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ORIGINAL PAPER

# Dynamic Expression and New Functions of *Early B Cell Factor 2* in Cerebellar Development

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## Abstract

The collier/Olf1/EBF family genes encode helix-loop-helix transcription factors (TFs) highly conserved in evolution, initially characterized for their roles in the immune system and in various aspects of neural development. The *Early B cell Factor 2* (*Ebf2*) gene plays an important role in the establishment of cerebellar cortical topography and in Purkinje cell (PC) subtype specification. In the adult cerebellum, *Ebf2* is expressed in zebrin II (ZII)-negative PCs, where it suppresses the ZII+ molecular phenotype. However, it is not clear whether *Ebf2* is restricted to a PC subset from the onset of its expression or is initially distributed in all PCs and silenced only later in the prospective ZII+ subtype. Moreover, the dynamic distribution and role of *Ebf2* in the differentiation of other cerebellar cells remain unclarified. In this paper, by genetic fate mapping, we determine that *Ebf2* mRNA is initially found in all PC progenitors, suggesting that unidentified upstream factors silence its expression before completion of embryogenesis. Moreover we show *Ebf2* activation in an early born subset of granule cell (GC) precursors homing in the anterior lobe. Conversely, *Ebf2* transcription is repressed in other cerebellar cortex interneurons. Last, we show that, although *Ebf2* only labels the medial cerebellar nuclei (CN) in the adult cerebellum, the gene is expressed prenatally in projection neurons of all CN. Importantly, in *Ebf2* nulls, fastigial nuclei are severely hypocellular, mirroring the defective development of anterior lobe PCs. Our findings further clarify the roles of this terminal selector gene in cerebellar development.

**Keywords** Ebf2 gene · COE transcription factors · Cerebellar development · Purkinje cell development · Purkinje cell subtype specification · Cerebellar cortex topography · Cerebellar nuclei development · Fastigial nuclei development · Cerebellar granule cell subtypes

## Introduction

The cerebellar cortex is built around a single output projection neuron, the Purkinje cell, which projects directly to the cerebellar and lateral vestibular nuclei. The major inputs to PCs come directly from the inferior olivary complex via climbing fibers and indirectly via the mossy fiber-granule cell pathways (and in specialized cases, with a glutamatergic unipolar brush cell interpolated in the pathway). This simple circuit is modulated by a suite of inhibitory interneurons—stellate, basket, Golgi, Lugaro, etc. Finally, the mature cerebellar cortex also houses a variety of glial cells, notably here the Bergmann glia (BG). In addition, embedded in the cerebellar white matter, three bilateral sets of nuclei receive mossy and climbing fiber collaterals and PC axons. The cerebellar nuclei (CN) contain large glutamatergic neurons, projecting to gray matter nuclei located in the pons, midbrain, and thalamus, as well as inhibitory interneurons and GABAergic nucleo-olivary neurons.

Despite its superficial uniformity, the cerebellar cortex houses an elaborate and stereotyped topography, built around a scaffold of PC subtypes [1–7]. The architecture is first compartmentalized into four transverse expression domains ("transverse zones": anterior (AZ), central, posterior, and

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nodular), each of which is further subdivided into a reproducible array of parasagittal "stripes." Cerebellar architecture is revealed in many ways, notably here the expression patterns for zebrin II/aldolase C [8, 9] and *Ebf2* (ZII+/*Ebf2*− and ZII −/*Ebf2*+) [10, 11].

During cerebellar embryogenesis, two distinct germinal, neurogenetic matrices form within the cerebellar primordium: the upper rhombic lip (URL) and the ventricular zone (VZ) (reviewed in [12]). All the inhibitory neurons of the cerebellar cortex and nuclei originate from *Ptf1a*+ progenitors in the cerebellar VZ [13, 14], while glutamatergic neurons—granule cells (GCs), unipolar brush cells and glutamatergic projection neurons of the CN—derive from *Atoh1*+ progenitors in the URL [15–18]. The newborn PCs migrate from the VZ to the cerebellar anlage, where they regroup into a stereotyped array of clusters (E13–17), that is, the forebear of the adult stripes (reviewed in [19]). At the same time, the precursors in the URL proliferate and spread across the cerebellar cortical surface to form the nuclear transitory zone, from which the excitatory component of the CN arises [16, 17], and a secondary germinal epithelium, the external granular layer (EGL), which generates GCs [16, 17]. Later in embryogenesis, glutamatergic interneurons dubbed unipolar brush cells (UBCs) spread across the cerebellar cortex from posterior lobules [18, 20].

The framework of PC transverse zones and parasagittal stripes guides the establishment of cortical topography. For example, zone-and-stripe boundaries restrict the terminal fields of afferent projections (reviewed in [21]), GC dispersion [22, 23], interneuron neurite extension [24], etc. ([25], reviewed in [26]), as well as Bergmann glial gene expression profiles (e.g., [27]).

The development of cerebellar topography reveals a central role for PC subtype specification. The *Early B cell Factor 2 (Ebf2)* gene plays an important role in the establishment of cerebellar cortical topography. The collier/Olf1/EBF family genes [28], encode helix-loop-helix TFs highly conserved in evolution [29–32], which were originally characterized for their roles in the immune system [33], and subsequently implicated in various aspects of neural development [34], including specification of motor neuron types and subtypes [35, 36], neuronal differentiation [31, 32, 37, 38], cell migration [39, 40], and axon guidance [41]. One member of this family, *Ebf2* [42–44], contributes to neuroendocrine [40], olfactory [44], and peripheral nerve development [40, 45, 46]. Various studies have also shown that EBF2 is important for PC survival [47], electrical activity and motor learning [48], and the transcriptional regulation of cerebellar genes involved in several aspects of neuronal function, including immunoglobulin superfamily adhesion molecules [49]. In postnatal and adult cerebella, *Ebf2* is only expressed in ZII-negative (*Ebf2*+/ZII−) PCs [11], in which it suppresses the prospective ZII+ phenotype [10]. *Ebf2* null mutants ($Ebf2^{LacZ/LacZ}$) [40] feature a transdifferentiation and death of the ZII− subtype, leading to disruption of the normal transverse zone and parasagittal stripe pattern [10, 11]. To date, the *Ebf2* mutation is the only known genetic manipulation capable of subverting PC subtype specification. However, many issues remain obscure. For example, it is unclear whether *Ebf2* is restricted to a subset of PC progenitors from the onset of its expression in early postmitotic precursors (~E11), or if it becomes spatially or lineage restricted at a later stage of development. Likewise, while in the adult CN *Ebf2*-positive neurons are confined to the medial nuclei and to a small domain within the interposed and vestibular nuclei [50], it has not been established whether, at early stages, this gene is expressed ubiquitously in the CN and then restricted to a subset of CN neurons. Finally, it is not clear to date whether *Ebf2* is transiently expressed in other neuronal and glial lineages of the cerebellar primordium. To address these issues, by Cre-mediated genetic fate mapping, we permanently labeled all cerebellar progenitors expressing *Ebf2* during development and analyzed their distribution in mature cerebellar lineages in adult wild-type and *Ebf2* null mice.

