Development 134, 2325-2335 (2007) doi:10.1242/dev.000620

Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins

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The genetic pathways that partition the developing nervous system into functional systems are largely unknown. The engrailed (En) homeobox transcription factors are candidate regulators of this process in the dorsal midbrain (tectum) and anterior hindbrain (cerebellum). *En1* mutants lack most of the tectum and cerebellum and die at birth, whereas *En2* mutants are viable with a smaller cerebellum and foliation defects. Our previous studies indicated that the difference in phenotypes is due to the earlier expression of *En1* as compared with *En2*, rather than differences in protein function, since knock-in mice expressing *En2* in place of *En1* have a normal brain. Here, we uncovered a wider spectrum of functions for the En genes by generating a series of En mutant mice. First, using a conditional allele we demonstrate that *En1* is required for cerebellum development only before embryonic day 9, but plays a sustained role in forming the tectum. Second, by removing the endogenous *En2* gene in the background of *En1* knock-in alleles, we show that *Drosophila en* is not sufficient to sustain midbrain and cerebellum development in the absence of *En2*, whereas *En2* is more potent than *En1* in cerebellum development. Third, based on a differential sensitivity to the dose of *En1/2*, our studies reveal a genetic subdivision of the tectum into its two functional systems and the medial cerebellum into four regions that have distinct circuitry and molecular coding. Our study suggests that an 'engrailed code' is integral to partitioning the tectum and cerebellum into functional domains.

KEY WORDS: Cerebellum, Foliation, Tectum, Patterning, *En1*, *En2*, Mouse

INTRODUCTION

The genetic regulation of patterning processes that regulate the size, cellular differentiation and morphology of regions of the developing nervous system is fundamental to establishing functional circuits that control different behaviors, emotions and basic bodily functions. The embryonic brain region that gives rise to the midbrain [mesencephalon (mes)] and anterior hindbrain [rhombomere (r1)] is an ideal model system for studying these genetic pathways in vertebrates. Anterior-posterior (A-P) patterning of the midbrain (Mb) and anterior hindbrain is orchestrated by an organizing center in the isthmus located between the mes and r1. Fgf8 is the key isthmic organizer molecule that acts between embryonic day (E) 8.5 and 13 to regulate the expression of genes that direct Mb and r1 development (Wurst and Bally-Cuif, 2001; Zervas et al., 2005). Expression of the engrailed transcription factors (En1 and En2) before E13 is regulated by Fgf8, and En1/2 are crucial for mes/r1 development. It has been challenging to determine the full spectrum of functions of each En gene in mouse because there is an early loss of the mes/r1 in *En1* mutants, and the two genes have overlapping functions. It is also unclear whether the two En proteins have equivalent functions in brain development.

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Accepted 11 April 2007

Following specification of the mes/r1 region during neural tube closure, the mouse tectum and cerebellum (Cb) develop from the dorsal mes and r1, respectively (Zervas et al., 2005). The tectum of the Mb forms as a layered structure that is divided morphologically and functionally into the anterior superior colliculus and posterior inferior colliculus that process visual and auditory information, respectively. Although expansion of the tectum along the A-P axis is tightly linked to the level of isthmic organizer signaling, the molecular basis of differential allocation of the inferior and superior colliculi is not understood. The Cb is the center for motor control. Differentiated cells of the mouse Cb begin to be generated at E10.5 and form a multi-laminar structure consisting of the deep nuclei surrounded by a dense layer of granule cells, a monolayer of Purkinje cells and an outer molecular layer. The granule cell precursors form a proliferative external granule layer at E13.5 and then migrate past the Purkinje cell layer to form the inner granule layer (IGL) from birth until postnatal day (P) 14. Beginning at E17.5, fissures form in a stereotyped manner and generate a highly foliated Cb. In terms of how early A-P patterning could influence the final structure of the Cb, it is important to note that a morphogenetic rotation of dorsal r1 transforms the A-P axis of r1 into the medial-lateral (M-L) axis of the Cb primordium by E12.5 (Sgaier et al., 2005) (see Fig. 8). Globally, the adult Cb is subdivided into a medial vermis and two lateral hemispheres, with the vermis divided along the A-P axis by 8-10 folia in different inbred mouse strains (referred to as I-X) and the hemispheres divided by 6 folia (Larsell, 1952). Preservation of the general pattern of folia across mammals suggests that there is an evolutionarily conserved genetic program that patterns folia of the Cb (Altman and Bayer, 1997; Herrup and Kuemerle, 1997).

The mouse *En1* and *En2* genes provide a unique tool for gaining access to the genetic regulation of Cb and tectum patterning. The dynamic expression patterns of the *En* genes (see Fig. 1) and their

mutant phenotypes reflect each successive stage of Cb and tectum development (Joyner, 1996). *En1* is first expressed in the mes/r1 at E8.5, ~12 hours before *En2*, and is later expressed in the absence of *En2* in a number of other tissues. *En1*-null mutant mice die at birth and have an almost complete deletion of the Mb and Cb owing to tissue loss by E9.5 (Wurst et al., 1994), which is caused, at least in part, by cell death (Chi et al., 2003). Thus, *En1* is required for the initial establishment of the mes/r1 region. By contrast, *En2*-null mutants have a mild phenotype – they are viable and have defects limited to growth of the Cb and patterning of particular folia (Joyner et al., 1991; Millen et al., 1994). An overlap in *En* gene function has been demonstrated by the complete absence of the tectum and Cb in *En1;En2* double mutants (Liu and Joyner, 2001; Simon et al., 2004), and a rescue of the *En1* mutant brain phenotype when *En1* is replaced with *En2* using gene targeting (Hanks et al., 1995). Surprisingly, we found that *Drosophila en* also can rescue the *En1* mutant brain defects in knock-in mouse mutants, although *en* cannot rescue other defects (Hanks et al., 1998). An important question is whether *En1* has any later roles in tectum and Cb patterning, as has been suggested by the Cb phenotype of *En1*-null mutants that survive on a C57BL/6 genetic background (Bilovocky et al., 2003), and the degree to which such functions overlap with *En2*.

In order to study the temporal requirement for *En1* in mes/r1 development, we generated a conditional mutant allele of *En1*. We find that if *En1* is removed at ~E9, only the posterior tectum is depleted, and two copies of *En2* are required to sustain Cb development in these conditional *En1* mutants. We next compared the function of *Drosophila* and mouse En proteins in the mouse brain using a sensitive genetic assay. We provide evidence that En2 is more potent at supporting Cb development than En1, and demonstrate that *Drosophila* En cannot rescue the *En1* mutant brain defects in the absence of endogenous En2. Curiously, our analysis of knock-in mutants and *En1/2* double-null mutants uncovered that both genes are preferentially required in particular functional domains of the tectum and cerebellum. We propose an 'En code' that divides the tectum and Cb into functional systems based on the dose of En required for the development of each domain.

MATERIALS AND METHODS

Generation of *En1^{flox}* knock-in mice and conditional ablation of *En1* in r1

The *En1^{flox}* targeting construct was produced by subcloning a 6.0 kb 5′ *Bam*HI *En1* sequence into the *Bam*HI site of pPNT*frt-Neo-frt-loxP* to generate pPNT*frt-Neo-frt-loxP*+*En1*-5′ arm. A 2.7 kb *Bam*HI-*Xba*I fragment of *En1* including most of exon 2 was subcloned into the *Xba*I site of the pGEM-11ZF vector to generate pGEM-11ZF+*En1*-3′ arm. A *loxP* sequence was inserted into the *Kpn*I site within the 3′ UTR of *En1* and the *En1-loxP*-3′arm was released by *Xba*I and *Sal*I digestion and subcloned into the *Hin*dIII site of pPNT*frt-Neo-frt-loxP*+*En1-5′* arm to generate the final targeting vector.

The *En1^{flox}* targeting construct was linearized with *Sal*I and electroporated into W4 embryonic stem (ES) cells (Auerbach et al., 2000) as described previously (Matise et al., 2000). Clones were screened by Southern blot analysis using 5′ external and 3′ internal probes to identify targeted clones (see Fig. S1 in the supplementary material). One positive clone was obtained and injected into C57BL/6 blastocysts to generate ES cell chimeric mice (Papaioannou and Johnson, 2000). Chimeric mice were mated with Black Swiss mice to generate *En1$^{flox\text{-}neo/+}$* mice. The *neo* cassette was removed by mating *En1$^{flox\text{-}neo/+}$* mice with *hACTB-Flpe* mice (Rodriguez et al., 2000), which expresses Flpe broadly under the control of the human β-actin promoter. The wild-type (337 bp) and *En1^{flox}* (380 bp) alleles were detected by PCR with the following primers: *En1*flox1A, 5′-GCCAAACTGCTT-ACGACCG-3′; *En1*flox1B, 5′-TGGGTGGGTAGAGAAGAGGC-3′.

