Genetic deletion of genes in the cerebellar rhombic lip lineage can stimulate compensation through adaptive reprogramming of ventricular zone-derived progenitors

Alexandre Wojcinski¹, Morgane Morabito¹, Andrew K. Lawton¹, Daniel N. Stephen¹ and Alexandra L. Joyner¹,²*

Abstract

Background: The cerebellum is a foliated posterior brain structure involved in coordination of motor movements and cognition. The cerebellum undergoes rapid growth postnatally due to Sonic Hedgehog (SHH) signaling-dependent proliferation of ATOH1+ granule cell precursors (GCPs) in the external granule cell layer (EGL), a key step for generating cerebellar foliation and the correct number of granule cells. Due to its late development, the cerebellum is particularly vulnerable to injury from preterm birth and stress around birth. We recently uncovered an intrinsic capacity of the developing cerebellum to replenish ablated GCPs via adaptive reprogramming of Nestin-expressing progenitors (NEPs). However, whether this compensation mechanism occurs in mouse mutants affecting the developing cerebellum and could lead to mis-interpretation of phenotypes was not known.

Methods: We used two different approaches to remove the main SHH signaling activator GLI2 in GCPs: 1) Our mosaic mutant analysis with spatial and temporal control of recombination (MASTR) technique to delete Gli2 in a small subset of GCPs; 2) An Atoh1-Cre transgene to delete Gli2 in most of the EGL. Genetic Inducible Fate Mapping (GIFM) and live imaging were used to analyze the behavior of NEPs after Gli2 deletion.

Results: Mosaic analysis demonstrated that SHH-GLI2 signaling is critical for generating the correct pool of granule cells by maintaining GCPs in an undifferentiated proliferative state and promoting their survival. Despite this, inactivation of GLI2 in a large proportion of GCPs in the embryo did not lead to the expected dramatic reduction in the size of the adult cerebellum. GIFM uncovered that NEPs do indeed replenish GCPs in Gli2 conditional mutants, and then expand and partially restore the production of granule cells. Furthermore, the SHH signaling-dependent NEP compensation requires Gli2, demonstrating that the activator side of the pathway is involved.

Conclusion: We demonstrate that a mouse conditional mutation that results in loss of SHH signaling in GCPs is not sufficient to induce long term severe cerebellum hypoplasia. The ability of the neonatal cerebellum to regenerate after loss of cells via a response by NEPs must therefore be considered when interpreting the phenotypes of Atoh1-Cre conditional mutants affecting GCPs.

Keywords: Cerebellum, SHH signaling, GLI2, Nestin-expressing progenitors, Neurogenesis, Atoh1-Cre, Regeneration

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Background

The cerebellum (CB) consists of 80% of the neurons in the human brain [1] (60% in mouse [2]), and is involved in balance and motor coordination, but also modulates language, reasoning and social processes via its connections throughout the forebrain [3–7]. The CB undergoes its major growth in the third trimester and infant stage in humans, and the first 2 weeks after birth in mice, primarily due to expansion of the granule cell precursor (GCP) pool in the external granule cell layer (EGL) [8–10]. Given the late development of the CB compared to other brain regions, the CB is particularly sensitive to environmental and clinical factors that impact on growth (or cause injury) around birth. Furthermore, CB hypoplasia and perinatal injury is the second leading factor associated with autism [11]. It is therefore important to identify genes that regulate cerebellum development. Many of the genes have been identified based on motor defects in homozygous null mutant mice, or in conditional mutants that remove genes in specific cell lineages. Intrinsic growth compensation mechanisms involving lineages where the gene does not function could however, obscure the normal function of a gene in cerebellar growth.