## Materials and Methods

Figures 2, 3, 4, 5, and 6 shown in this paper represent the results of experiments conducted at least in triplicate.

**Animal Welfare** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experimental plan was designed in agreement with the stipulations of the San Raffaele Institutional Animal Care and Use Committee (permit number 336). Surgical procedures were performed on mice anesthetized with Avertin (Sigma, St. Louis, MO, USA).

**Generation of Ebf2::Egfp-iCre Transgenic Mice** A BAC clone spanning the mouse *Ebf2* locus (clone RP24-283N8 from https://bacpacresources.org) was modified by homologous recombination in *E. coli* (reviewed in 51) by inserting the *Egfp-iCre* fusion gene into exon 2, replacing the first in-frame ATG codon of the *Ebf2* coding sequence (exon 2). The transgene is followed by an *Sv40* polyadenylation sequence. Thanks to this fusion transgene, cells expressing *Ebf2* display a nuclear green fluorescence. The modified BAC plasmid was injected into the male pronucleus of fertilized oocytes (FVB/N strain); the microinjected zygotes were implanted in the fallopian tubes of pseudo-pregnant recipient females. The progeny were analyzed by genotyping (PCR and Southern blotting) to select founders. Since the line was characterized and established, it has been maintained on a FVB/N background.

**Mouse Genetics** *Ebf2* wt and *Ebf2* null comparisons were carried out on F1 hybrids obtained by crossing $Ebf2^{LacZ/+}$; *Ebf2::Egfp-iCre* pure-bred FVB/N females with $Ebf2^{LacZ/+}$

[40], $R26^{YFP/YFP}$+/+ congenic C57BL/6N males. All studies were conducted using coisogenic control littermates. This hybrid strain was chosen to obviate the low fertility and poor maternal behavior of pure C57BL/6J $^{Ebf2LacZ/+}$ mothers. Embryos were staged counting the morning of vaginal plug detection as embryonic day 0.5.

**Tissue Preparation** Postnatal mice were anesthetized with Avertin (Sigma) and perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA). Embryos were fixed overnight by immersion in 4% PFA. Fixed brains and embryos were rinsed three times in 1x PBS, cryoprotected in 30% sucrose overnight, embedded in OCT (Bioptica), and stored at − 80 °C, before cryotome sectioning. For vibratome sectioning, fixed brains were embedded in 4% low-melt agarose at 40 °C and allowed to solidify on ice. Individual 100 μm sections were collected in 24 multi-well plates and processed for LacZ staining.

**In Situ Hybridization** Digoxygenin-labeled riboprobes were transcribed from plasmids containing *Ebf2*, *iCre*, and *Atoh1* cDNAs. In situ hybridizations were performed as described by Pringle and Richardson (www.ucl.ac.uk/~ucbzwdr/doubleinsituprotocol.htm).

**Immunofluorescence** For immunofluorescence, 18 μm cryosections were rinsed three times in 1x PBS, preincubated in 10% goat serum and 0.3% triton X-100 in 1x PBS, and incubated overnight at 4 °C with the primary antibodies. Sections were then washed 3 × 10 min in PBS and incubated for 2 h at room temperature with appropriate secondary antibodies (Alexa 488 anti-rabbit Ig, 1:1000 or Alexa 488 anti-chicken Ig, 1:1000 for anti-GFP antibodies or Alexa 546 anti-mouse Ig—all diluted 1:1000); after 3 washes, the cryosections were stained with DAPI (Sigma) and mounted in fluorescent mounting medium (Dako).

Cryosections were immunostained with the following primary antibodies:

- Rabbit anti-GFP (1:500, Invitrogen no. A11122)
- Chicken anti-GFP (1:1000, Abcam, no. ab13970)
- Rabbit anti-calbindin (1:500, Swant, no. cb38a)
- Mouse anti-S100beta (1:400, Proteintech, no. 15146-1-AP)
- Rabbit anti-Pax2 (1:500, Invitrogen/Thermo, no. 71-6000)
- Rabbit anti-GABA (1:2000, Sigma, no. A2052)
- Mouse anti-Smi32 (1:500, Covance, no. SMI-32P)
- Goat anti-parvalbumin (1:1000, Swant, no. PVG231)
- Rabbit anti-TBR2 (1:500, Abcam, no. ab23345)
- Mouse anti-NeuN (1:100, Chemicon/Millipore, no. MAB377)

**Count of CN Neurons in Ebf2 Null vs Ebf2 wt Cerebella** In the adult cerebellum, Smi32 labels heavy MW neurofilament-positive neurons, namely, PCs and large CN projection neurons. The number of Smi32+ cerebellar nuclear projection neurons was obtained by analyzing 18 μm frontal sections from *Ebf2*−/− and control P60 cerebella. The cerebella were serially sectioned and one of two series per animal was analyzed ($n = 3$). Statistical analysis was conducted using the unpaired two-tailed Welch's $t$ test ($p = 0.0045$ for medial nuclei).

## Results

**Generation of an Ebf2-iCre BAC Transgenic Line** To trace the *Ebf2*-positive lineage in the embryonic, postnatal, and adult cerebellum, we generated transgenic mice by fertilized oocyte injection of the transgene described in Fig. 1, which was obtained by BAC recombineering [51, 52] from a ~ 170 kb BAC clone containing the first 7 exons of the mouse *Ebf2* gene (Fig. 1a). In this construct, a green fluorescent protein-improved Cre [53] fusion gene (*Gfp-iCre*) was used to replace the first coding exon (E2 in Fig.1b) of the *Ebf2* gene and terminated with an SV40 polyadenylation site (Fig. 1b). Transcription of this gene results in nuclear green fluorescence. This line (MGI code, Tg(Ebf2-Egfp-icre)1Ggc), was originally produced in our laboratory (AB, FCh, GGC) and used in two previously published fate mapping studies [39, 54]. This construct permits permanent tagging of cells using Cre-dependent reporters. Moreover, while the transgene is being expressed, it can be revealed thanks to the nuclear signal produced by eGFP fused to iCre. Therefore, the line can be used to map both current and historical expression patterns. The transgene will be hereon referred to as *Ebf2::Cre.* In this paper, the *Ebf2::Cre* line was crossed with two Cre-activated indicator lines: a ROSA26 β-galactosidase reporter [55] and a ROSA26 YFP reporter [56]. The double-transgenic progeny of these crosses will be hereon referred to as Cre/βgal and Cre/YFP, respectively.