Mes/r1-specific *En1* conditional mutant mice (*En1$^{flox/Cre}$*) were generated by crossing *En1$^{flox/+}$* mice with *En1$^{Cre/+}$* mice. *En1$^{Cre/Cre}$* and *En1$^{flox/Cre}$* mice were generated within the same litter by crossing *En1$^{flox/Cre}$* mice with *En1$^{Cre/+}$* mice. *En1$^{flox/+}$;En2$^{-/+}$* were bred to *En2$^{-/+}$* mice to generate *En1$^{flox/+}$;En2$^{-/-}$* and *En1$^{Cre/+}$;En2$^{-/+}$* were bred to *En1$^{flox/+}$;En2$^{-/-}$* mice to generate *En1$^{flox/Cre}$;En2$^{-/+}$* and *En1$^{flox/Cre}$;En2$^{-/-}$* mice. To eliminate the possibility that the observed mild phenotype in *En1$^{flox/Cre}$* mice was due to a genetic background effect, we generated *En1$^{Cre/Cre}$*-null mutants in the same litters as *En1$^{flox/Cre}$* mice by mating *En1$^{flox/Cre}$* mice to *En1$^{Cre/+}$* mice. We screened a total of 39 mice from seven separate litters in which the expected frequency of all genotypes is 25%. Whereas nine *En1$^{flox/Cre}$* mice were found, only one *En1$^{Cre/Cre}$* mouse that survived to adulthood was observed (2.6%).

Breeding and genotyping of *En1/En2* double mutants

En2^{ntd} (Millen et al., 1994), *En2$^{tau\text{-}lacZ}$* (Sgaier et al., 2005), *En1^{lki}* (Hanks et al., 1995), *En1^{Cre}* (Kimmel et al., 2000) or *En1^{CreERT1}* (Sgaier et al., 2005) mutant alleles were used as *En2*- or *En1*-null alleles on an outbred Swiss Webster (SW) genetic background. Various combinations of *En1*- and *En2*-null alleles were interbred to generate the required genotypes. *En1^{2ki}* (Hanks et al., 1995) and *En1^{Denki}* (Hanks et al., 1998) mice were bred with *En2^{ntd}* and *En2$^{tau\text{-}lacZ}$*, respectively, on an outbred SW genetic background to generate the required genotypes. For embryonic analysis, noon of the day on which a vaginal plug was detected was designated as E0.5. Genotyping was carried out by PCR. The *R26R*, *En1^{Cre}* and *En1^{CreERT1}* alleles were detected as previously described (Li et al., 2002; Sgaier et al., 2005; Soriano, 1999). The primers used for genotyping the *En2* wild-type and *En2^{ntd}* alleles were: 1, 5′-TGCTCTTTGACGCTTCGGTG-3′; 2, 5′-CCTTGGATGGAGT-GCTCAAAGC-3′; and 3, 5′-TCATGCTGGAGTTCTTCGCC-3′. PCR with primers 1 and 2 detected a 300 bp wild-type allele, whereas primers 1 and 3 detected a 500 bp *En2^{ntd}* mutant allele. The primers used to genotype *En1* wild-type, *En1^{2ki}* and *En1^{Denki}* alleles were: A, 5′-AGCT-GCACCGCACCACCAAC-3′; B, 5′-GCACACAAGAGCGAGGCAGC-3′; C, 5′-CCCTGTGCCTTCGCTGAGG-3′; D, 5′-TGCCTGGCGCC-TGTAGGACC-3′; and E, 5′-TTGTAGGGTAATGGGGCTGGG-3′. PCR with primers A and B detected the 232 bp *En1* wild-type allele, whereas primers C and D detected the 230 bp *En1^{2ki}* allele, and primers E and C detected the 280 bp *En1^{Denki}* allele.

Histological analysis, β-gal histochemistry and RNA in situ hybridization

Tissue processing, RNA in situ hybridization and β-galactosidase (β-gal) analysis were performed as described on the Joyner website (http://www.mskcc.org/mskcc/html/75282.cfm) using *Fgf8* (Crossley and Martin, 1995), *Fgf17* (Xu et al., 1999), *Spry1* (courtesy of Gail Martin), *Otx2* (Simeone, 1993), *Gbx2* (Bouillet, 1995), *Wnt1* (Parr, 1993), *En1* (Joyner and Martin, 1987) antisense RNA probes.

Fate mapping

En1$^{CreERT1/+}$;En2$^{-/+}$;R26R/R26R adult males were bred with 5- to 6-week-old *En2$^{-/+}$* females and tamoxifen (Sigma T-5648) administered at 5 mg per 40 g of body weight via gavage at 18.00 h of E10.5 (Sgaier et al., 2005).

RESULTS

En1 is not required after ~E9.0 for development of the Cb and superior colliculus

Our finding that *En2* can fully rescue the *En1* mutant phenotype in *En1^{En2ki}* knock-in mice (Hanks et al., 1995) indicates that the two En proteins are functionally interchangeable and that the brain defects in *En1* and *En2* single mutants arise from cells that express only one En gene at critical time points in development. To address the question of whether En2 can compensate for En1 after the mes/r1 is specified, we first delineated the dynamic expression patterns of the En genes using *En1^{lacZ}* (Hanks et al., 1995; Matise and Joyner, 1997) and *En2$^{tau\text{-}lacZ}$* (Sgaier et al., 2005) knock-in alleles. *En1-lacZ* expression was first detected at the two-somite stage broadly spanning the mes/r1 region (Fig. 1A) and then progressively narrowed around the mes/r1 junction

(isthmic organizer) (Fig. 1B-D). *En2-tau-lacZ* expression commenced at the five-somite stage in a subdomain of the *En1*-positive region (Fig. 1G) and quickly broadened (Fig. 1G-J). Within the bilateral wing-like structure of the E12.5 cerebellar primordium (CbP), *En1-lacZ* was restricted to the medial-most region (vermis anlage), whereas *En2-tau-lacZ* was expressed in all but the most-lateral CbP (Fig. 1E,K). Similarly, in the Mb, *En2-tau-lacZ* expression extended more rostrally than *En1-lacZ* (into the anlage of the superior colliculus). Furthermore, expression of both En genes formed a mirror image double gradient across the mes/r1 region, with highest levels in the isthmus. Starting at E15.5, *En1* and *En2* expression changed to a sagittal striped pattern along the M-L axis of the CbP, owing to regional downregulation (Fig. 1F,L) (Millen et al., 1995). Thus, the En genes have dynamic gene expression patterns, but after ~E9 (including postnatal stages; data not shown) the *En1* expression domain appears to be encompassed within the *En2* expression domain.

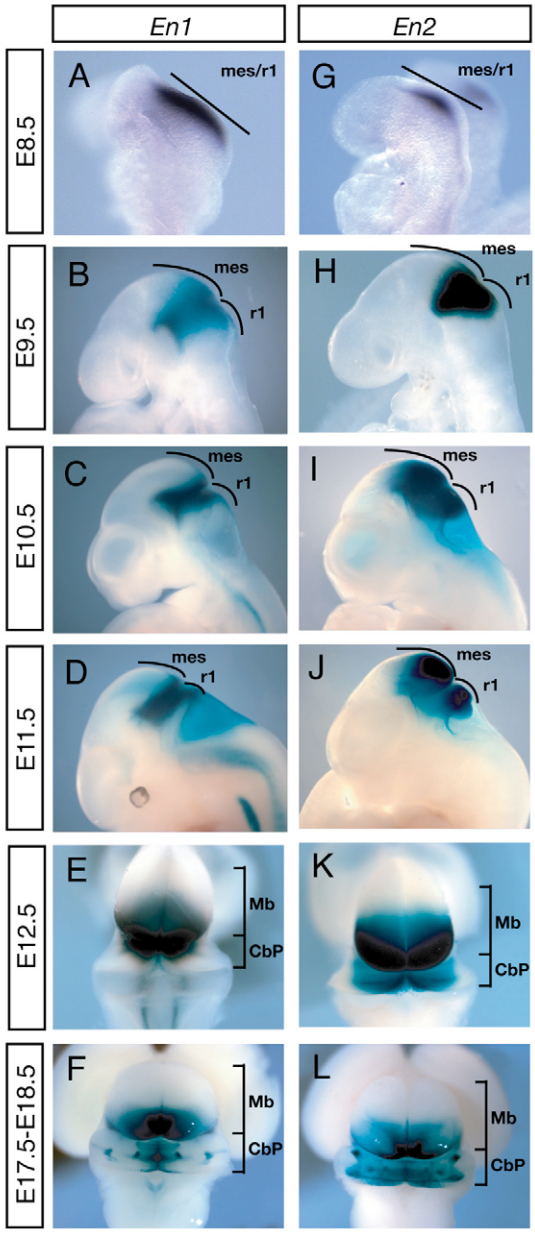

Fig. 1. Dynamic expression of *En1*^{lacZ} and *En2*$^{tau\text{-}lacZ}$ in the mes/r1 of mouse embryos. *En1* (**A**) and *En2* (**G**) mRNA expression detected by whole-mount RNA in situ hybridization. (**B-F**) *En1-lacZ* and (**H-L**) *En2-tau-lacZ* expression at the indicated stages detected by β-gal analysis. *En1* is induced earlier and becomes more restricted than *En2* by E10.5. Staining in the fourth ventricle in I and D is a result of probe trapping. CbP, cerebellar primordium; mes, mesencephalon; Mb, midbrain; r1, rhombomere 1.

