The Engrailed homeobox genes determine the different foliation patterns in the vermis and hemispheres of the mammalian cerebellum

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SUMMARY

Little is known about the genetic pathways and cellular processes responsible for regional differences in cerebellum foliation, which interestingly are accompanied by regionally distinct afferent circuitry. We have identified the Engrailed (En) homeobox genes as being crucial to producing the distinct medial vermis and lateral hemisphere foliation patterns in mammalian cerebella. By producing a series of temporal conditional mutants in En1 and/or En2, we demonstrate that both En genes are required to ensure that folia exclusive to the vermis or hemispheres form in the appropriate mediolateral position. Furthermore, En1/En2 continue to regulate foliation after embryonic day 14, at which time Fgf8 isthmic organizer activity is complete and the major output cells of the cerebellar cortex have been specified. Changes in spatially restricted gene expression occur prior to foliation in mutants, and foliation is altered from the onset and is accompanied by changes in the thickness of the layer of proliferating granule cell precursors. In addition, the positioning and timing of fissure formation are altered. Thus, the En genes represent a new class of genes that are fundamental to patterning cerebellum foliation throughout the mediolateral axis and that act late in development.

KEY WORDS: Fgf8, Granule cells, Sonic hedgehog, Morphogenesis, En, Mouse

INTRODUCTION

A key question in developmental biology is how tissues acquire their overall shape, as proper tissue morphology underlies normal organ function. The anatomy of the cerebellum (Cb) exemplifies the relationship between differences in morphology and neural circuitry. In most vertebrates, the outer surface of the Cb folds during development creating mediolateral (ML) fissures surrounding folia (called lobules). The enlarged surface area created by the lobules allows for an increase in the number of neurons organized into a layered surface cytoarchitecture (called the cortex), and thus in the complexity of neural circuits and the range of behaviors controlled by the Cb. Whereas the Cb of most vertebrates has one foliation pattern along the ML axis, mammals have a central core, called the vermis, which has a foliation pattern that is distinct from the two surrounding hemispheres and the most lateral floculi-paraflocculi (Altman and Bayer, 1997). Furthermore, each region of the cerebellum receives distinct neural inputs. Most of the proprioceptive and sensory inputs project to the vermis, whereas the hemispheres are enriched with circuits arising from the cortex (Altman and Bayer, 1997; Purves et al., 2004; Sillitoe and Joyner, 2007). Moreover, in humans, compared with in mouse, the hemispheres are much larger than the vermis, possibly reflecting a greater cerebellum involvement in cortex-associated functions (Altman and Bayer, 1997; MacLeod et al., 2003). It is crucial to determine how the distinct morphology of each region is regulated during development.

Most mammals have a basic pattern of ten major lobules in the vermis (I-X anterior to posterior) and four in the hemispheres (Simplex, CrusI, CrusII and Paramedian). The foliation pattern rapidly transitions from the vermis to the hemispheres in the paravermis. The extent to which the major lobules are further subdivided by fissures varies between species (Larsell, 1970) and to a small degree between mouse strains (Inouye and Oda, 1980). There is a degree of continuity between the hemispheres and the vermis, as two of the vermis lobules (VI and VII) are continuous with the four lobules of the hemispheres. However, the morphology of the lobules is distinct in each ML region, and so is the degree of subdivision of the lobules. The genetic pathways that regulate the formation of distinct lobules in the vermis and the hemispheres are not known.

The Cb cortex consists of a dense layer of granule cells, an overlying monolayer of Purkinje cells and Bergmann glia, and an outer cell-sparse molecular layer (Goldowitz and Hamre, 1998). Although the Purkinje cells are derived from the ventricular zone of dorsal rhombomere 1 by embryonic day (E) 13.5 in mouse, the granule cells are generated from E18.5 to postnatal day (P) 16.5 by a progenitor layer covering the surface of the Cb (the external granule layer, EGL). Foliation occurs simultaneously with, and is dependent on, granule cell production (Lauder, 1974). At the base of each fissure, Purkinje cells and Bergmann glial fibers have a distinct cellular arrangement that could allow them to act as

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‘anchoring centers’, so that proliferation of granule cells between anchoring centers would result in outward growth of lobules (Sudarov and Joyner, 2007). An important question is what genes regulate the formation of anchoring centers in the appropriate spatial and temporal manner to produce a normal foliation pattern. Of the factors known to be required for Cb development, fibroblast growth factor 8 (Fg8/Cre) is expressed from E8.5 to E12.5 by the isthmus organizer, and is required to specify the Cb primordium (Chi et al., 2003). Sonic hedgehog (Shh) secreted by the Purkinje cells after E16.5 is required to maintain granule cell precursor proliferation (Corrales, 2006; Lewis, 2004). By contrast, there is evidence that the two engrailed (En) homeobox transcription factors (Joyner and Martin, 1987) regulate the pattern of at least some lobules. In En2 mutants, vermis lobule VIII is abnormally shifted posterior and only three lobules form in the hemispheres (Joyner et al., 1991; Millen et al., 1994). By contrast, En1 null mutants on most genetic backgrounds are perinatal lethal and lack the cerebellum (Wurst et al., 1995). Furthermore, En1 null mutants on most genetic backgrounds are perinatal lethal and lack the cerebellum (Wurst et al., 1994; Bilovocky et al., 2003). However, when En1 is conditionally ablated at ~E9, the Cb forms and some mutants (En1lox/Cre) have normal foliation (Sgai et al., 2007). These results raised the question of whether En1 is required for the initial specification of the Cb primordium and En2 is required subsequently for foliation. An alternative is that in En1lox/Cre mutants En2 compensates for the lack of En1 in foliation, as En2 can replace En1 in specification of the Cb (Hanks et al., 1995). Furthermore, lobules I-V and VIII are greatly reduced in size in En1lox/–; En2lox/– mutants (Sgai et al., 2007). The extent to which the two En genes regulate foliation throughout the ML axis of the Cb is not clear because of the early requirement for En1 in specification of the Cb.