**Ebf2::Cre Transgene Distribution Closely Reflects the Expression of the Endogenous Ebf2 Gene** To determine how faithfully *Ebf2::Cre* transgene expression recapitulates the distribution of the endogenous *Ebf2* gene in the cerebellar primordium, we compared *iCre* and endogenous *Ebf2* transcript distribution by in situ hybridization (ISH) in adjacent sections from *Ebf2::Cre* hemizygotes on embryonic days (E) 12.5 and 13.5 and on postnatal day (P) 1 (Fig. 2). Our results clearly indicate that the transgene's expression provides a faithful representation of endogenous *Ebf2* distribution at all developmental stages examined. Importantly, transcription of the *Ebf2::Cre* transgene at birth is mainly restricted to anterior lobe PCs (see below), in agreement with its selective expression in the zebrin II negative (ZII−) population.

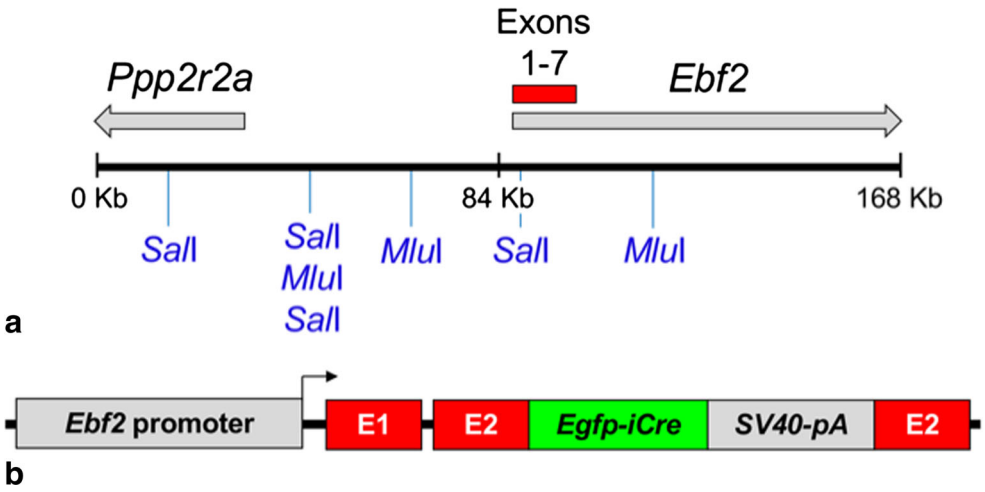


**Fig. 1** The *Ebf2::Egfp-iCre* transgene. **a** The BAC clone described in this paper contains the entire 5′ sequence of the *Ebf2* gene, the first seven exons (E), and a part of intron 7. **b** The *Egfp-iCre* fusion gene followed by the SV40 polyadenylation signal was inserted into exon 2, replacing the translation initiation codon of the *Ebf2* coding sequence

**The Cre-Activated Reporter Labels All PCs** To determine whether *Ebf2* is restricted to the ZII− population from the onset of its expression, Cre/βgal mice were analyzed by LacZ staining. In the progeny, the reaction product was uniformly distributed in all PCs located in all lobules (Fig. 3), including lobule X, known to contain almost exclusively ZII+ PCs. This indicates that, at the onset of its expression in the cerebellum, *Ebf2* labels all postmitotic PC progenitors, and only later is its expression restricted to ZII− PCs. This novel finding implies that other factors, promoting *Ebf2* downregulation in ZII+ PCs, play a major role in cerebellar cortical patterning at a stage subsequent to the birth of all PCs from the cerebellar VZ (see "Discussion").

**The Ebf2::Cre-Activated Reporter Selectively Labels Early-Born GCs** *Ebf2* is expressed in *Atoh1*+ progenitors, including prospective GCs, which begin leaving the rhombic lip at around E12.5 (Fig. 4b). At this stage, *Ebf2* and *iCre* expression overlap with the *Atoh1*+ domain, while later-born *Atoh1*+ progenitors (E13.0) do not coexpress *Ebf2*. To study the distribution of Ebf::Cre-tagged cells, we performed LacZ staining in Cre/βgal adult cerebella (14 μm sagittal sections). In keeping with published data [16], GCs born at around E12 and tagged by *Ebf2-iCre* mostly populate the anterior lobe, with a sharp antero-posterior boundary around the primary fissure (prf, the AZ/central zone (CZ) boundary: Fig. 4b).

**The Ebf2::Cre-Activated Reporter Does Not Tag Cerebellar Cortical Interneurons** Next, by dual immunofluorescence, we analyzed YFP reporter distribution and cell fate in Cre/YFP animals (Fig. 5). We analyzed postnatal day 0 (P0) cerebellar sections for the GABAergic interneuron marker Pax2 (Fig. 5a). Very little colocalization of the cytoplasmic reporter with nuclear Pax2 was observed, indicating that cerebellar inhibitory interneuron progenitors never express *Ebf2*. To confirm this notion and extend it to the postnatal cerebellum, we analyzed adult (P60) sections immunostained for parvalbumin, a cytoplasmic marker of molecular layer (ML) interneurons, namely, basket and stellate cells. No colocalization was observed between parvalbumin and the *Ebf2*+ lineage revealed by YFP (Fig. 5b), indicating that *Ebf2* does not normally participate in the development of molecular layer interneurons. Likewise, URL-derived Tbr2-positive UBCs [18], a late-born glutamatergic interneuron type that settles in the granular layer during late gestation [20], were also YFP negative (Fig. 5c), arguing against a direct lineage relationship.

**Some Bergmann Glia Derive from Ebf2+ Progenitors** While cerebellar cortical interneurons are almost entirely unrelated to *Ebf2*+ lineages, a non-neuronal population deriving from the cerebellar VZ, namely, Bergmann glia (BG), appears to derive from *Ebf2*+ progenitors. BG initially emerge from the VZ as radial glia that lose their apical process and later proliferate to match the concurrent expansion of the cerebellar cortex (reviewed in [57]). Our fate mapping results (Fig. 5d, magnification in **D′**) show that some S100β+ BG (~5%) intermingled with PC cell bodies are YFP immunopositive in wt Cre/YFP cerebella (not shown), indicating that they likely derive from bipotential (PC and astroglia) *Ebf2*+ precursors. While *Ebf2* contributes to the specification and survival of ZII− PCs, our results indicate that its mutation does not hamper BG cell specification.