The expression analysis of *En1* and *En2* suggests that *En1* might only be required in the mes/r1 before ~E9. To determine whether this is the case, we generated a conditional mutant allele of *En1* (*En1*^{flox}) in which the coding sequences in the second exon are flanked with *loxP* sites (see Fig. S1 in the supplementary material). Germline chimeras were bred to Black Swiss mice to avoid rescue of the *En1* mutant phenotype by the C57BL/6 background (Bilovocky et al., 2003). In order to delete *En1* at ~E9.0, we combined the *En1*^{flox} allele with the null *En1*^{Cre} knock-in allele (Kimmel et al., 2000). Within the mes/r1 region, Cre function is first detected at the five-somite stage and is active in all mes/r1 cells by 15 somites (E9.0) (Li et al., 2002). In *En1*$^{flox/Cre}$ mice, the second exon of the *En1*^{flox} allele thus begins to be deleted shortly after its expression is induced, and *En1* is expressed for only approximately 24 hours (Fig. 2A).

Indeed, *En1*$^{flox/Cre}$ mice were found to be viable and survive to adulthood. *En1*$^{flox/Cre}$ mutants had a limb phenotype similar to the rare *En1* mutants that survive (Loomis et al., 1996) (data not shown). The brains of all but one adult *En1*$^{flox/Cre}$ mouse analyzed (n=8) appeared grossly normal in whole-mount (Fig. 2B,C). The one mouse that was different had a partial deletion of the Mb and Cb (data not shown). Analysis of sagittal sections of the remaining *En1*$^{flox/Cre}$ mice revealed that the inferior colliculus (posterior tectum) was partially truncated (in all eight) (Fig. 2E,F and Table 1; compare also with Fig. 3B,I). In addition, five of the seven *En1*$^{flox/Cre}$ mice had a mild foliation defect in the anterior vermis (medial Cb), and the overall size of the vermis was slightly smaller than normal. The fissure between the anterior-most folia (I/II and III) either failed to form (in two of five) (Fig. 2F), or was shallower than normal (in three of five) in these mutants. Of significance, in two of the seven mutants analyzed, the fissure between folia I/II and III appeared as deep as in wild-type brains (Fig. 2D,E). One likely possibility for the variable rescue in the Cb is that in the two *En1*$^{flox/Cre}$ mutants that had a normal Cb, *En1* was ablated at a slightly later stage. Interestingly, in all eight of the *En1*$^{flox/Cre}$ mice analyzed, the superior colliculus and the hemispheres (lateral Cb) appeared normal (Fig. 2D-I). Thus, our analysis of the requirement for *En1* after ~E9 demonstrates that two copies of *En2* are sufficient to support Cb development, but despite being expressed in a broader domain of the tectum than *En1*, *En2* alone is not able to fully regulate inferior colliculus development.

One copy of *En2* is not sufficient to support development of the remaining inferior colliculus or the Cb when *En1* is deleted by E9

To determine whether one copy of *En2* is sufficient to support development of the superior colliculus and Cb when *En1* is removed at ~E9, we removed one copy of *En2* on the *En1*$^{flox/Cre}$ background. Strikingly, *En1*$^{flox/Cre}$;*En2*$^{-/+}$ mutant mice were not found at weaning (42 mice were analyzed from five litters of a cross between *En*$^{Cre/+}$;*En2*$^{-/+}$ and *En*$^{flox/+}$;*En2*$^{-/+}$ mice). We therefore analyzed the phenotypes of *En1*$^{flox/Cre}$ embryos lacking one *En2* allele at E18.5, and compared them with *En1*$^{-/-}$ and *En1*$^{-/-}$;*En2*$^{-/-}$ double mutants (Fig. 3). As expected, at E18.5, the vermis of *En1*$^{flox/Cre}$;*En2*$^{+/+}$ mice was either normal or slightly delayed in forming folia (Fig. 3B,I and Table 1). By contrast, *En1*$^{flox/Cre}$;*En2*$^{-/+}$ mice (Fig. 3J) displayed a complete deletion of the Cb that was very similar to *En1*$^{-/-}$;*En2*$^{-/+}$

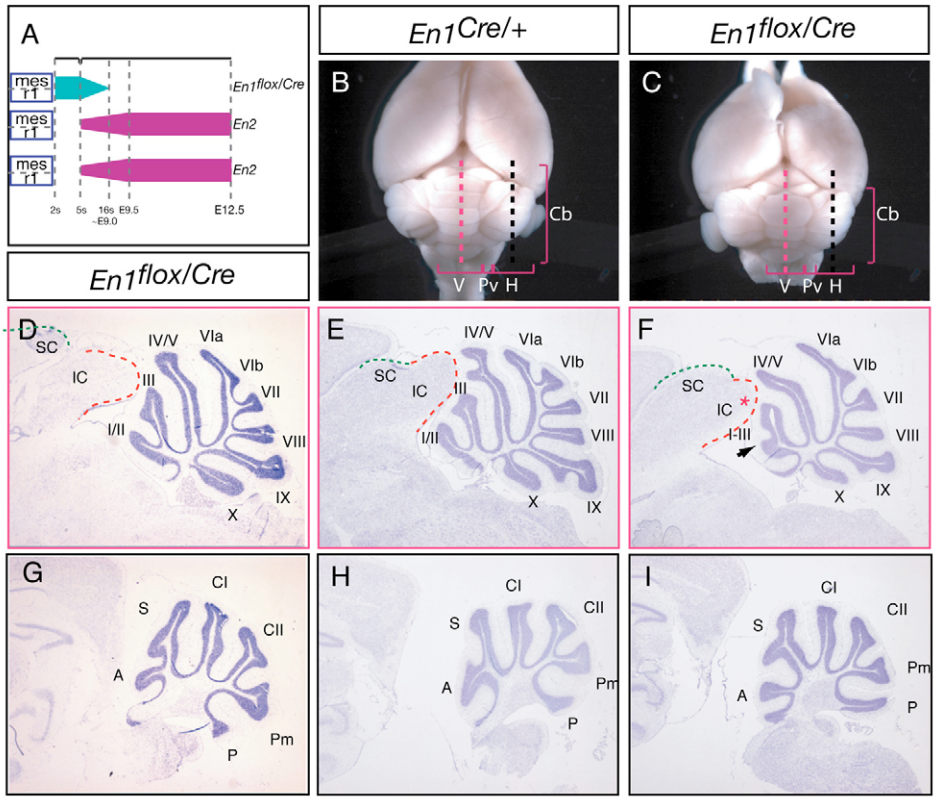

Fig. 2. *En1* is required prior to ~E9.0 to pattern the anterior cerebellum and after E9.0 to form the posterior midbrain. (**A**) Schematic illustrating the expression profile of En alleles in the mes/r1 region of *En1*$^{flox/Cre}$ mice. Note that only one functional *En1* allele is present because the *En1-Cre* allele is an *En1*-null. (**B**,**C**) Dorsal view of posterior adult brains of (B) *En1*$^{Cre/+}$ and (C) *En1*$^{flox/Cre}$ mice. (**D-I**) Cresyl Violet-stained sagittal sections of the brains were taken at the level of (D-F) the vermis and (G-I) hemispheres as indicated in B,C by the red and black dashed lines, respectively. E,H and F,I are sections taken from brains in B and C, respectively. Arrow in F indicates the predominant phenotypes of fusion of vermis folia I-II and III and the red asterisk indicates a slight truncation of the inferior colliculus. In some *En1*$^{flox/Cre}$ mice, a normally foliated Cb was also seen (D,G). H, hemisphere; V, vermis; Pv, paravermis; Cb, cerebellum; IC and SC are the inferior and superior colliculus, respectively outlined by red and green dashed lines. Vermis and hemisphere folia are indicated by roman numerals (I-X) and letters (A, S, Cl, CII, Pm, P), respectively.