We produced a temporal series of En2 and En1/En2 conditional mutant mice and discovered that the two genes act together to preferentially promote formation of lobules that are specific to the hemispheres and the vermis. Furthermore, En1/En2 are required even after Fg8 expression has ended for Cb foliation to be patterned normally. En1/En2 regulate both the position and the timing of formation of fissures in the vermis. Also, regional differences in the thickness of the EGL along the anteroposterior (AP) axis are altered in unison with changes in AP spatially restricted gene expression patterns. Thus, En1/En2 are high in the genetic hierarchy that regulates the morphology of the entire Cb.

MATERIALS AND METHODS
Generation of mouse strains
All animal studies were performed under an approved IACUC animal protocol according to the institutional guidelines at the Memorial Sloan-Kettering Cancer Center (MSKCC). Gene targeting in ES cells (Matise et al., 2000) was used to generate mice containing En1lox, En2lox, En2GFPloxIRES and Rosa26CreERT2 alleles (see Fig. S1 in the supplementary material). At least two targeted ES clones were identified for all the alleles. Southern blot analysis (data not shown) and injected into C57BL/6J blastocysts. Some chimeras were bred with Tc-Cre (Bai et al., 2002) or hACTB-FlpE deleter mice (Rodriguez et al., 2000) to remove neo or to create a deleted allele. Mice were maintained on an outbred Swiss Webster background. The En2lox allele functions as wild type (WT), as En2lox mice are normal (Fig. 2C,D), and the deletion allele (En2lox) is null, as En2lox alleles and En2lox mice display an En2– phenotype (Joyner and Martin, 1991). Phenotype (data not shown; see Fig. 2E,F). En2lox/–; En2lox/–; En2lox/– mice appear normal (data not shown). The En2GFPloxIRES allele was found to express GFP in the same pattern as En2 (data not shown) and to have a normal Cb when homozygous. The En2GFPloxIRES allele is hypomorphic, as En1lox/–; En2GFPloxIRES mice have a more extreme phenotype than do En1lox/–; En2lox mice (see Fig. 3).

Tamoxifen administration, and genotyping
Noon on the day a vaginal plug was detected was designated as E0.5. Tamoxifen (TM) was administered by oral gavage to pregnant females at 17.00 hours on E9.5 or E10.5 (3.5-5 mg/40 g of body weight), or twice at 17.00 hours on E11.5 and E12.5 or E13.5 and E14.5 (4 mg + 3 mg). Genotyping was carried out using published PCR protocols (Joyner et al., 1991; Soriano, 1999; Sudarov and Joyner, 2007) or with modifications (see Fig. S1 in the supplementary material).

Histology, RNA in situ hybridization, β-galactosidase and immunohistochemistry analysis
Standard methods in situ hybridization were used, and detailed protocols are available at http://www.mskcc.org/mskcc/html/57282.cfm. In general for histological analysis, 12-µm serial cryostat sections were obtained and analyzed at 96-µm intervals. The Gli1 (Mileen et al., 1995), Fgf8 (Crossley and Martin, 1995), Rnx (Shirasawa et al., 2000) and Onx2 (gift from J. Rossant, Hospital for Sick Children, Toronto) antisense RNA probes were as described previously. Bright-field images were collected using MagnaFire or Volocity software. Fluorescent images were obtained with Openlab 3.5 or Volocity.

The primary antibodies used were: rabbit anti-Enh (Davis et al., 1991), mouse anti-BrdU (1:500; Becton Dickinson) and rabbit anti-Pax6 (1:1000, Chemicon). Secondary antibodies from Molecular Probes (donkey anti-rabbit, mouse IgG-Alexa488, donkey anti-rabbit IgG-Alexa555) were diluted 1:1000.

Quantification
To assay proliferation, pregnant females were administered 100 µg BrdU/g body weight 60 minutes prior to sacrifice. The percentage of BrdU-positive cells at E18.5 and P2 was calculated as the percentage of Pax6-positive cells that were also BrdU positive within the EGL below a 100 µm length (ImageJ software, NIH, was used to trace the length) of the outer surface at the top of lobules IV-V, or top of lobule VI. For E18.5 embryos, the three most medial sections (12 µm thick) and, for P2 animals, the six most medial sections were quantified from three brains each. The thickness of the EGL was calculated as the number of cell layers at the top of lobules IV-V and VI. A Student’s t-test was performed for statistical analyses.