**A Majority of Cerebellar Nuclear Neurons Derives from Ebf2+ Precursors** While *Ebf2* only decorates the medial nucleus and a small territory of the interposed nucleus in the adult cerebellum [50], it is not clear whether its expression spans all intermediate and lateral nuclei at early stages of development. By LacZ staining of adult (P60) cerebella harvested from Cre/βgal mice, we show that medial, interposed, and lateral nuclei are all positive for LacZ, indicating that a large majority of CN neurons transiently expresses *Ebf2* during cerebellar development (Fig. 6a). In Cre/YFP cerebella at the same stage, strong YFP expression is

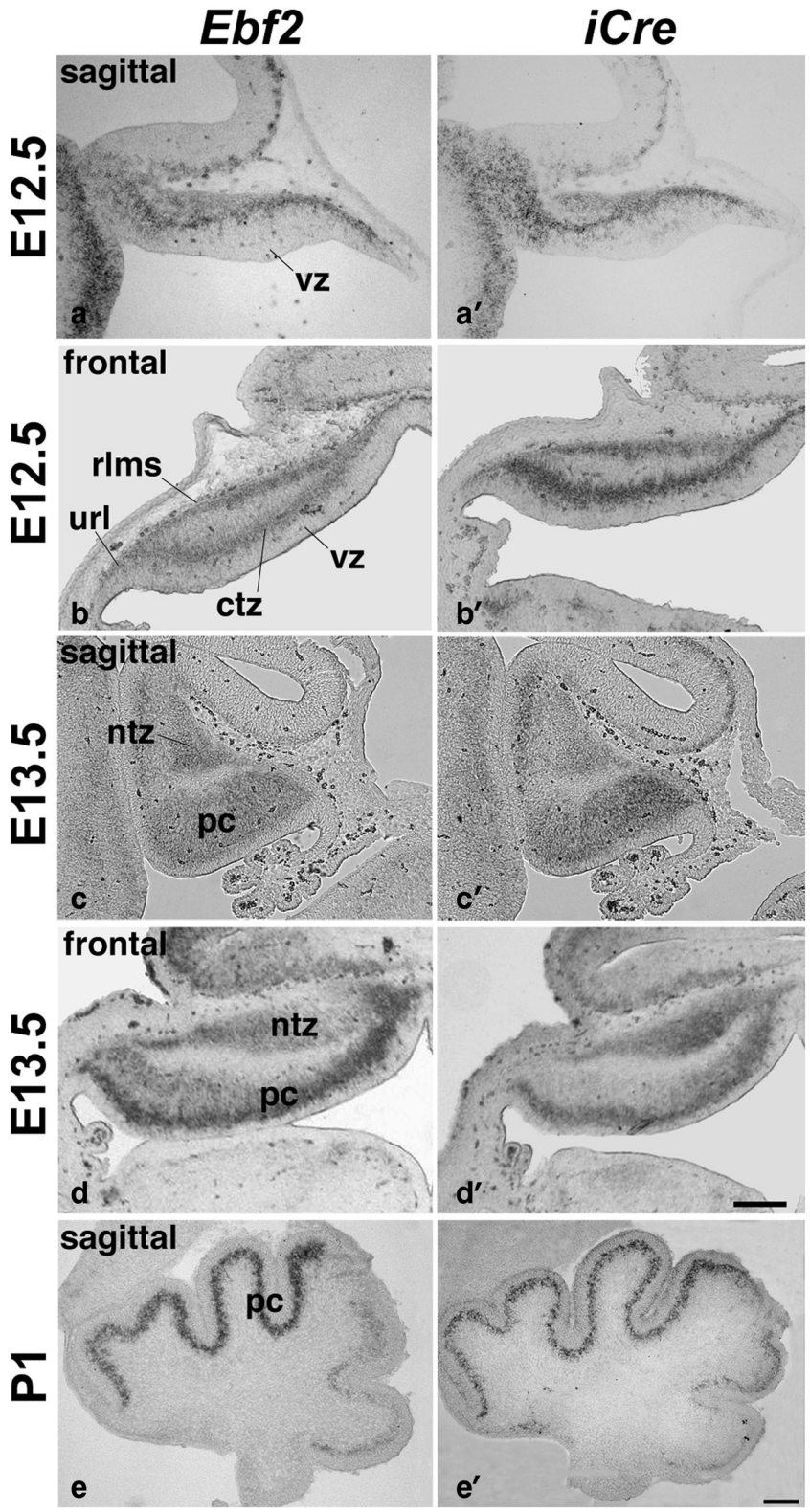


**Fig. 2** *Ebf2::Cre* expression overlaps with *Ebf2* transcript distribution in the cerebellar primordium. In situ hybridization with *Ebf2* (**A**–**E**) or *iCre* c-RNA probes (**A′**–**E′**) on adjacent sections of *Ebf2::Cre* embryos at different stages of cerebellar development (sectioning plane as indicated). vz, ventricular zone; ctz, cortical transitory zone; url, upper rhombic lip; rlms, rhombic lip migratory stream; ntz, nuclear transitory zone; pc, Purkinje cells. Size bars 100 μm (**A**–**D′**); 200 μm (**E**, **E′**)

observed in large projection neurons (Fig. 6b) positive for Smi32, which binds to a non-phosphorylated epitope of heavy MW neurofilament proteins (168 and 200 KDa subunits). The reporter is also active in some smaller-size GABA+ cell bodies (Fig. 6c). While hardly any colocalization is seen in cortical interneurons, this finding indicates that *Ebf2* may be expressed in early-born CN interneurons. Taken together, our results and those published previously indicate that *Ebf2* is initially expressed in all