(Fig. 3G) and *En1*$^{-/-}$;*En2*$^{-/-}$ (Fig. 3H) mutant embryos and more severe than *En1*$^{-/-}$;*En2*$^{+/+}$ mutants (Fig. 3F) that had some lateral Cb tissue remaining (see also Table 1). However, unlike *En1*$^{-/-}$;*En2*$^{-/+}$ (Fig. 3G) and *En1*$^{-/-}$;*En2*$^{-/-}$ mutants (Fig. 3H), which have no tectum, in *En1*$^{flox/Cre}$;*En2*$^{-/+}$ mutant mice (Fig. 3J) some superior colliculus tissue remained, similar to the phenotype of *En1*$^{-/-}$;*En2*$^{+/+}$ mice (Fig. 3F and Table 1). By contrast, *En1*$^{flox/Cre}$;*En2*$^{-/-}$ mutants had a complete deletion of the Cb and tectum (Fig. 3K and Table 1). In summary, when *En1* is expressed until ~E9.0 (*En1*$^{flox/Cre}$), two copies of *En2* can support superior colliculus and Cb development

Table 1. Summary of *En1/2* and *En1* conditional mutant phenotypes

Genotype		Phenotype					
					Cb subregions		
En1 alleles	*En2* alleles	SC	IC	Cb size	aV (I-V)	pV (VIII)	pH (CII/PM)
–/–	–/–	x	x	x			
–/–	–/+						
flox/Cre	–/–						
Denki/Denki	–/–	x/√	x	x			
flox/Cre	–/+	√√	x	x			
–/–	+/+	√√/√	x	(√)			
–/+	–/–	√√√	√/x	√	√	√/x	√√*
Denki/+	–/–	√√√	√√	√√	√√	x	√√*
(Ψ) *Denki/Denki*	+/–	√√√	√√	√√	√√	x	√√√
2ki/–	–/–	√√√	√√	√√	√√√	√	√√
+/+	–/–	√√√	√√√	√√	√√√	√	√√
flox/Cre	+/+	√√√	√	√√√	√√/√√√	√√√	√√√
–/+	–/+	√√√	√√√	√√√	√√	√√	√√√
2ki/2ki	–/–	√√√	√√√	√√	√√√	√√√	√√
2ki/2ki	–/+						
2ki/2ki	+/+						
Denki/Denki	+/+	√√√	√√√	√√√	√√√	√√√	√√√
+/+	–/+						
–/+	+/+						
+/+	+/+						

En1/2 alleles: +, wild type; –, null. SC/IC: √√√, normal superior/inferior colliculus; √√, reduced in size; √, little formed; ×, not formed. Cb size: √√√, normal; √√, ~1/3 overall reduction; √, ~1/2 overall reduction; (√), only the most lateral tissue remains; ×, not formed. Anterior vermis (aV): √√√, normal folia I-V; √√, fusion and smaller folia I-III; √, fusion and smaller folia I-V. Posterior vermis (pV): √√√, folium VIII is associated normally with folium VII; √√, folium VIII is positioned between lobule VII and IX; √, folium XIII is smaller and associated with folium IX; ×, little or no folium VIII. Posterior hemisphere (pH): √√√, crusII and paramedian folia are normal; √√, crusII and paramedian folia are fused; √√*, one mutant had a partial fissure separating crusII and paramedian. Ψ, denotes the mutants that survived into adulthood and that were analyzed.

and partial development of the inferior colliculus, whereas one copy of *En2* is not sufficient to support any development of the inferior colliculus or Cb, and in the absence of all *En2* the superior colliculus also does not form.

Particular regions of the Mb and Cb are sensitive to the dose of *En1* and *En2*

Since our analysis of *En1* conditional mutants showed that En1 is preferentially required in the inferior colliculus and folia I-III, we further explored the requirement for each mouse En gene in mes/r1 development by analyzing viable *En1* and *En2* double mutants. Surprisingly, although the Cb of $En1^{-/+}$ or $En2^{-/+}$ mice on an outbred background is normal (data not shown), section analysis of $En1^{-/+};En2^{-/+}$ double heterozygotes (Fig. 4A,E,C,G) revealed two foliation defects in the vermis. One was a variable but consistent (n=7 of 8) partial fusion of the three anterior-most folia (I-III), similar to the phenotype seen in some $En1^{flox/Cre}$ mutants (Fig. 2F and see Table 1). $En1^{-/+};En2^{-/+}$ mice (Fig. 4G) had an additional slight posterior shift in the position of folium VIII that was milder than in $En2^{-/-}$ mutants (Fig. 4F), in which folium VIII is associated

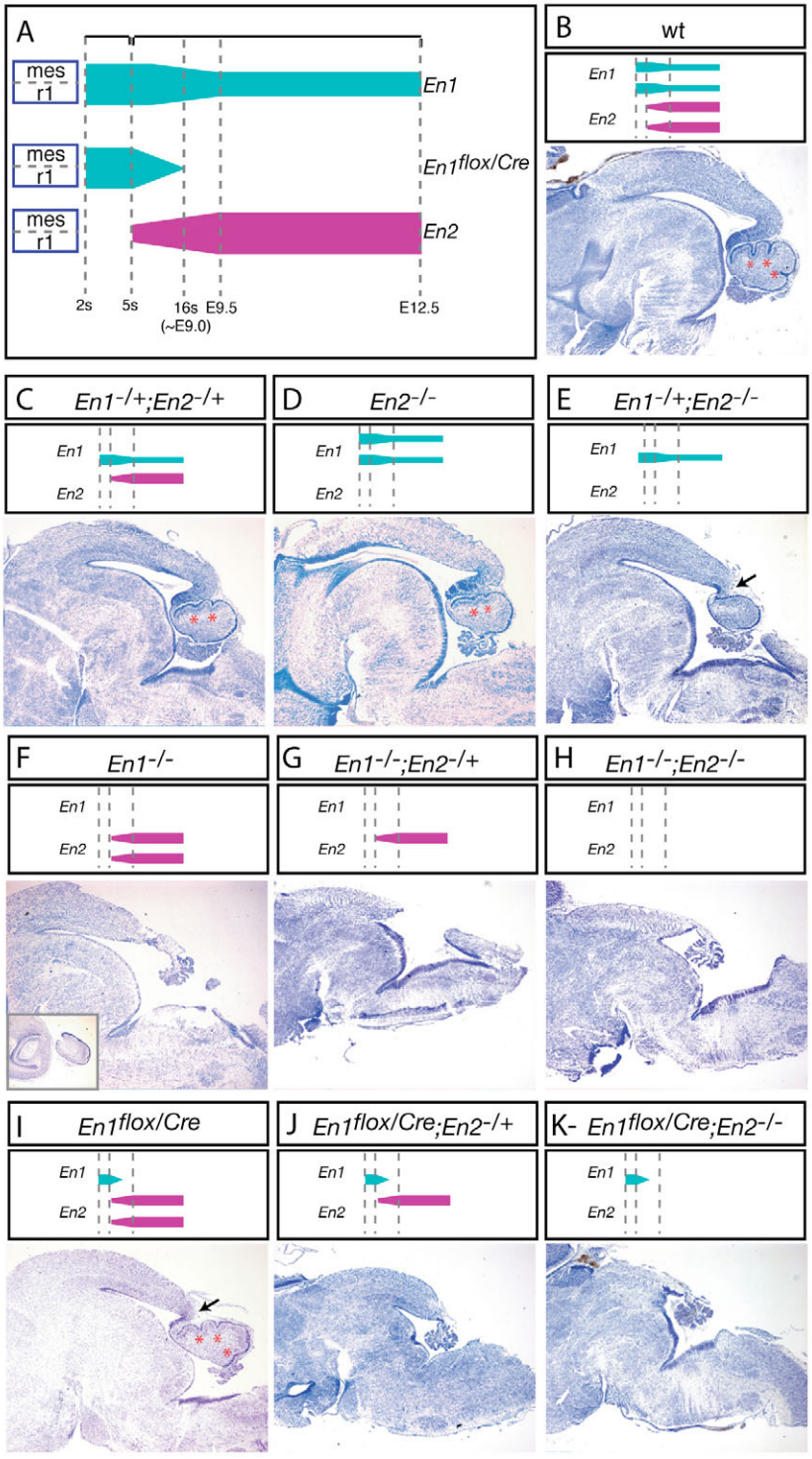

Fig. 3. Differential requirement of En genes for mouse midbrain and cerebellar development. (**A**) Schematic illustrating the detailed expression profile of each En allele (*En1*, *En2* and $En1^{flox/Cre}$) within the mes/r1 region. (**B-K**) Cresyl Violet-stained mid-sagittal sections of E18.5 brains with the corresponding genotypes and all the functional En allele profiles indicated at the top. Asterisks indicate forming fissures. Black arrows indicate truncated inferior colliculus. The inset in F shows the lateral Cb tissue that forms in the $En1^{-/-}$ mutants.

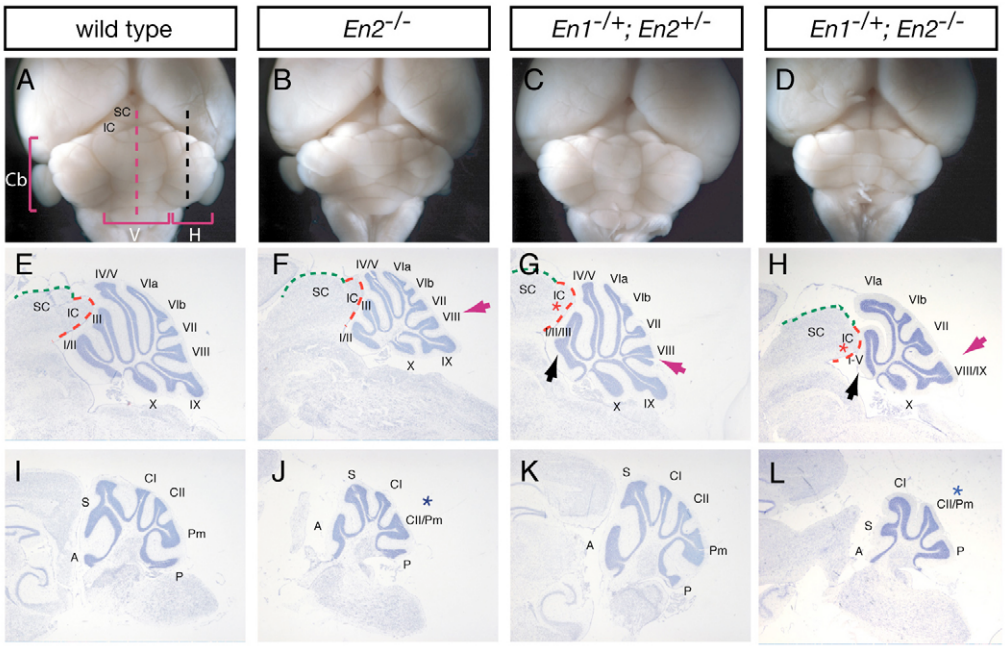

Fig. 4. Viable *En1* and *En2* double-mutant adult mice have midbrain and cerebellar defects. (**A-D**) Dorsal posterior views of adult brains of (A) wild-type, (B) $En2^{-/-}$, (C) $En1^{-/+};En2^{-/+}$ and (D) $En1^{-/+};En2^{-/-}$ mutant mice. (**E-L**) Cresyl Violet-stained sagittal sections of the cerebella shown in A-D at the level of the vermis (E-H) and hemispheres (I-L), as indicated in A by the red and black dashed lines, respectively. Black and red arrowheads indicate the anterior and posterior foliation defects, respectively. Blue and red asterisks indicate hemisphere foliation defects and truncation in the IC, respectively. See Fig. 2 legend for midbrain and cerebellum annotation.

