), and *En2^{GFP}* and PLCβ4 (I)

Fig. 6.

Schematic representation of *En1*- and *En2*-expressing Purkinje cells at E17.5 relative to early-onset stripe markers. Schematic diagrams illustrating the expression patterns of *En1* (A–D) and *En2* (A'–D') (green dots) with respect to the early-onset stripe markers EPHA4 (A and A'), NG (B and B'), CALB (C and C'), and PLCβ4 (D and D') (red dots) in E17.5 coronal cerebellar sections. Yellow dots indicate overlap between either *En1* or *En2* with the indicated stripe marker. Primary data shown in Fig. 5

Fig. 7.

EN1/2 expression in Purkinje cells at P21 is patterned along the M-L axis but does not strictly obey ZII domains. **A** Coronal sections through anterior and posterior zones of P21 cerebellum showing the striped pattern of ZII expression in *green* and EN1/2 localization detected with a pan-EN antibody [36] in *red*. **B** Higher magnification images of regions *boxed* in (**A**) demonstrating that EN-positive Purkinje cells (identified by large nuclei in PCL and highlighted by *red arrowheads*) fall within ZII⁺ as well as ZII⁻ domains. ZII⁺ cells that lack EN expression also are detected (highlighted by *black arrowheads with green outline*).

A 500 μm, **B** 50 μm

Fig. 8. Patterned expression of EN1/2 in Purkinje cells at P21 shows a weak correlation with ZII M-L domains. **A** Representative tracings of Purkinje cells from lobules III and IX. ZII⁺ Purkinje cell somata are represented by *green circles*, ZII⁻ by *gray circles*, and EN⁺ Purkinje cell nuclei designated by *red crosses*. *Red lines* traced next to the PCL represent the EN⁺ domains and *green lines* ZII⁺ domains. **B** Percentage of EN⁺ and ZII⁺ Purkinje cells in lobules III and IX. **C** Quantification of EN⁺ Purkinje cell distribution within ZII⁺ and ZII⁻ domains shows a trend toward co-localization of the two markers (Purkinje cells in lobule III, $n=693$; Purkinje cells in lobule IX, $n=893$; $n=3$ mice)

Fig. 9.

EN1/2 expression in Purkinje cells at P21 does not strictly correlate with the HSP25 boundaries. **A** Coronal sections containing the central and nodular zones showing striped pattern of HSP25 expression in *green* and EN1/2 localization detected with a pan-EN antibody in *red*. **B** Higher magnification images of regions *boxed* in (**A**) demonstrate that EN⁺ Purkinje cells (*red arrowheads*) do not correlate well with boundaries of HSP25 expression. HSP25⁺ cells that lack EN expression were abundant as well (*black arrowheads with green outline*). Scale bars are 500 μ m (**A**) and 100 μ m (**B**)