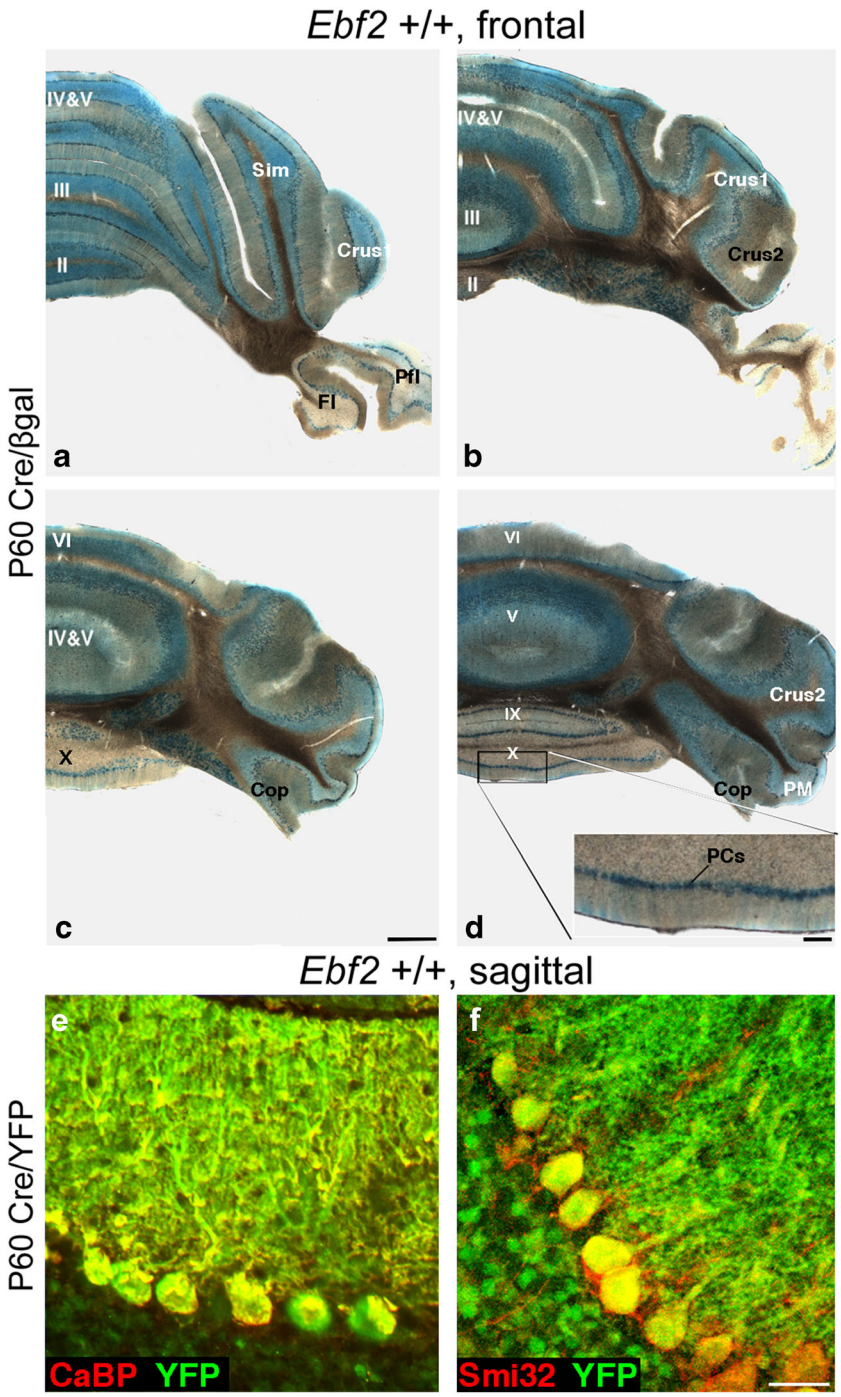


**Fig. 3** The Cre/βgal transgene is expressed in all postmitotic PC progenitors at P60. **a**–**d** X-gal staining on vibratome-sectioned (100 μm) Cre/βgal cerebella on a wild-type *Ebf2*+/+ background. Cre/βgal labels all PCs at all antero-posterior levels examined, including lobule X PCs (inset in **d**), which do not express *Ebf2* in the adult cerebellum. **e**, **f** Immunofluorescence on Cre/YFP transgenic cerebella on a wild-type *Ebf2*+/+ background. Ebf2 is coexpressed with CaBP and Smi32, two markers of PCs. Cop, copula pyramidis; Fl, floccule; Pfl, paraflocculle; Sim, simplex lobule; PM, paramedian lobule. Size bars: **a**–**d** 500 μm (bar in **c**); inset in **d**, 200 μm; **e**, **f** 50 μm

prospective CN and that its restriction to a subset of CN neurons [50] takes place during development.

**Medial Nuclei Are Reduced in the Ebf2 Null Cerebellum** By dual immunofluorescence for Smi32, we analyzed *Ebf2* wt and mutant cerebella. In agreement with the fact that the *Ebf2* transcript is restricted to the medial nuclei and a small territory in the interpositus nuclei in adult cerebella [50], we found the medial nuclei to be especially affected in the mutant (Fig. 6d, e), displaying a ~ 55% decrease in the number of Smi32+ projection neurons ($p < 0.005$, plotted in Fig. 6f).

## Discussion

In the cerebellum, *Ebf2* has been implicated in PC subtype specification and survival [47, 58], in the control of cerebellar