with folium IX instead of with VI/VII. Furthermore, there was a general delay in fissure formation in the vermis of E18.5 $En1^{-/+};En2^{-/+}$ mutants (Fig. 3B,C), similar to but milder than the delay in $En2^{-/-}$ mutants (Fig. 3D) (Millen et al., 1994).

$En1^{-/+};En2^{-/-}$ mice, which have a smaller Mb and Cb than normal at E18.5 (Simon et al., 2005), were found to survive to adulthood. $En1^{-/+};En2^{-/-}$ mutants (Fig. 3B,E) had a more severe delay in Cb foliation than $En2^{-/-}$ mutants (Fig. 3D). Consistent with this, the Cb of adult $En1^{-/+};En2^{-/-}$ mutants (Fig. 4D,H,L) had a variable but greater size reduction and more foliation defects than $En2^{-/-}$ (Fig. 4B,F,J) or $En1^{-/+};En2^{+/-}$ mice (Fig. 4C,G,K and see Table 1). In the majority of the mutants (six out of eight) the five anterior-most folia (I-V) of the vermis were replaced by a single fold. In addition, folium VIII was more defective than in $En2^{-/-}$ mutants: it was absent or decreased in size and misaligned with folium IX. The inferior colliculus also was partially truncated. In one of the mutants, folia I-V were almost completely lost and fused to a more profoundly truncated tectum (data not shown). One other $En1^{-/+};En2^{-/-}$ mutant had an even more severe phenotype that included a complete deletion of the vermis and inferior colliculus (data not shown). In contrast to the vermis, although the overall size of the $En1^{-/+};En2^{-/-}$ hemispheres (Fig. 4I,L) was reduced compared with $En2^{-/-}$ mutants (Fig. 4J), the foliation defect was the same (fusion of the crusII and paramedian folia) or, paradoxically, partially rescued in one mutant. Interestingly, in all the viable *En1/2* mutants the relative thickness of the remaining molecular and granule cell layers and Purkinje cell density were normal. Our analysis of viable *En1/2* mutants provides additional evidence that the anterior vermis (folia I-V) and inferior colliculus are most sensitive to a reduction in *En1*, and also reveals redundant functions for *En1* and *En2* in folia I-V and VIII.

En2 can sustain more extensive Cb vermis development than *En1*

In order to directly compare the in vivo functions of En1 and En2 proteins, we took advantage of our $En1^{En2ki}$ knock-in mice (Hanks et al., 1995). A sensitive assay for comparing protein function is to combine knock-in alleles with loss-of-function alleles (Wang and Jaenisch, 1997). If the two En proteins have equivalent properties, then removal of *En2* in $En1^{En2ki/En2ki}$ mice ($En1^{En2ki/En2ki};En2^{-/-}$) should only cause the foliation defects seen in $En2^{-/-}$ mice. Indeed, when expressed in two copies from the *En1* locus, *En2* is sufficient to support development of the inferior colliculus. Unlike $En1^{flox/Cre};En2^{+/+}$ mice, which have a partial truncation of the tectum, the inferior colliculus appeared normal in $En1^{En2ki/En2ki};En2^{-/-}$ adult or early postnatal mice (Fig. 5A,C and data not shown). Furthermore, consistent with *En1* expression being restricted to the primordium of the vermis, as compared with the broader *En2* expression in the hemispheres after ~E9.5, the hemispheres of $En1^{En2ki/En2ki};En2^{-/-}$ mice (Fig. 5G,I) had the characteristic $En2^{-/-}$ phenotype (Fig. 4J). However, unlike $En2^{-/-}$ mice (Fig. 4F), in which folium VIII is associated with folium IX, in three out of four adult $En1^{En2ki/En2ki};En2^{-/-}$ mice (Fig. 5A,C), folium VIII was normal. This shows that the vermis foliation defects in $En2^{-/-}$ mice can be rescued by expressing the *En2* gene, but not the *En1* gene, from the *En1* locus. This suggests that En1 and En2 proteins are not equivalent, but rather that En2 activity is either specifically required in the posterior Cb (folium VIII) or more active in the Cb.

We further tested whether En2 is generally more potent than En1 by comparing the phenotype of $En1^{-/+};En2^{-/-}$ mice with $En1^{En2ki/-};En2^{-/-}$ mice. Strikingly, we found that $En1^{En2ki/-};En2^{-/-}$ mice (n=3) had a much milder anterior Cb phenotype than $En1^{-/+};En2^{-/-}$ mutants. The anterior vermis of $En1^{En2ki/-};En2^{-/-}$ mutants (Fig. 5A,D) had three distinct folia (I/II, III and IV/V) compared with one in $En1^{-/+};En2^{-/-}$ mice (Fig. 4H). In addition, in the posterior vermis of $En1^{En2ki/-};En2^{-/-}$ mice (Fig. 5D), folium VIII was only partially displaced toward folium IX, in contrast to $En1^{+/-};En2^{-/-}$ mutants (Fig. 4H), which have a substantial deletion of folium VIII. This sensitive dosage assay of one copy of *En2* or *En1* expressed from the *En1* locus in the absence of all other En function thus indicates that En2 is generally more active than En1 in regulating Cb development. Finally, the posterior tectum of $En1^{En2ki/-};En2^{-/-}$ mice (Fig. 5D) was partially truncated (Fig. 4H and data not shown), seemingly less than in $En1^{-/+};En2^{-/-}$ mutants

(Figure 4H and data not shown), indicating that the dose of both En proteins contributes to the truncation of the inferior colliculus in the various En mutants.

Drosophila en expressed in place of *En1* cannot support mes/r1 development in the absence of *En2*

Our finding that one or two copies of *En2* expressed from the *En1* locus in the absence of endogenous *En2* can support tectum and Cb development prompted us to determine whether *Drosophila en* could do the same. To determine whether *Drosophila en*, when expressed in place of *En1*, can sustain mes/r1 development in the absence of *En2*, we made use of knock-in mice ($En1^{Denki}$) that express *Drosophila en* in place of *En1* (Hanks et al., 1998). Whereas $En1^{En2ki/En2ki};En2^{-/-}$ and $En1^{Denki/Denki};En2^{+/+}$ mice are viable, we did not detect any $En1^{Denki/Denki};En2^{-/-}$ mice after birth. Section analysis of E18.5 $En1^{Denki/Denki};En2^{-/-}$ embryos revealed either the same phenotype as $En1^{-/-};En2^{-/-}$ embryos (n=1 of 3) or a similar phenotype to $En1^{flox/Cre};En2^{-/+}$ mutants (n=2 of 3; data not shown). Interestingly, half the expected number of $En1^{Denki/Denki};En2^{-/+}$ mice survived to adulthood, indicating that *Drosophila* En has some En1-like activity as $En1^{-/-};En2^{-/+}$ die at birth. Histological analysis of the brains of three $En1^{Denki/Denki};En2^{-/+}$ mice (Fig. 5A,E and Table 1) that survived surprisingly revealed that two of the mutants had a normal tectum and Cb, whereas one had a partial deletion of the posterior tectum and a vermis foliation defect similar to $En1^{+/-};En2^{-/-}$ mutants (Fig. 4H). The five $En1^{Denki/+};En2^{-/-}$ mice analyzed had a phenotype that was milder than $En1^{-/+};En2^{-/-}$ mice, with a distinct IV/V folium (Fig. 5A,F and Fig. 4H). All but one of the five $En1^{Denki/+};En2^{-/-}$ mutants analyzed had an $En2^{-/-}$ foliation pattern in the hemispheres (Fig. 4J, Fig. 5G,L). These results indicate that *Drosophila* En function can partially replace normal En1 function in the initiation of normal *En2* expression (before ~E9.0) in $En1^{Denki/Denki};En2^{+/+}$ mice, after which mouse En2 contributes a necessary function for continued normal development of mes/r1-derived structures.