Magnetic resonance microimaging
Magnetic resonance microimaging (micro-MRI) was performed on a 7T micro-MRI (Bruker Biospec) with 750 mT/m actively shielded gradients (BGA09S, Bruker) using a 25-mm (i.d.) quadrature Litz coil (Doty Scientific) and a protocol modified from Johnson et al. (Johnson et al., 2002) (see movie legends in the supplementary material for details).

RESULTS
Strategy for time-specific activation of En2 or inactivation of En1/En2 function
As an approach to determine when the pattern of foliation is determined, an En2 conditional knock-out (CKO) allele (En2CKO) was generated with loxP sites surrounding the homeodomain encoding region (see Fig. S1A in the supplementary material; Fig. 2A), as well as a hypomorphic conditional allele that expresses GFP and En2 before but only GFP after loxP-mediated deletion of exon 1 (En2GFPloxIRES) (see Fig. S1B in the supplementary material). As a complement to temporal ablation, we generated an allele (En2lox) in which En2 function is silent until activated with Cre by inserting floxed Tau-lacZ sequences into the 5’ UTR of En2 (see Fig. S1C in the supplementary material). We also generated a new R26CreERT2 (Zambrowicz et al., 1997) knock-in allele (Rosa26CreERT2) that expresses the T2 form of CreER (Indra et al., 1999) with neo removed to ensure strong expression (see Fig. S1D in the supplementary material).

The recombination efficiency of our R26CreERT2 allele was characterized using the R26R lacZ reporter allele (Soriano, 1999). X-galactosidase (X-gal) staining of sagittal sections of R26CreERT2R embryos showed expression in most cells of the Cb when TM was
administered at E9.5 (Fig. 1A,B) or E10.5 (Fig. 1C,D), in many cells at E11.5 and E12.5 (Fig. 1E,F), and in a few cells at E13.5 and E14.5 (Fig. 1G,H). In adult brains of R26CreERT2^{-/-} embryos (TM on E10.5), β-gal activity was detected in all the major Cb cell types (Fig. 1K,L; data not shown). Efficient recombination was also detected in all other regions of the brain (data not shown). Importantly, no or only rare β-gal-expressing cells were detected in brains in the absence of TM (Fig. 1I,J).

To further assess recombination efficiency, En2 protein expression was examined in the adult Cb of En2 conditional mutants, as En2 is expressed in most granule cells and in the interneurons within the molecular layer where En1 is largely absent (Davis et al., 1988; Millen et al., 1995) (see also Fig. 2E). In En2^{lox/-} / R26CreERT2^{+/-} mutants, quantitative analysis of recombination efficiency in interneurons of mutants with an En2 null phenotype (n=2 for TM at E9.5, n=2 for TM at E10.5, n=2 for TM at E11.5 and E12.5) showed that compared with control mice, only ~10-30% of the interneurons expressed En2 in mutants (see Table S1 in the supplementary material) (compare Fig. 2E,H with 2K,N). Similarly, in En2^{loxlz/-} / Rosa26CreERT2^{+/-} mutants treated with TM on E10.5, or on E11.5 and E12.5, recombination efficiency was high (up to 88%) (Fig. 2Q; data not shown), indicating a broad restoration of En2

Fig. 1. The Rosa26CreERT2 allele produces efficient recombination in the embryonic brain until late gestation. (A-L) X-galactosidase staining of Cb sagittal sections of Rosa26CreERT2^{-/-} embryos (A-L) or adults (K,L) treated with tamoxifen (TM) at the times indicated. Panels on right are higher powered images of the areas outlined in the left panels. Anterior is to the left. Scale bars: 100 μm in A,C,E,G,J; 25 μm in B,D,F,H; 400 μm in K; 50 in μm L.

Fig. 2. En2 is required after ~E12 to regulate the Cb foliation pattern. (A,B) Schematics (not drawn to scale) of conditional En2lox/- (A) or En2^{loxlz/-} conditional (B) alleles and the corresponding experimental time-course used to ablate or activate En2, respectively. Green rectangles and red lines represent the periods when En2 was expressed or ablated, respectively; arrows indicate the time of TM administration. Hematoxylin and Eosin (H&E)-stained sagittal sections are shown of the vermis (C,F,J,O) and hemispheres (D,G,I,M,P) of the mutants indicated. Inactivation of En2 up to ~E12 produces an En2 null mutant phenotype, whereas activation at ~E12 rescues the foliation defects. (E,H,K,N,Q) Images of immunostaining for En2 protein in the boxed vermis regions. Defective folia are outlined by dotted lines. S, Simplex; CI, CrusI; CII, CrusII; Pm, Paramedian. Scale bar in E: 200 μm for C,D,F,G,I,J,L,M,O,P; 25 μm for E,H,K,N,Q.
expression. To further assess the timing of functional ablation, we performed western blot analysis and analyzed the initiation of molecular and morphological phenotypes. Analysis of En2 protein in brain extracts from En2lox/–;R26CreERT2/+ compared with En2+/+ and En2–/– control embryos showed that a decrease in protein was apparent at 36 hours (n=2) post-TM treatment at E10.5, and at 72 hours En2 was barely detectable in mutants (see Fig. S2 in the supplementary material). Furthermore, the Cb primordium was reduced in size and Fgf8 expression decreased by 48 hours post-TM treatment at E10.5 in En1/En2 double CKOs (see below). We conclude that functional ablation of En1/En2 occurs around 36 hours after TM treatment and thus designate gene ablation as ~36 hours post-TM.