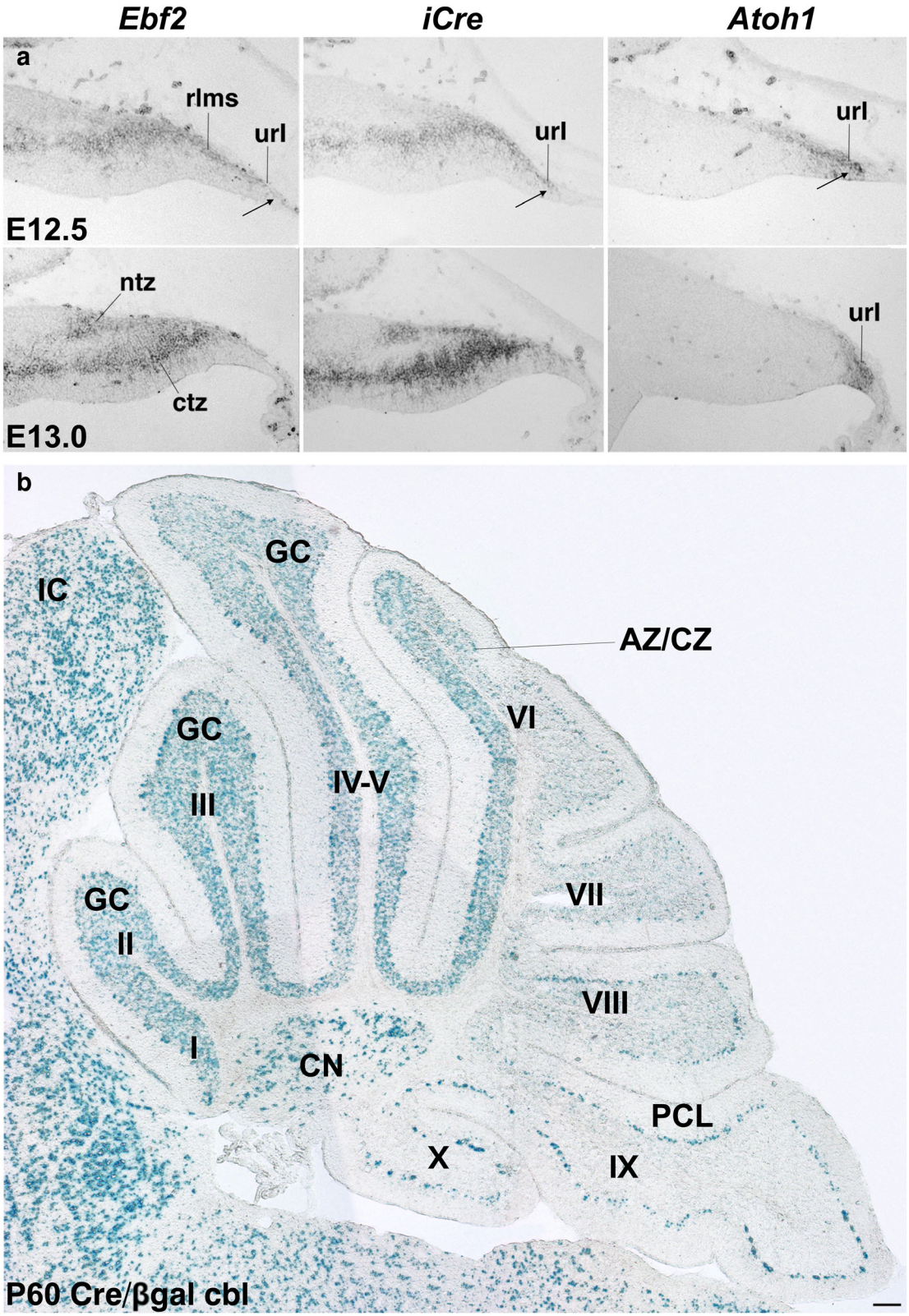


**Fig. 4** The *Ebf2::Cre* transgene is expressed in *Atoh1*+ progenitors. **a** In situ hybridization with *Ebf2*, *iCre*, or *Atoh1* c-RNAs on adjacent sagittal sections of *Ebf2::Cre* embryos at two stages of cerebellar development. *Ebf2* and *iCre* expression only overlap with the *Atoh1*+ domain at E12.5 (arrows). At this stage, *Atoh1*+ progenitors include early-born GCs. **b** X-gal stained sagittal section (20 μm) of the Cre/βgal cerebellum on an *Ebf2*+/+ genetic background. LacZ+ GCs, located in the anterior lobe and rostral half of lobule VI, correspond to the GCs born at around E12.5. The transgene is also expressed in all PCs and CN neurons. url, upper rhombic lip; rlms, rhombic lip migratory stream; ctz, cortical transitory zone; ntz, nuclear transitory zone; IC, inferior colliculus; GC, granule cells; prf, primary fissure; CN, cerebellar nuclei neurons; PCL, PC layer. Size bars 100 μm (**a**); 200 μm (**b**)

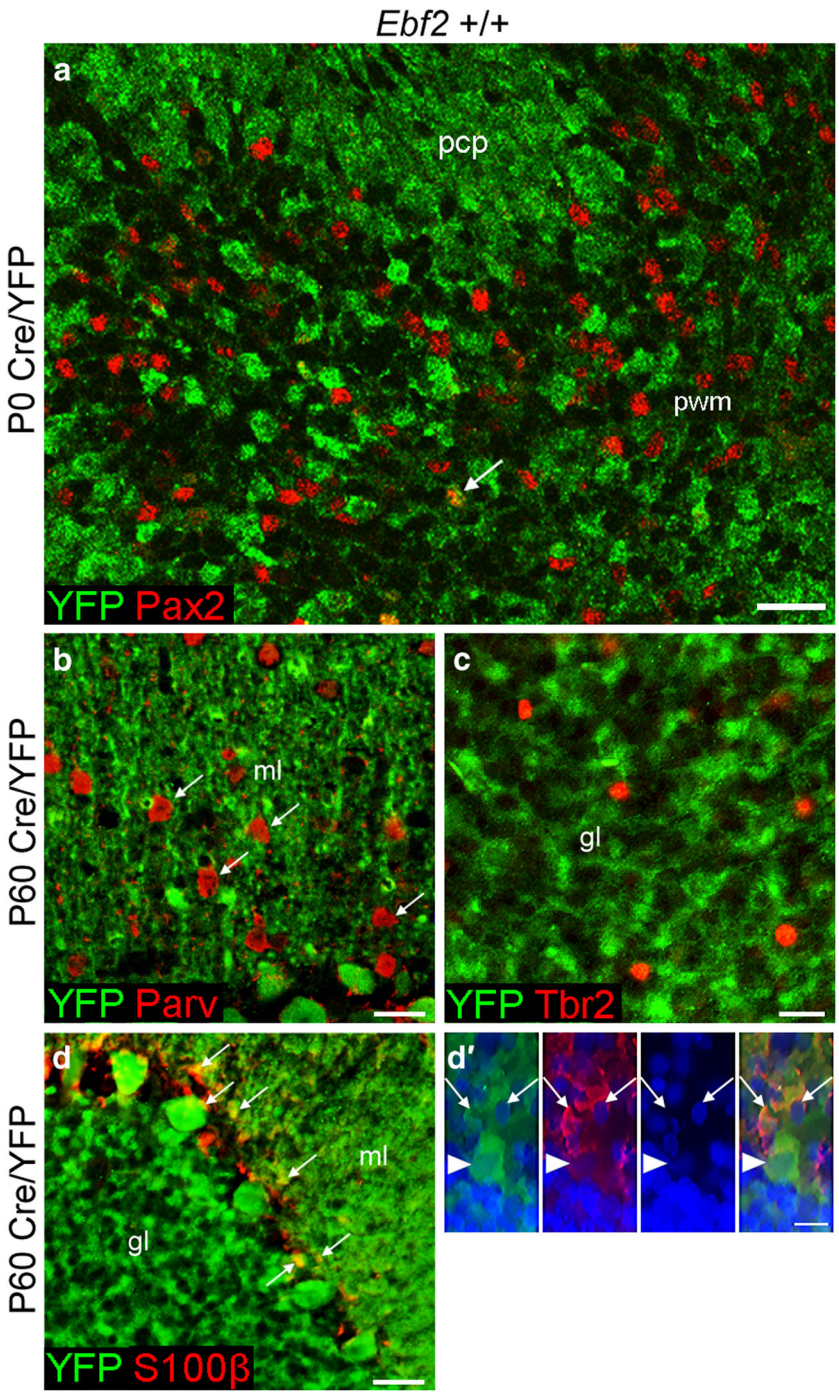


**Fig. 5** *Ebf2::Cre* lineage tracing labels Bergmann glia but not cortical interneuron precursors. **a** Pax2+ GABAergic interneuron progenitors (red nuclei) in newborn Cre/YFP transgenic mice. Arrows point to rare Pax2+ progenitors positive for YFP (yellow). **b** Parvalbumin-positive molecular layer interneurons (basket and stellate cells, red signal, arrows) are negative for YFP, like Tbr2+ unipolar brush cells (red nuclei) located in the GCL of lobule X (**c**). **d** Arrows point to S100β+ Bergmann glia (BG) immunopositive for YFP, indicating that BG precursors express *Ebf2*. gc, granule cell layer; ml, molecular layer; pcp, PC plate; pwm, prospective white matter. **D′** Colocalization of YFP (green) and S100 β (red) in presumptive BG cells (arrows); arrowhead points to PC soma. Size bars 40 μm (**a**); 25 μm (**b**, **d**); 30 μm (**c**); 25 μm (**D′**)