Medial-lateral patterning of the Cb appears to be altered in $En1^{-/+};En2^{-/-}$ mutants

One possible reason for the loss of the posterior tectum and anterior vermis in $En1^{-/+};En2^{-/-}$ adult mutants is specific loss of the cells that give rise to the these two regions (caudal mes and rostral r1) (Sgaier et al., 2005; Zervas et al., 2004). To determine whether this is the case, we fate mapped the posterior mes and anterior r1 in $En1^{-/+};En2^{-/-}$ mutants by genetic inducible fate mapping (GIFM) (Joyner and Zervas, 2006) using our null $En1^{CreERT1/+}$ allele (Sgaier et al., 2005) and the *R26R lacZ* reporter allele (Soriano, 1999). When tamoxifen (TM) is administered at 18.00 h to E10.5 wild-type ($En1^{CreERT1/+};En2^{+/+};R26R/+$) embryos (marking cells at E11-12), the posterior mes and medial-most domain of the E12.5 CbP are marked (Fig. 6A) and give rise to the vermis and inferior colliculus of the adult, respectively (Fig. 6G,I) (Sgaier et al., 2005). GIFM in $En1^{CreERT1/+};En2^{-/-};R26R/+$ mutants also marked the posterior mes and medial-most domain of the E12.5 CbP (Fig. 6B). A difference that was apparent between the mutants and wild types was that despite an overall reduction in the size of the mes and r1 in the mutants, the size of the initial marked domain in the mes/r1 at E12.5 was similar to that in the wild type, and the regions devoid of marked cells were smaller in the mutants as compared with the wild type (Fig. 6A,B). At E16.5, the size of the marked population of cells in the Cb appeared wider in $En1^{CreERT1/+};En2^{-/-};R26R/+$ embryos (Fig. 6D) than in wild types (Fig. 6C). By contrast, the size of the marked

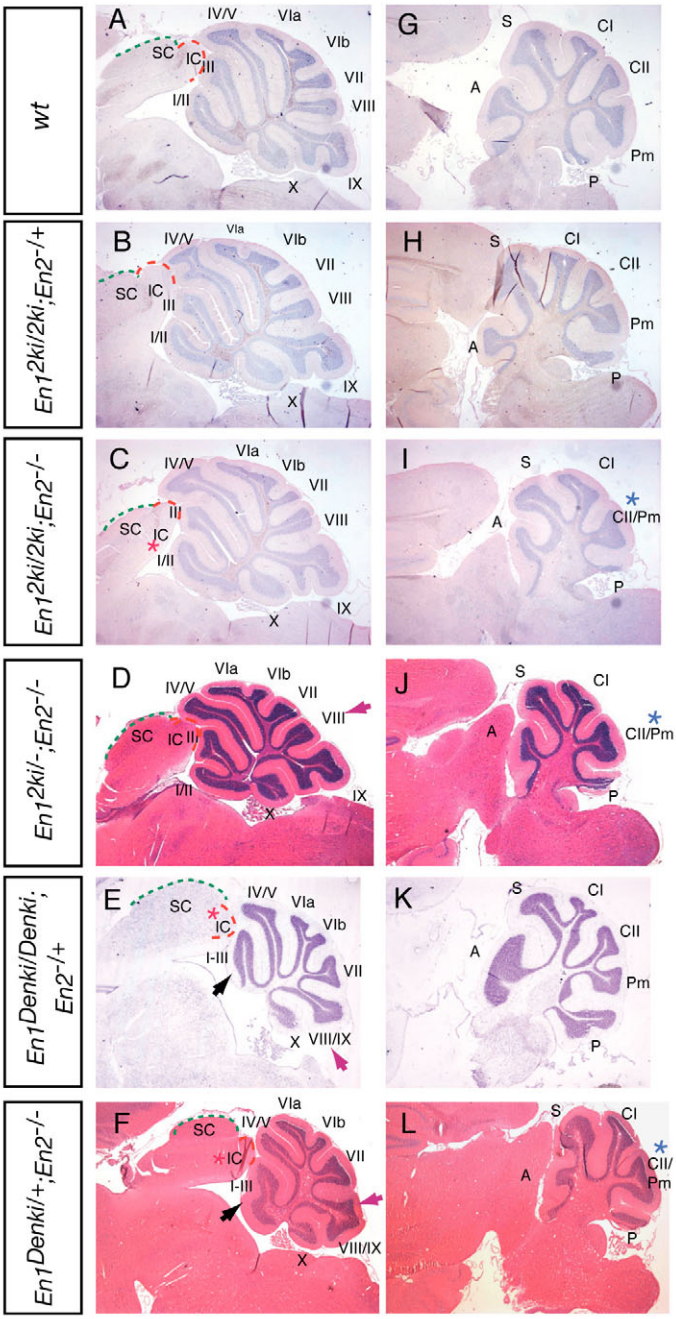

Fig. 5. In the absence of *En2*, expression of mouse *En2* but not *Drosophila en* in place of *En1* can rescue mes/r1 defects. Stained sagittal sections of wild-type (*wt*) (**A**,**G**), $En1^{2ki/2ki};En2^{-/+}$ (**B**,**H**), $En1^{2ki/2ki};En2^{-/-}$ (**C**,**I**), $En1^{2ki/-};En2^{-/-}$ (**D**,**J**), $En1^{Denki/Denki};En2^{-/+}$ (**E**,**K**), and $En1^{Denki/+};En2^{-/-}$ (**F**,**L**) at the level of the vermis (A-F) and hemispheres (G-L). A-C,E,G-I,K are stained with Cresyl Violet; D,F,J,L with Hematoxylin and Eosin. For comparison, the $En2^{-/-}$ phenotype is shown in Fig. 4. See Fig. 2 legend for midbrain and cerebellum annotation, and Fig. 4 legend for key to asterisks and arrows.

domain in the tectum was smaller in mutants than in wild types (Fig. 6C-F). Similarly, in adult $En1^{CreERT1/+};En2^{-/-};R26R/+$ mutants, the marked domain in the Cb was broader than normal, whereas the size of the marked domain in the tectum was greatly reduced compared with wild types as it was restricted to the remaining inferior colliculus (Fig. 6G-J). The fate mapping results in the mes suggest

that in *En1/2* mutants, the posterior mes cells marked at E12.5 do not expand normally and this results in a smaller inferior colliculus in the adult. By contrast, because the domain of marked cells in the adult Cb is larger than normal even though the vermis is reduced in size, this suggests that the anterior r1 cells marked at E12.5 are not only retained but contribute to more lateral regions of the vermis than normal. Thus, the loss of tectum and vermis tissue in $En1^{-/+};En2^{-/-}$ adult mutants is not simply owing to loss of the cells that give rise to the these two regions.

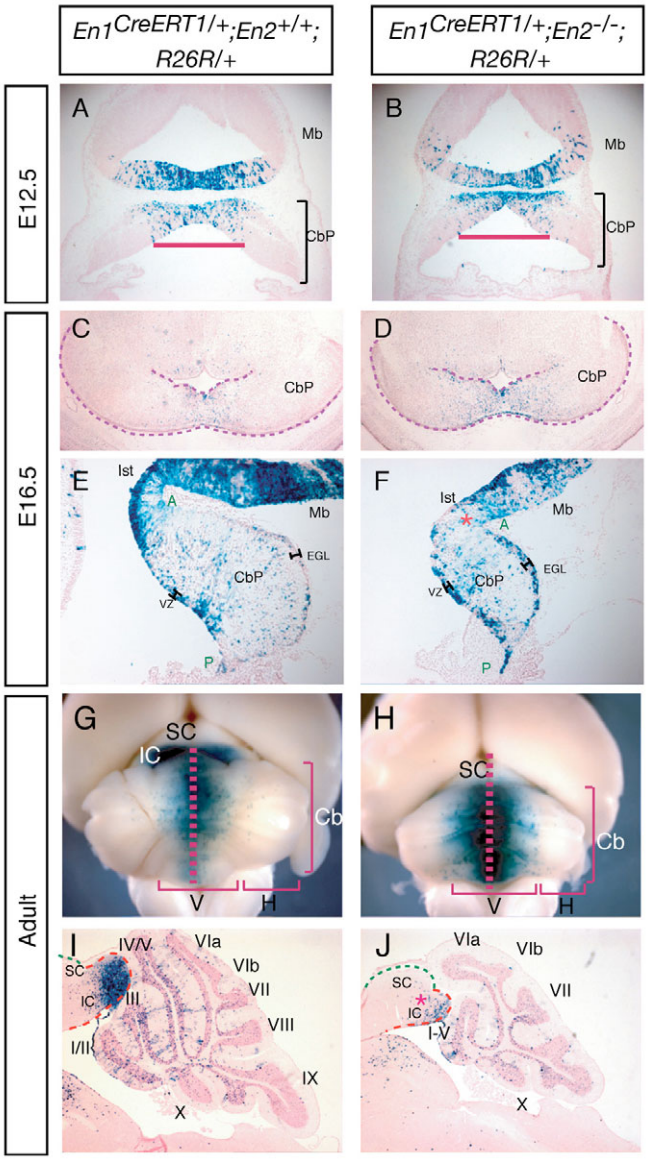

Fig. 6. mes and r1 cells behave differently in $En1^{-/+};En2^{-/-}$ mutants. (**A-J**) Tamoxifen was administered to mouse embryos carrying the indicated alleles at 18.00 h on E10.5. (A,B) Coronal sections of E12.5 brains and (C,D) horizontal and (E,F) midline sagittal sections of E16.5 brains stained for β-gal activity. (G,H) Dorsal views of β-gal-stained adult brains. (I,J) Sagittal sections of adult brains taken at the level of the vermis and stained for β-gal. The domain of marked cells was comparable in the $En1^{CreERT1/+};En2^{-/-}$ and $En1^{CreERT1/+};R26R/+$ embryos at E12.5, but decreased in the tectum and increased in the Cb of $En1^{CreERT1/-};En2^{-/-};R26R/+$ embryos by E16.5. CbP, cerebellar primordium; EGL and associated bar, external granule layer. Ist, isthmus; Mb, midbrain; VZ, ventricular zone; A, anterior; P, posterior; M, medial; L, lateral. Red asterisks indicate truncation of the isthmus/inferior colliculus. CbP is outlined by a purple dashed line in C,D; in I,J, the IC and SC are outlined by dashed red and green lines, respectively. The red bar in A,B indicates the size of the CbP domain with marked cells.