The CB develops from two germinal zones. The ventricular zone (VZ) gives rise to all the inhibitory neurons, including Purkinje cells (PCs) [12] as well as Nestin-expressing progenitors (NEPs) that expand in the cerebellar cortex after birth to produce astrocytes, including specialized Bergmann glia, and late born interneurons of the molecular layer [13, 14]. Ptf1aCre mice have been used to delete genes in inhibitory neurons and some glia [15]. Excitatory neurons including granule cells (GCs) originate from the upper rhombic lip [16–18]. In mice, the EGL is established between embryonic day (E) 13.5 and E15.5. Atoh1-expressing GCPs then proliferate and expand in the EGL until postnatal day (P) 15 in response to Sonic Hedgehog (SHH) secreted by PCs [19–21]. When GCPs become postmitotic, they migrate down Bergmann glial fibers to form the internal granule cell layer (IGL). Interestingly, in rodent models the developing CB has been found to have a remarkable ability to recover from some injuries [22–24]. Indeed, we recently found that proliferating cerebellar GCPs can be replaced via adaptive re-programming of NEPs after an acute depletion of the perinatal EGL due to irradiation [25–27]. Thus, NEPs in the neonatal CB have highly plastic behaviors. However, whether NEPs are harnessed to replenish cells lost in developmental mutants that lack key factors required for expansion and survival of GCPs has not been addressed.

One of the major pathways driving CB development is HH signaling. There are three hedgehog (Hh) genes in mammals, Indian (Ihh), Desert (Dhh) and Shh [28, 29]. Shh, the most widely expressed Hh gene, is required for development of most organs [29] by regulating a variety of cell behaviors including cell death, proliferation, specification and axon guidance. The cellular context (i.e. tissue, developmental stage, convergence of other signaling pathways) and concentration of SHH are thought to determine the particular response of a cell to SHH. HH signal transduction is mediated by the receptors Patched1 (PTCH1) and Smoothened (SMO) [28–30]. In the absence of HH signaling, PTCH1 constitutively represses SMO activity, whereas HH binding relieves this inhibition, in part by allowing accumulation of SMO in cilia [31]. The GLI/Ci transcription factors are the effectors of the HH pathway. In mammals, the transcriptional activator (A) and repressor (R) functions of the GLIs have been divided between the three proteins [32]. A general rule is that high levels of HH signaling induce the formation of a GLI2 activator (GLI2A) and this leads to transcription and translation of an addition activator, GLI1A, while a reduction or absence of the ligand allows for the formation of a GLI3 repressor (GLI3R). Importantly, we demonstrated that Gli1 expression is dependent on GLI2/3A, and thus is only expressed in cells receiving a high level of HH signaling [33, 34]. The three Gli genes, Shh, Smo, Patch1 and Patch2 are expressed in the CB and all but Patch2 are required for CB development [20, 21, 35–37]. In particular, we have shown that SHH functions by inducing GLI1A/2A and is required for expansion of GCPs, primarily after birth [20, 38], whereas Gli3 is not required in the cerebellum after E12.5 [36]. In addition to the crucial role of SHH in generating the pool of GCs, expansion of NEPs and thus production of NEP-derived interneurons and astroglia (astrocytes and Bergmann glia) also require SHH-signaling [13, 25, 39]. Furthermore, HH-signaling in NEPs is crucial for expansion of NEPs, recovery of the EGL and scaling of interneuron numbers after injury to the EGL [25].

Here we report that deletion of Gli2 in the vast majority of the GCPs is not sufficient to induce major cerebellar hypoplasia. Using our MASTR technique [40] in a mosaic mutant analysis of the effect of deleting Gli2 in scattered GCPs, we found that HH/Gli2-signaling is indeed necessary to maintain GCPs in an undifferentiated and proliferative state and to promote their survival. However, and similar to when the EGL is depleted using irradiation, we uncovered that NEPs are harnessed to repopulate the EGL and then wild type progenitors differentiate into GCs when Gli2 is deleted in most GCPs using an Atoh1-driven constitutive Cre [41]. Our results not only provide more evidence for the unusual ability of the CB to recover from perinatal stress, but also reveal that NEP-dependent compensation should be taken into account when studying genes implicated in GCP development or survival and when using the Atoh1-Cre transgene.
Methods

Animals

The following mouse lines were used: Gli2<sup>flox/flox</sup> [20], Atoh1-Cre [41], Atoh1-FlpoER, Nestin-FlpoER (a transgene similar to that described in [40]) and Rosa26-MASTR(frt-STOP-frt-GFPcre) [40], Atoh1-GFP [42], Nes-CFP [43], Rosa26<sup>FTRT-STOP-FTRT-DTom</sup> (Jackson Laboratory, 021875). The Atoh-FlpoER line was made using the FLPoER1 cDNA described in [40] by subcloning it into the Atoh1 expression construct described in [17]. All mouse lines were maintained on an outbred Swiss Webster background and both sexes were used for the analysis. Animals were housed on a 12 h light/dark cycle and were given access to food and water ad libitum. All experiments were performed using mice ages P0–P30.