cortical patterning [10, 11], and in the regulation of PC firing and motor learning [48]. In the cerebellar cortex, this gene is expressed mainly in the anterior vermis. In the present paper, we present the results of genetic fate mapping used to dissect the spatio-temporal distribution of the *Ebf2* transcript in the developing cerebellum.

**Ebf2 Expression in the PC Lineage Is Ubiquitous at First, Then Becomes Spatially Restricted During Development** *Ebf2* belongs to a phylogenetically conserved family of terminal selector genes, a class of regulatory factors that bestow cell fates on early postmitotic precursors. Selector genes may play a role in the generation of neuronal types and subtypes both through their molecular interaction with factors restricted to specific subterritories [35] and through their own temporally dynamic expression, regulated by feed-forward loops to specify unique cell fates or suppress alternate ones. This regulation is used by many stem cell lineages to generate diversity [58, 59].

PC progenitors undergo terminal cell division in the subventricular zone of the 4th ventricle between E10

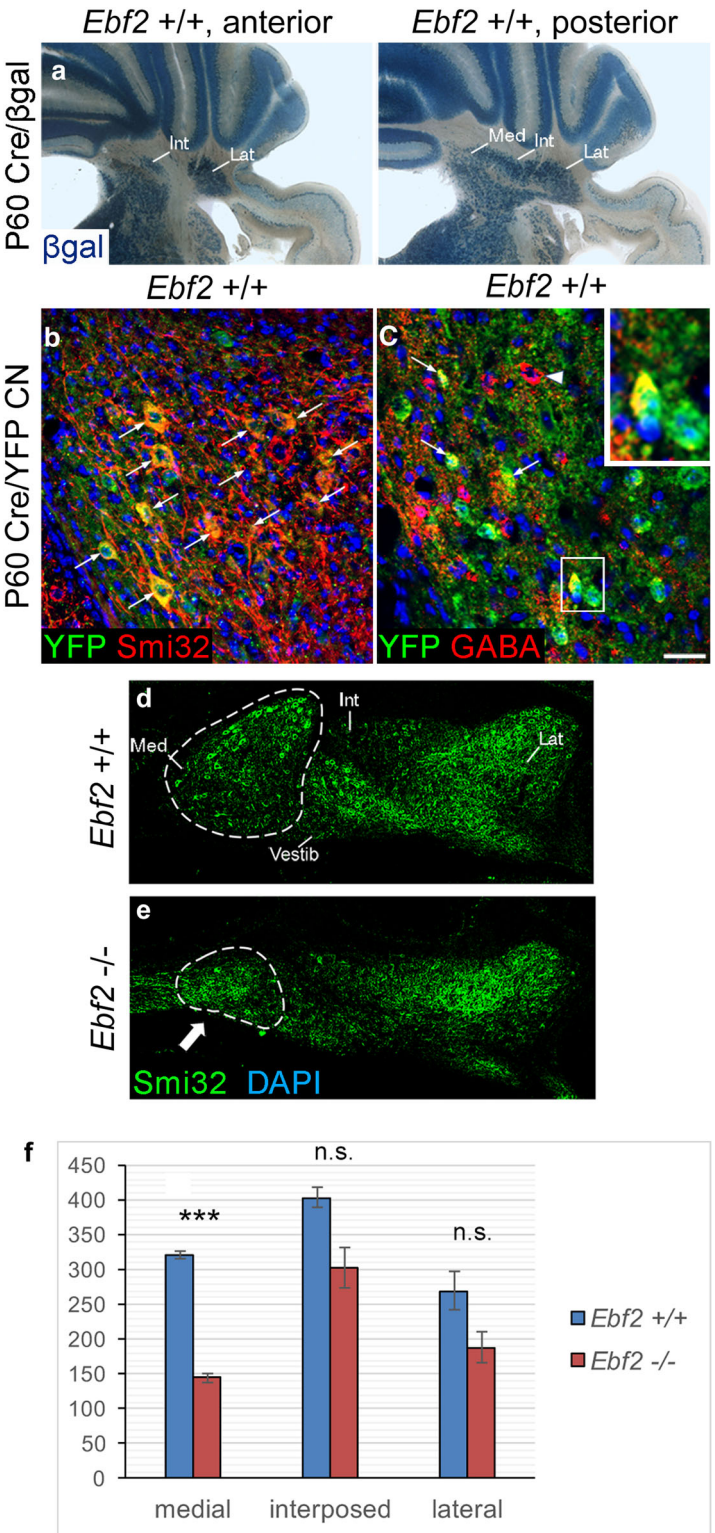


**Fig. 6** The medial nuclei contain fewer Smi32+ projection neurons in the *Ebf2* null mouse. **a** Two consecutive LacZ-stained frontal sections (150 μm) of a Cre/βgal cerebellum on a wild-type background reveal that lateral, interpositus, and medial nuclei are tagged by *Ebf2::Cre*. **b** Smi32- and YFP-immunostained sagittal sections of the Cre/YFP CN. Arrows point to double-positive cells, indicating that a majority of Smi32 CN neuron precursors express *Ebf2*. **c** GABA- and YFP-immunostained sagittal sections of the Cre/YFP CN. Arrows point to double-positive cells, indicating that some GABAergic CN neuron precursors express *Ebf2* during development. See inset in **c**. **d**, **e** Smi32-immunostained frontal sections of the wild-type and *Ebf2* null CN, respectively, medial to the left. Note the decreased number of immunopositive cells, in the medial nucleus (solid white arrow). **f** Cell counts performed in wt and *Ebf2*−/− CN confirm the evidence shown in **b**, demonstrating a significant decrease in the number of Smi32+ projection neurons in the medial nuclei. $n = 3$; data are mean ± s.e.m.; Welch's $t$ test, $^{***}p = 0.0045$. Int, interpositus; Med, medial (fastigial); Lat, lateral. Size bars 30 μm (**b**, **c**); 200 μm (**d**, **e**)

and E13. The earliest-born PCs (E10–11.5) are mainly destined to become the *Ebf2*−/ZII+ population in the adult. The later-born cohort becomes the *Ebf2*+/ZII− subset. From previous studies of PC phenotype specification in the *Ebf2* null mutant, it was concluded that a key role of *Ebf2* was to suppress the ZII+ phenotype [10, 11]. However, the data presented here clearly show that all PCs express *Ebf2* at an early stage of development. This is reminiscent of fate specification mechanisms operating in mouse motor neurons [36] and in *Caenorhabditis*