**En2 is required after ~E12.5 to regulate patterning of folia**

We first addressed whether En2 is required for foliation during the time the Cb primordium is being generated (prior to E13.5). Unlike the wild-type foliation pattern (Fig. 2C,D) seen in some En1lox/Cre mutants, all En2lox/–;Rosa26CreERT2/+ CKO embryos administered TM on E9.5 (En2-E9.5 CKO, n=3) had a phenotype similar to that of En2 null mutants (Fig. 2F,G,I,J). Furthermore, when TM was administered later than E9.5, most of the En2 CKO mutants (2/3 for TM at E10.5; 3/4 for TM at E11.5 and E12.5) displayed a foliation pattern similar to that of En2 null mutants (Fig. 2L,M ; data not shown). Thus, En2 is required for Cb foliation after ~E13.

We next activated En2 function at different developmental time points using our En2 loxlz allele to investigate whether En2 is dispensable prior to ~E13. Indeed, the adult hemisphere Cb foliation pattern of all Rosa26CreERT2/+;En2loxlz/loxlz mice (9/9) and the vermis of the majority of mice (2/3 for TM at E9.5, 3/4 for TM at E10.5, 1/2 for TM at E11.5 and E12.5) was rescued compared with in En2loxlz/loxlz mice and resembled that of the wild type (Fig. 2O,P). Thus, En2 is required for Cb foliation after ~E13.

**En1 and En2 act together after E11 to produce diversity in foliation in the vermis and hemispheres**

Although En1 is not required after E9 for Cb foliation in the presence of two normal En2 alleles, it is possible that En1 and En2 act together after this time point to regulate foliation, and that patterning of the entire Cb is dependent on both genes. We therefore analyzed En1/En2 double CKOs on a double heterozygous background in which only some animals have a mild anterior foliation defect (Sgaier et al., 2007). Strikingly, sagittal sections (n=4) or micro-MRI imaging (n=1) of the Cb of the En1/2-E10.5 CKO mutants that survived past P13 revealed that neither the vermis nor the hemisphere foliation patterns resembled those of controls (Rosa26CreERT2/+;En1loxlz/loxlz;En2loxlz/loxlz mice treated or not treated with TM; see Fig. 3A,B, Fig. 4; data not shown) or En2–/– mice. En1/2-E9.5 CKOs died at birth and had a major deletion of the midbrain and medial Cb (data not shown), which is likely to be due to the early requirement for En1/En2 in specification of the Cb primordium.

We took two approaches to determine the nature of the lobules remaining in the mutants. Photographs of a sagittal series of sections were examined for each mutant (see Fig. S3 in the supplementary material), and 3D reconstructed micro-MRI images of a P14 En1/2-

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**Fig. 3. Inactivation of both En1 and En2 preferentially affects the lobules specific to the vermis and the hemispheres** (A-J) H&E-stained sagittal sections of adult Cb from the mutants indicated. (K) Schematics of idealized surface renderings of foliation of a flattened Cb based on sections and micro-MRI of all mutants. (L) A series of lateral to sagittal sections of adult wild-type (WT) Cb are shown with shading of lobules I-V (yellow), VI-VIIa (green), VI-IX (red) and X (blue). The earlier En1/En2 expression is removed, lobule I-V and VIII in the vermis are reduced in size, remnants of lobules I-V and VIII/IX are extended more laterally than normal, lobules VI/VII in the vermis are relatively larger than normal, and CII and Pm in the hemispheres are fused. Scale bar: 200 µm.
E10.5 CKO and P21 Rosa26$^{CreER}{\text{T2/2}}$;En1$^{lox/-}$;En2$^{lox/-}$ mutant and littermate controls were analyzed (Fig. 4; see also Movies 1-4 in the supplementary material). In wild-type mice, the vermis has nine or ten lobules depending on the strain, with lobules I and II being fused in most strains, including in the outbred background used in these studies. Lobules I-V, referred to as the anterior vermis, do not extend into the hemispheres (yellow, Fig. 3L). The central vermis consists of lobules VI and VII, which are continuous with the hemisphere lobules. In many strains, lobule VI is divided by a shallow fissure into two sublobules called VIA and VIB (green, Fig. 3L). Sublobe VIA is continuous with lobule Simplex in the hemispheres, lobule VIB with CrusI, and lobule VII with CrusII and Paramedian. Lobule VIII in the posterior vermis diminishes in size as it extends laterally (called the copula pyramidis in the hemispheres), and lobules IX and X do not extend into the hemispheres (blue, Fig. 3L).