The isthmic organizer is not lost in $En2^{-/-}$ and $En1^{-/+};En2^{-/-}$ mutants

Since *Fgf8* expression is lost in *En1/En2* double-homozygous mutant embryos by E9 (Liu and Joyner, 2001), one possible reason for the defects in $En2^{-/-}$ and $En1^{-/+};En2^{-/-}$ mutants is a disruption of the isthmic organizer (decrease in Fgf signaling). However, our previous whole-mount RNA in situ analysis of $En1^{-/+};En2^{-/-}$ embryos from the six-somite stage to E9.5 did not reveal any obvious changes in the expression of *Fgf8* or mes/r1 morphology (Liu and Joyner, 2001). Based on section analysis, $En2^{-/-}$ embryos had a slight reduction in the size of the Cb by E11.5, and $En1^{-/+};En2^{-/-}$ mutants had a greater reduction in the CbP and truncation of the tectum (see Fig. 7). We therefore performed RNA in situ analysis at E11.5, a day before *Fgf8* expression is normally terminated in the isthmus. Consistent with the fate mapping study, we found that the expression domain of *En1* was not obviously altered along the A-P axis in the mutants (Fig. 7G-I). The only obvious difference in the expression domains of *Fgf8* and *Fgf17* (a related organizer gene) and of the direct target gene *Spry1* (Liu et al., 2003) between $En2^{-/-}$ and $En1^{-/+};En2^{-/-}$ mutants as compared with wild-type mice ($En1^{-/+}$) was a slight reduction in the size of the domains, which correlated with the reduction in mes/r1 tissue in each mutant (Fig. 7A-F and data not shown). Consistent with retention of organizer activity, the expression domains of two mes genes (*Otx2* and *Wnt1*) and one r1 gene (*Gbx2*) regulated by Fgf8, were not altered (data not shown).

DISCUSSION

In this study, we have analyzed a series of mouse En conditional knock-in and null mutants to decipher the overlapping and individual functions of the two highly conserved En genes in mes/r1 development. Overall, we found that the inferior colliculus of the tectum and three regions of the Cb are particularly sensitive to the level of En genes (see below and Table 1). Interestingly, the anterior vermis and tectum defects we observed in $En1^{flox/cre}$, $En1^{Denki/Denki};En2^{-/+}$ and $En1^{-/+};En2^{-/+}$ mutant mice have similarities to $Fgf8^{-/+};Fgf17^{-/-}$ mutants (Xu et al., 2000), raising the possibility that a key role of *En1/2* is to maintain *Fgf8* expression (Liu et al., 2003). We found that *Fgf8* expression is maintained as long as one allele of *En1* is present, although there are subtle decreases in *Fgf8/17* expression. Since *En1/2* expression persists after E12.5, when *Fgf8* expression is terminated, and $En1^{-/+};En2^{-/-}$ mutants have a much more severe loss of the tectum and Cb folia than $Fgf8^{-/+};Fgf17^{-/-}$ mutants, it is possible that *En1/2* do not control mes/r1 development solely through regulating *Fgf8/17* expression.

En1/2 differentially regulate retention of cells in the mes and r1

By determining the fate of the *En1*-expressing cells at ~E11, which normally give rise to the vermis and inferior colliculus, in $En1^{-/+};En2^{-/-}$ mutants using GIFM, we uncovered an unexpected differential role for *En1/2* in regulating growth and survival of cells in the tectum versus the Cb. In *En1/2* mutants, the posterior mes cells marked at E12.5 do not expand normally and this results in a smaller inferior colliculus in the adult. By contrast, the anterior r1 cells marked at E12.5 are not only retained but contribute to more lateral regions of the vermis than normal. If the lineage restriction at the

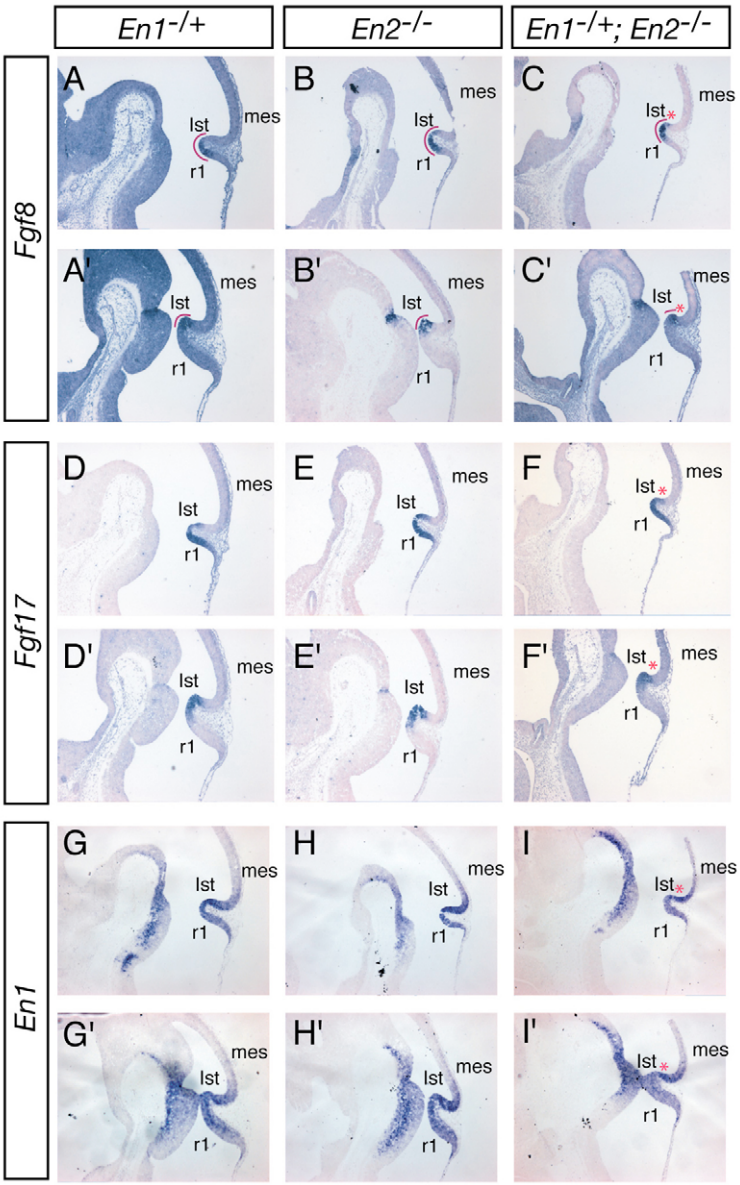

Fig. 7. *Fgf8*, *Fgf17* and *En1* are expressed normally in *En2*$^{-/-}$ and *En1*$^{-/+}$;*En2*$^{-/-}$ mutant E11.5 mouse embryos. RNA in situ hybridization of *Fgf8* (**A-C'**), *Fgf17* (**D-F'**) and *En1* (**G-I'**) on sagittal sections of *En1*$^{-/+}$ (A,D,G), *En2*$^{-/-}$ (B,E,H) and *En1*$^{-/+}$;*En2*$^{-/-}$ (C,F,I) E11.5 mutant embryos. Upper (A-I) and lower (A′-I′) panels show medial and lateral sections, respectively. The *Fgf8/17* expression domains are positioned normally but are slightly reduced in size in mutants (C,F). *En1* is expressed normally in mutants (H,I). The isthmus corresponds to the *Fgf8* expression domain and is outlined in red. Red asterisks indicate truncation of the isthmus/inferior colliculus. r1, rhombomere 1; Ist, isthmus, mes, mesencephalon.

mes/r1 border that restricts mes and r1 cells from mixing (Zervas et al., 2004) is disrupted in *En1/2* mutants, then it is possible that the population of marked mes cells in *En1*$^{CreERT1/+}$;*En2*$^{-/-}$;*R26R/+* embryos move into r1 and expand the marked population in the Cb. Another possibility is that the precursors of the lateral Cb are selectively lost in the mutants. However, this is not in accordance with our observation that the hemispheres of *En1*$^{-/+}$;*En2*$^{-/-}$ adults are less compromised than the vermis. Although the ultimate overall loss of cells in the mes and r1 of *En1*$^{-/+}$;*En2*$^{-/-}$ mutants could be accounted for by cell death, similar to the situation in *En1* mutants (Chi et al., 2003), our fate mapping study shows that it is not as simple as the cells being lost equally on either side of the isthmus.