*elegans* and *Drosophila*, involving the *Ebf2* gene orthologs *unc-3* and *col*, respectively [35, 58, 59]. Our results suggest that subtype specification via *Ebf2* may involve an initial step of activation and a subsequent step at which *Ebf2* is repressed. In prenatal Purkinje neuron progenitors, the restriction of *Ebf2* gene expression likely occurs before E17 (see Fig. 2 E, I, [11]), and further studies are required to uncover the nature of regulatory signals (transcriptional and/or epigenetic regulators) repressing *Ebf2* transcription in prospective ZII+ PC precursors. Unpublished evidence (L.C. and G.G.C.) indicates that *Ebf2* mRNA levels are highly upregulated in the *Ebf2* null mutant cerebellum, suggesting negative autoregulation. Moreover, *Ebf2* expression could be downregulated by Notch signaling both at the transcriptional level [31] and via protein-protein interactions (see [32, 60]). Likewise, ZFP423, a molecular interactor of all three EBF TFs [60–62], maintains the stem cell progenitor pool that supports the birth of ZII− PC precursors and promotes their differentiation [63].

**Ebf2 Is Transiently Expressed in a Subset of Early-Born GC Progenitors Populating the Anterior Lobe** Our *Ebf2* fate mapping results show labelling of a subset of GCs restricted to the anterior cerebellum (AZ), with a transition in the granular layer located at the AZ/CZ boundary (Fig. 4b). A boundary at this location has been described from expression data (e.g., Otx1/2 [64]), lineage distributions in embryonic stem cell chimeras [65], the effects of mutations (e.g., NeuroD [66]), and the results of birthdating experiments [16]. GCs born by E12.5 and labeled by *Ebf2::Cre* mostly populate the anterior lobe, with a sharp anteroposterior boundary around the primary fissure. It is intriguing that the restriction of the *Ebf2*-tagged GC population to the AZ mirrors that of the adult *Ebf2*+ PCs (see Fig. 2 and [11]).

GCs are generated from the upper rhombic lip. GC precursors migrate out from the rhombic lip across the cerebellar surface to completely cover it with a secondary proliferative zone, the external granular layer (EGL). From there, postmitotic GCs descend through the nascent molecular layer to form the granular layer (~E17-P20). *Ebf2* expression by the anterior GC lineage seems already active early in EGL development. Ebf2::Cre+ EGL precursors are already found in the Atoh1+ upper rhombic lip at E12.5 (Fig. 2B). No *Ebf2*+ GCs are observed in adult cerebella (see [10, 11]), indicating that the gene is transiently expressed in the rhombic lip migratory stream and is downregulated thereafter (presumably before P1, since there is already no evidence of *Ebf2* expression in the newborn EGL (e.g., Fig. 2E).

Finally, the number of anterior lobe GCs is clearly reduced in the anterior lobe of the *Ebf2* null cerebellum, in which both PCs and GCs are tagged by *Ebf2::Cre* [11]. We have not characterized this aspect in detail because the number of adult GCs depends on both cell-autonomous factors (e.g., [67]) and the lack of mitogen released by PCs .

**Ebf2 Gives No Cell-Autonomous Contribution to Cortical Interneuron Progenitor Development, but Is Transiently Expressed in BG Precursors** Our results indicate that inhibitory interneuron progenitors of the cerebellar cortex never express *Ebf2*, ruling out a cell-autonomous contribution of this gene to their development. Forced expression of *Ebf2* in *Ascl1*+ or *Pax2*+ progenitors would be necessary to determine if forced *Ebf2* expression in interneuron progenitors interferes with their ability to leave the VZ, invade the prospective white matter, and start evolving into various types of GABAergic interneurons under the guidance of local extracellular cues. Likewise, *Ebf2* provides no cell-autonomous contribution to UBC development, although it has been established that the distribution of UBCs in the cerebellar cortex depends critically on the establishment of a correct cortical topography, which in turn requires EBF2 function.

At odds with cortical interneurons, a small number of BG progenitors do transiently express *Ebf2*, although BG development and proliferation are not obviously affected by *Ebf2* mutation (data not shown). Thus the role, if any, of this gene in BG development remains unclear. Taken together, these results show that within the pool of *Ptf1a*+ progenitors, *Ebf2* expression is shared by the PC and BG precursor lineage, but not by *Pax2*+ cells fated to generate GABAergic interneurons of the cerebellar cortex.

**Ebf2 Mutation Affects the Number of Medial Nuclear Neurons** *Ebf2* mutation reduces the number of neurons in the medial CN. *Ebf2::Cre* tagging reveals that while in the adult cerebellum *Ebf2* is restricted to the anterior part of the medial nucleus, plus a small territory in the interpositus nucleus, this gene is expressed throughout the CN during development. This mirrors the progressive restriction of expression seen in the PC population.

In the *Ebf2* null cerebellum, this restriction is accompanied by an overall reduction in the number of Smi32+ cells, i.e., mostly glutamatergic, nucleo-fugal projection neurons, especially in the medial nuclei. Interestingly, the anterior part of the medial nucleus is likely the main target area for Ebf2+/ZII− corticonuclear afferents, with the ZII+/Ebf2− projections concentrated in the more posterior part. This suggests that defects in the corticonuclear PCs of the predominantly Ebf2+/ZII− anterior vermis (AZ), plus cell-autonomous defects in the anterior medial nucleus, may combine to eliminate a neuronal subpopulation.

## Conclusions

In summary, *Ebf2*, a terminal selector gene that controls PC development and survival as well as cerebellar cortical patterning, contributes dynamically to the patterning of the cerebellar cortex and nuclei, affecting development of the latter. *Ebf2* may be part of a complex combinatorial code operating at different stages in cerebellar phenotype specification, differentiation, and the formation of connectivity.

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### Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experimental plan was designed in agreement with the stipulations of the San Raffaele Institutional Animal Care and Use Committee (permit number 336).

**Conflict of Interest** The authors declare that they have no conflicts of interest.

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