Based on the examination of a series of histological sections and micro-MRI images of the Cb, only the most posterior lobule of En1/2-E10.5 CKO mutants had a normal appearance; this was therefore designated as lobule X. The three vermis lobules anterior to lobule X had a very abnormal morphology for this region of the vermis (red in Fig. S3 and Movie 2 in the supplementary material). We designated these lobules as VII, VIII and IX, as lobule VII was continuous with CrusII/Paramedian in the hemispheres. Lobule VIII in the posterior vermis extended more laterally than normal, as did lobules I-V, VI-VII, VIII-IX and X, which were designated as lobule X. The three vermis lobules anterior to lobule X do not extend into the hemispheres (blue, Fig. 3L).

One possible contributing factor to the reduction in size of anterior lobules in En1/2-E10.5 CKO mutants was the reduced Fgf8 expression, as mutants with reduced Fgf8 signaling have fewer anterior lobules (Xu et al., 2000; Basoon et al., 2008; Sato et al., 2009) and which can convert the hindbrain into a midbrain (Broccoli et al., 1999), was not expanded posterior in the dorsal axis. Volumetric micro-MRI data was used to generate dorsal and oblique ventral (3D) views of surface rendered images, as well as coronal sections (2D) of the cerebellum at P14 (A) and P21 (B). Coloring is as in Fig. 3. Arrowheads indicate approximate morphological borders between vermis and hemispheres (dorsal views); asterisks indicate lateral extension of posterior lobules in mutants (coronal sections). All mice analyzed with micro-MRI were treated with TM at E10.5.

En1/En2 are required for robust expression of Fgf8 in the isthmic organizer after E11

One possible contributing factor to the reduction in size of anterior lobules in En1/2-E10.5 CKO mutants is the reduced Fgf8 expression, as mutants with reduced Fgf8 signaling have fewer anterior lobules (Xu et al., 2000; Basoon et al., 2008; Sato et al., 2009) and which can convert the hindbrain into a midbrain (Broccoli et al., 1999), was not expanded posterior in the dorsal axis.
midline (Fig. 5E,F). Thus, En1/En2 are required to maintain full expression of Fgf8 after ~E11, but the Cb primordium is not converted into a midbrain phenotype in En1/En2 CKOs.

**En1 and En2 regulate vermis foliation after Fgf8 expression ends**

As an approach to determine whether the decrease in Fgf8 expression alone accounts for the reduced size of lobules I-V, and to further explore when En1/En2 are required for patterning foliation, we reduced the activity of En1/En2 after ~E11. In adult En1/2-E13.5+E14.5 CKO mutants (n=2), the vermis had a foliation pattern that was a milder version than that observed in the En1/2-E10.5 CKO mutants (compare Fig. 3E with 3L3; see also Fig. S2 in the supplementary material). In the anterior region, lobules IV/V were present and lobules I-III were fused into one lobule, and in the posterior region lobule VIII was shifted posterior and fused with dorsal lobule IX. The hemisphere foliation pattern appeared similar to that of wild type except that lobules I-V and VIII/IX of the vermis were present more laterally than normal (Fig. 3F; Fig. S2 in the supplementary material).

To further test the requirement for En1/En2 in regulating foliation after ~E15, we took advantage of our hypomorphic En2GFPloxIRES allele to provide a genetic background further depleted for En1/En2. In the vermis of En1lox–;En2GFPloxIRES control mice, only one or two major anterior lobules were present, and lobule VIII was very small and fused with dorsal lobule IX (Fig. 3G). In the hemispheres, CrusII and Paramedian were only partially separated (Fig. 3H). As expected, En1/2GFP-E13.5+E14.5 mutants (Rosa26CreERT2/+;En1lox–;En2GFPloxIRES treated with TM) had a more extreme phenotype in both the vermis and the hemispheres (Fig. 3L). In summary, En1/En2 are required independently of Fgf8 isthmic organizer activity and after at least ~E15 to promote the distinct foliation patterns in the vermis and the hemispheres.

**En1/En2 regulate the order in which fissures form**

We recently demonstrated that lobule VIII is smaller and shifted posteriorly in En2 mutants, because the fissure separating lobule VIII from lobule IX (secondary, se) forms later than normal, producing a shallower fissure, whereas the fissure between lobules VIII and VII (prepyramidal, ppy) forms earlier than normal creating a deeper fissure (Sudarov and Joyner, 2007). This is in contrast to mutants in which there is a general decrease in granule cell proliferation or an increase in cell death, as the formation of fissures is delayed but the order in which they form is normal and in extreme mutants the last fissures do not form (e.g. Corrales et al., 2006). We therefore tested whether the timing of fissure formation in the vermis of En1/En2 temporal CKO mutants correlated with the altered foliation pattern in adults.