En1 is required after E9 only for development of the inferior colliculus

Consistent with the *En1* expression domain being encompassed by the *En2* domain after E9, we found that *En1*$^{CreERT1/flox}$ conditional mutants are viable and have a normal Cb and superior colliculus. However, despite strong expression of *En2* in the posterior mes after E9 when *En1* is deleted in these mutants, the inferior colliculus does not develop normally in *En1*$^{flox/Cre}$ mutants. These results indicate that the En1 protein has a different function from En2, or that En1 and En2 are expressed differently in the tectum after E9. We demonstrated that the latter is the case, because the tectum develops normally when En2 is produced from the *En1* locus in the absence of endogenous En2 (*En1*$^{En2ki/En2ki}$;*En2*$^{-/-}$ mice). Based on our expression analysis, the crucial difference must be that En1 is transiently expressed around E9 in a broader domain than En2, or that En1 is later produced at a higher level than En2. Regardless, our studies have uncovered a differential requirement for the two En genes in the superior and inferior colliculi (see Table 1 and Fig. 8A).

En1 and *En2* are differentially required in subregions of the Cb

Our analysis of *En1/2* double-mutant combinations (null, knock-in and conditional) uncovered additional differential requirements for *En1* and *En2* in specific regions of the Cb (see Table 1 and Fig. 8B). *En1/2* functions are normally uncoupled in the hemispheres as only *En2* is required to divide the posterior region into two folds (crusII and paramedian). However, the partial rescue of the hemisphere

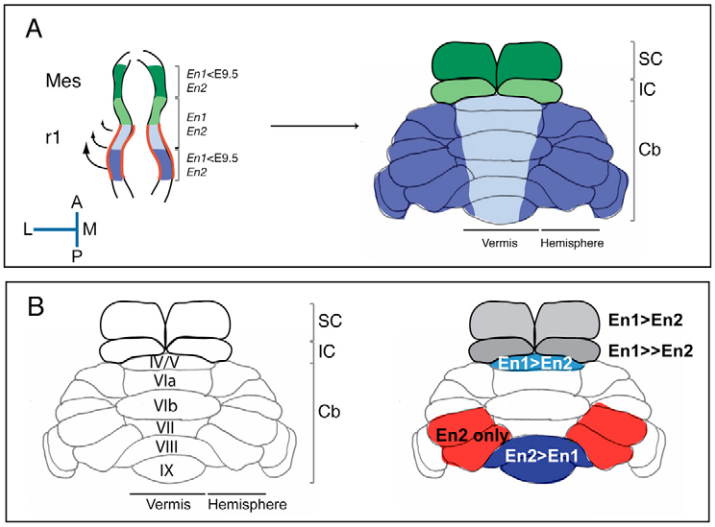

Fig. 8. An 'En code' can account for genetic partitioning of the midbrain and cerebellum vermis into functionally related domains. (**A**) Schematic illustrating the domains (color coded) within the mouse early neural tube (left) along the A-P axis that express either or both of the En genes, and the regions of the adult cerebellum and midbrain (right) that they give rise to owing to a rotation of the neural tube. The red-outlined region delineates rhombomere 1. (**B**) Schematic illustrating the normal gene dosage requirement for *En1* and *En2* in sustaining development of distinct domains of the tectum and Cb. Note that based on a sensitive knock-in assay, En2 protein appears to be more active throughout the vermis than En1. Thus, the gene dosage effects are likely to reflect differences in *En1* and *En2* gene expression. Cb, cerebellum; Mes, mesencephalon; SC, superior colliculus; IC, inferior colliculus; A, anterior; P, posterior; M, medial; L, lateral.

phenotype in rare $En1^{-/+};En2^{-/-}$ and $En1^{Denki/+};En2^{-/-}$ mutants indicates that En1 can support hemisphere development when expressed more laterally than normal. A comparison of the phenotypes of these mice with $En1^{flox/Cre};En2^{+/+}$ mutants (which have normal posterior foliation) indicated that *En2* plays a greater role than *En1* in formation of folium VIII. We demonstrated that this difference is not owing to a difference in gene expression, but instead to a difference in protein activity because the vermis foliation defect seen in $En1^{+/+};En2^{-/-}$ mutants is rescued in $En1^{En2ki/En2ki};En2^{-/-}$ mice. Furthermore, $En1^{En2ki/-};En2^{-/-}$ mice have a milder phenotype than $En1^{-/+};En2^{-/-}$ mutants. Thus, En2 appears to be more effective in promoting development of the vermis (folia I-V and VIII) than En1. We further discovered that the two En genes act concomitantly to divide the anterior Cb into five folia. $En1^{-/+};En2^{-/+}$ double heterozygotes and the majority of $En1^{flox/Cre};En2^{+/+}$ mutants have a fusion of the anterior three folia (I-III) and the anterior defect is greatly exaggerated in $En1^{-/+};En2^{-/-}$ mutants (fusion of folia I-V), despite $En2^{-/-}$ mutants having normal anterior foliation. Since some $En1^{flox/Cre}$ mice have normal anterior folia, this indicates a crucial requirement for expression of *En1* only at ~E9, when *En1* expression is fading out in the mutants and *En2* is initiating. To our knowledge, this is the first evidence that the pattern of Cb folia can be influenced by genetic events that occur at such an early embryonic stage.

It is revealing to compare the early *En1/2* expression patterns and the broad regions of the mes/r1 that differentially require the two genes. Based on our recent fate map of the mes/r1 using GIFM (Sgaier et al., 2005; Zervas et al., 2004), the anterior and posterior mes give rise to the superior and inferior colliculi, respectively (Fig. 8A). Consistent with strong and sustained expression of the En genes in the primordium of the inferior colliculus, this region is most sensitive to the dose of En genes, and in particular to that of *En1*. However, by using the sensitive assay of $En1^{2ki}$ knock-in alleles combined with removal of the endogenous *En2* gene, we found that En2 is at least as potent as En1 at promoting inferior colliculus development. Given the transient expression of the En genes in the superior colliculus, it is perhaps surprising that this region is dependent at all on the combination of the two genes. This indicates a requirement for a short burst of En function (En1 or En2) before E9.5. The remaining inferior colliculus tissue in En mutants is likely to correlate with tectum cells that are normally in the low end of the En gradient, suggesting that they are least sensitive to loss of En alleles. There also is a general correlation between the domains of En gene expression and the requirement for each gene in the Cb (vermis versus hemispheres). After E9.5, *En1* is only maintained in anterior r1 and the medial Cb primordium (the anlage of the vermis), consistent with no function in the hemispheres. The limit of the *En2* expression domain extends more posterior early in r1 and laterally later in the CbP, correlating with a role in the hemispheres. It is not clear, however, why the En genes do not play a major role in the anterior hemispheres or in folia VI/VII and IX/X in the vermis.

An 'En code' divides the tectum and Cb into subregions

Taken together, our analysis of a series of En mutants provides evidence that functional domains of the Cb are genetically encoded by the engrailed genes, as specific regions of the tectum and Cb have differential sensitivities to reducing En gene dosage. The phenotypes of multiple mutants point to a genetic division of the tectum into two regions and of the Cb into six. We propose that this represents an 'En code' that is used to partition the mes/r1 region into domains that in the adult regulate related neural functions (Fig. 8B). The two functional divisions of the tectum, the inferior and superior colliculi, are delineated based on a temporal requirement for *En1* and sensitivity to the overall dose of En protein. Within the vermis of the Cb, the anterior five folia (I-V) and folium VIII are particularly sensitive to a reduction of En genes, and preferentially to *En2*, thus dividing the vermis into four broad regions (folia I-V, VI/VII, VIII, IX/X). Strikingly, this division of the Cb is very similar to the transverse zones recently proposed based on four different domains of parasagittal gene expression (Armstrong et al., 2005; Ozol et al., 1999). The fact that two independent genetic measures of regionalization of the vermis (mutant phenotypes and gene expression) point to the same subdivisions of the vermis strongly argues that patterning of the folia is fundamental to organization of Cb function. Consistent with this, each transverse zone receives afferent inputs from distinct regions of the spinal cord and/or particular hindbrain nuclei. We predict that, likewise, the division of the hemispheres into regions based on a need for *En2* only in two folia (crusII and paramedian) (Fig. 8B) represents genetic partitioning into related functional systems.

We thank the NYU Transgenic/ES Cell Chimera Facility for the blastocyst injections; Dr P. Soriano for providing the *R26R* reporter mice; Drs G. Martin, R. Sillitoe, S. Blaess, M. Zervas, J. Li, and Y. Cheng for discussions and comments on the manuscript. This work was supported in part by a grant from NINDS.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/12/2325/DC1

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