In our control mice, each fissure in the vermis formed in a reproducible temporal series, with the preculminate (pcu), primary (pr) and se fissures forming almost simultaneously at E17.5 to divide the cerebellum into lobules I-III, IV/V and VI-X, followed by formation of the most posterior fissure (posterolateral, po), which separates lobule X from IX, by E18.5 (Fig. 6A). The ppy fissure then formed by P1 to separate VIII from VI/VII, followed by the precentral (pct) fissure separating lobules I/II from III by P2, the

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**Table 1. Comparison of regional volumes of the cerebellum when TM is administered at E10.5**

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**Fig. 5. En1/En2 are required for robust expression of Fgf8 in the isthmic organizer after ~E11.** (A-D) Medial (A,B) and slightly lateral (C,D) sagittal sections showing that Fgf8 RNA expression is reduced in the dorsal medial mid/hindbrain junction at E12.5 in En1/2-E10.5 CKOs compared with in controls. (E,F) Expression of Otx2 protein does not extend into the hindbrain in mutants. Cb primordium is outlined with a white dotted line. Scale bar: 25 μm.
En regulates regional diversity of foliation

intercral (itc) fissure (dividing lobules VIb/VII) and then, finally, the superior posterior (sp) fissure (dividing lobules VIa/VIb; see Fig. 6D,G, Fig. 7C). By contrast, the fissures in the hemispheres that are continuous with sp and itc in the vermis form nearly simultaneously by P1 (Fig. 6A,D”), and the hemisphere-specific ansoparamedial (ans) fissure separating lobule CrusII from Paramedian forms much later (~P5) (Millen et al., 1994). In the paravermis, the itc, ppy and se fissures form by P1 (after the pr, pcu and po fissures; Fig. 6J).

Like mutants with a general defect in the production of granule cells, we found that En1/En2 temporal CKO mutants had a smaller Cb and a delay in fissure formation, and that the delay was progressively longer as the phenotype worsened (Fig. 6). However, unlike mutants with a general granule cell defect, the order of fissure initiation was altered in En1/En2 temporal CKOs. We concentrated on analyzing the vermis, as it was not possible to determine the position of the primordia of the paravermis and hemispheres in mutants. To identify the verrm fissures, and thus the intervening lobules, in En1/En2-E10.5 CKOs, we analyzed the expression of markers with restricted expression domains along the AP axis of the developing vermis. Strong expression of Otx2 in the external granule layer (EGL) of control mice marks lobules IX and X (Frantz et al., 1994) (Fig. 7E,G). Thus, the anterior border of Otx2 expression demarcates the se fissure. Rnx (Shirasawa et al., 2000), by contrast, is expressed strongly in the EGL of lobules VII-IX (Fig. 5I,K), and thus demarcates the po fissure posteriorly and the itc fissure anteriorly. We found that the Otx2 and Rnx expression domains had similar relative positions in En1/En2-E10.5 CKO mutants at E18.5 and P2 (Fig. 7E-L), although the relative size of the domains was altered (see below). Based on the borders of Otx2 and Rnx expression in En1/En2-E10.5 CKOs, the two fissures that are present at P2 are the pr and the itc (Fig. 7L). The se and po fissures then form by P3 (Fig. 6H). However, interestingly, the pr fissure is deeper than the itc fissure at P3, consistent with the greater depth of the pr fissure in adult mutants. The order of fissure formation in the En1/2-E10.5 CKO vermis is therefore more similar to the lateral positions in the wild-type Cb, although formation of the ppy fissure is delayed (Fig. 6J,K). Thus, En1/En2 regulate the temporal sequence by which fissures form throughout the vermis, and the changes seen in En1/En2 mutants are likely to contribute to the altered adult foliation pattern.

En1/En2 regulate the position at which fissures form along the AP axis

To determine whether the change in the relative volumes of lobules seen in the adult vermis are present when foliation first initiates in the En1/2-E10.5 CKOs mutants, or whether the changes develop later, we measured the length of the outer surface of lobules I-V, VI, VII-IX and X at P2 and in the adult. In the P2 mutants, lobules I-V represented only 22% of the P2 midline Cb compared with 46% for the wild type (Fig. 7U), similar to lobules I-V encompassing 24% and 44% of the P14 mutant and control vermis, respectively (Fig. 7U). The length of the outer surface of lobule X in the mutants occupied a similar percentage of the Cb as in the wild type at P2 (~8%). In addition, lobule VI was much larger than normal in P2 En1/2-E10.5 CKOs (30.6% length in the mutant compared with 13.6% for the wild type), and lobules VI-IX were slightly larger in the mutants (Fig. 7U). Similar alterations in the proportion of the volume of the vermis occupied by each region were observed in the P14 En1/2-E10.5 CKOs (Fig. 7U). Thus, in En1/En2 mutants, although the relative positions of the domains of AP markers are preserved in the vermis, the relative sizes of the domains are changed, correlating with an alteration in the positioning of fissures along the AP axis. Therefore, En1/En2 regulate both the timing of fissure formation and their position along the AP axis.

Fig. 6. En1/En2 determine the timing of fissure formation. (A-I) H&E staining of vermis and hemisphere sagittal sections of the developing Cb in the mutants indicated compared with controls. (J,K) Outlines of a medial-to-lateral (left to right) series of sagittal sections of P1 wild type (J) and P3 En1/2-E10.5 CKOs (K) illustrate foliation pattern. Regions highlighted as in Fig. 3L. Scale bar in A: 100 μm for A-H,I; 25 μm for G,G’.
En1/En2 regulate regional gene expression and EGL thickness prior to and during foliation

We next examined the molecular and cellular basis of the changes in foliation at E18.5 and P2 in En1/2-E10.5 CKOs. Surprisingly, at E18.5 prior to the initiation of foliation in En1/2-E10.5 CKOs, Otx2 and Rnx were nevertheless expressed in regionally restricted domains, similar to at P2 (Fig. 7F,J). In the wild-type cerebellum at E18.5 and P2, the absence of the itc and ppy fissures in the region of lobules VI and VII is accompanied by a thinner EGL compared to in the region encompassing lobules I-V (Altman and Bayer, 1997; Corrales et al., 2004). We therefore investigated whether the delay in foliation in En1/2-E10.5 CKOs is associated with a general thinning of the EGL, and whether the relatively earlier foliation in the central region of the mutants compared to the anterior region is accompanied by a change in the relative thickness of the EGL in the two regions. The expression domains of Rnx and Gli1 (see below) were used as landmarks to identify the presumptive lobules I-V and VI in mutants. Whereas the wild-type EGL at E18.5 was much thicker at the top of lobules IV/V than lobule VI (top of lobule VI/VII contained ~30% less cells than the top of IV), in En1/2-E10.5 CKOs the EGL thickness was more similar in the two regions (the top of lobule VI contained ~15% less cells than the top of I-V; see Table 2). Curiously, the thickness of the EGL was 1.5-2 times greater in the mutants than in wild types at E18.5 (Table 2), although the overall size of each region, and thus total number of cells, was greatly reduced in the mutants. At P2 in the controls, the EGL at the top of lobule IV/V continued to be thinner than in lobule VI by ~25%, but it had nearly doubled in thickness in both regions (Table 2). Strikingly, in the En1/2-E10.5 CKOs at P2 the EGL thickness was similar to at E18.5 and thinner than in the controls. The difference in thickness of the mutant EGL at the top of lobule VI compared to at the top of lobules I-V was ~22%. Thus, the relative thickness of the EGL in the anterior and central regions of the Cb were more similar to each other in En1/2-E10.5 mutants than in wild type, and the overall thickness was greater in the mutants at E18.5 but thinner by P2 compared with in controls.

We next sought to determine whether the differences in EGL thickness are due to differences in the percentage of granule cells proliferating (see Fig. S4A-F in the supplementary material). The percentage of BrdU positive (proliferating) cells in the EGL (Pax6+) in the controls at E18.5 tended towards being slightly higher at the top of lobules IV/V (~21.0%) than at the top of lobule VI (~18.2%). By contrast, in the En1/2-E10.5 CKOs the opposite trend was seen (21.8% at the top of lobules I-V compared with 23.8% at the top of lobule VI; Fig. 7V). If the total number of cells in each region is taken into account, then in the wild type there were more proliferating cells per 100 μm of Cb surface in the anterior lobules than in the central lobules, and a more
similiar total number in the two regions of the mutants. At P2, the percentage of BrdU-positive EGL cells in wild-type and mutant mice was more similar in the two regions than at E18.5 (Fig. 7V).

Finally, since Shh stimulates granule cell proliferation after E17.5, we investigated whether Shh signaling was altered in En1/2-E10.5 CKOs \( (n=3) \) compared with in wild types \( (n=3) \) by analyzing expression of the direct target gene Gli1 (Bai et al., 2002). Interestingly, as in wild types, Gli1 expression was detected in En1/2-E10.5 CKOs at E18.5 and there was a slight reduction in the level of Gli1 in the central region, between the pr and sec fissures present in the wild type (Fig. 7M,N). Interestingly, consistent with the change in the relative sizes of the anterior and central regions in mutants, the region with lower expression in mutants comprised a larger percentage of the total length of the surface of the Cb compared with controls (9.3% in controls compared with 26.3% in mutants). At P2, Gli1 expression in the EGL was more even along the AP axis in both the controls and mutants, with a slight decrease anterior to the itc fissure. Thus, a reduction in Fgf8 and Fgf17 expression in the adult Cb (Sillitoe and Joyner, 2008).

Table 3. Summary of the times during development when conditional ablation of En1 and/or En2 results in foliation defects

<table>
<thead>
<tr>
<th>Gene ablated</th>
<th>Time gene function is ablated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>En1</td>
<td>E9</td>
</tr>
<tr>
<td>En2</td>
<td>Yes</td>
</tr>
<tr>
<td>En1+En2</td>
<td>nd</td>
</tr>
</tbody>
</table>

*For alleles produced using Cre, time is given as hours after expression of Cre; for alleles produced using CreER, time is given as hours after treatment with TM (first dose when TM given twice). N/A, not applicable, as mutants die at birth. nd, not determined.
later role in promoting the regional diversity of Cb morphology. Interestingly, the *Drosophila engrailed* gene is required to both posteriorize the wing disc and promote growth in the posterior compartment (Guillen et al., 1995; Hidalgo, 1994).

**En1/En2 regulate regional differences in gene expression and EGL thickness, as well as timing and positioning of fissures**

The question arises as to how En1/En2 regulate the pattern of foliation throughout the Cb. We found that when both En genes were conditionally ablated the stereotyped sequence of fissure formation was changed, and furthermore some fissures did not form at all, while others formed at inappropriate positions in the AP and ML axes (Fig. 8). The two most anterior fissures in the vermis formed much later than normal relative to other fissures, or did not form at all, whereas the itc fissure between lobules VI and VII in the central region formed relatively earlier. These changes in timing of fissure formation could well account for the more prominent lobules in the central region relative to the anterior-most lobules.

Significantly, in En1/2-E10.5 CKO mutants at P2 when fissures begin to form and at P14 when foliation is nearly complete (and in the adult), lobule VI in the central region occupied a greater relative proportion of the vermis than normal and the anterior region (lobules I-V) a smaller relative proportion. Thus, En1/En2 are involved in defining the position along the AP axis where lobules will form and the relative sizes of lobules from the onset of foliation. Moreover, three days before foliation begins in En1/2-E10.5 mutants, the expression domains of genes that normally mark particular lobules of the vermis (AP domains) were altered in anticipation of the subsequent changes in patterning of the foliation. Interestingly, *Gli1*, which is induced in proliferating granule cells by Shh signaling from Purkinje cells, was among the genes with an altered expression pattern. Furthermore, despite the delay in foliation in mutants, the onset of Shh signaling was not delayed. Moreover, the molecular changes in granule cell precursors were accompanied by significant regional changes in the thickness of the EGL. Correlating with the increase in the relative size of the central region in En1/2-E10.5 mutants, the normal thinning of the EGL in the central region was attenuated. The trend towards an increase in the number of proliferating cells in the anterior EGL of normal embryos also was less pronounced in En1/2-E10.5 CKOs. Thus, the En1/En2 genes appear to produce the distinct foliation patterns in the vermis and hemispheres by regulating spatially restricted gene expression, which leads to regional differences in the cellular processes that govern the expansion of the EGL, as well as the positioning and timing of fissure formation.

**En1 and En2 represent a new class of genes required to generate distinct foliation patterns in the vermis and hemispheres**

Our study is the first to identify a gene family involved in generating the two distinct foliation patterns in the vermis and hemispheres. As En function was reduced both in dose and length of activity, the Cb progressively took on a foliation pattern with less regional diversity. The vermis phenotype did not simply result from a deletion of some of the tissue that would normally form the vermis, but instead involved a change in the relative proportions of different AP regions of the Cb, such that the anterior region was diminished and central region expanded relative to the overall size of the vermis. In addition, the three hemisphere lobules that remained in mutants did not maintain their typical morphology, and remnants of the vermis-specific anterior and posterior lobules were present more laterally than normal. It is possible that the lateral extension of vermis lobules into the hemispheres in En1/En2 CKOs resulted from an early change in cell fate, as in En1/+;En2−/− mutants it was found, using fate mapping, that the cells at E12.5 that normally give rise to the vermis contribute to the hemispheres as well as to the vermis (Sgaier et al., 2007). While an increasing number of mutations cause a decrease in the complexity of Cb foliation, the mutants to date that do not have impaired differentiation of particular cell types appear to suffer primarily depletion of the vermis or to have a foliation pattern that resembles an early developmental stage when the overall pattern is simpler. For example, removal of the transcription factor Gbx2 after E9 (Li et al., 2002), or a frame shift mutation in *Wnt1* (Thomas et al., 1991), preferentially results in reduction of the vermis. Mutations in the *Fgf8* signaling pathway also affect only growth of the vermis, and preferentially the anterior vermis (Basson et al., 2008; Xu et al., 2000). In mutants such as *Meander tail*, the anterior vermis also is depleted, but as a result of abnormal differentiation (Ross et al., 1990). On the contrary, an allelic series of mutations in the *Shh* signaling pathway (Corrales et al., 2006), or loss of cyclin D1/D2 (Ciembrych et al., 2003), result in reduced granule cell proliferation and this leads to simplified vermis and hemisphere foliation patterns that resemble specific stages in early postnatal development. Progressive reduction in En1/En2 function, by contrast, moves Cb morphology towards a homogeneous foliation pattern throughout the ML axis. Thus, En1/En2 represent the first genes identified that are required to distinguish the vermis foliation pattern from that of the hemispheres. We hypothesize that En1/En2 were co-opted during evolution to produce the morphological differences between the vermis and the hemispheres, thus providing a basis for developing additional neural circuits involving the Cb in mammals.

**Fig. 8. Summary of the altered timing of vermis fissure formation in En1/2-E10.5 CKOs.** The order of fissure formation is color coded and indicated numerically above the arrowheads, and the times when the fissures form in the wild type (WT) and in En1/2-E9.5 CKOs are indicated in brackets.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at [http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.027045/-/DC1](http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.027045/-/DC1)
En regulates regional diversity of foliation

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