Tamoxifen (Tm, Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich) at 20 mg/ml. P2 Atoh1-FlpoER/+; R26<sup>MASTR/+</sup>; Gli2<sup>flox/flox</sup>, Atoh1-FlpoER/+; R26<sup>MASTR/+</sup>; Gli2<sup>flox/flox</sup> and P0 Nes-FlpoER/+; R26<sup>MASTR/+</sup>; Gli2<sup>flox/flox</sup> and P0 Nes-FlpoER/+; R26<sup>MASTR/+</sup>; Atoh-GFP/+ mice as well as Nestin-FlpoER/+; R26<sup>MASTR/+</sup>; Gli2<sup>flox/flox</sup> and Atoh1-Cre/+, Gli2<sup>flox/flox</sup> and Nestin-FlpoER; R26<sup>MASTR/+</sup>; Atoh1-Cre/+, Gli2<sup>flox/flox</sup> mice and littermate controls received one 200 μg/g dose of Tm via subcutaneous injection. 50 μg/g 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) was administered via intraperitoneal injection (10 mg/ml in sterile saline) one hour before the animals were sacrificed.

Tissue processing, immunohistochemistry (IHC) and transcript detection

For animals younger than P4, they were anaesthetized by cooling and brains were dissected out and fixed in 4% paraformaldehyde overnight at 4°C. Animals P4–30 received 50 μl intraperitoneal injections of ketamine and received ice-cold PBS via transcardial perfusion followed by 4% paraformaldehyde. Brains were collected and submerged in 4% paraformaldehyde overnight at 4°C. Tissues were processed for frozen embedding in optimal cutting temperature (OCT) compound and sectioned in the parasagittal plane on a Leica cryostat at 12 μm. For IHC, sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-Ki67 (Thermo Scientific, RM-9106-S0), mouse anti-P27 (BD Pharmingen, 610,241), rabbit anti-PAX6 (Millipore, AB2237), goat anti-GLI2 (R&D System, AF3635), Goat anti-SOX2 (R&D System, AF2018), rabbit anti-GFP (Life Technologies, A11122), rat anti-GFP (Nacalai Tesque, 04404–84), mouse anti-NeuN (Millipore, MBA377) diluted in PBS with 5% BSA (Sigma-Aldrich) and 0.3% Triton X-100 (Fisher Scientific). Sections were then exposed for 2 h at room temperature to secondary species-specific antibodies conjugated with the appropriate Alexa Fluor (1:500; Invitrogen). EdU was detected using a commercial kit (Life Technologies) after the IHC reactions. TUNEL staining and in situ hybridization were performed according to standard protocols. Cre and Gli1 cDNAs were used as the template for synthesizing digoxigenin-labeled riboprobes. Images were collected on a DM6000 Leica microscope and processed using Photoshop software.

Live imaging

Ex vivo cerebellar slice culture was done as previously described [25]. Briefly, P8 cerebella were embedded in 2.5% low-melting point agarose and sagittally sliced at 250 μM on a Vibratome. Slices were immediately taken to either a Leica TCS SP8 or SP5 confocal microscope platform. Slices were maintained in Eagle’s Basal Medium with 2 mM L-glutamine, 0.5% glucose, 50 U/ml Penicillin-streptomycin, 1xB27 and 1xN2 supplements at 37°C and 5% CO₂. Image stacks were acquired every 5 min for ~4 h. Cell tracking was performed using Imaris software. The autoregressive tracking function was employed with a sport size of 6 μM and a step size of 7 μM. Manual correction was performed.

Quantifications and statistical analyses

ImageJ software was used to measure the area (mm²) of cerebellar sections near the midline. For all IHC staining, cell counts were obtained using ImageJ and NeuroLucida Software. For each developmental stage, three sections were analyzed per animal and ≥3 animals. Statistical analyses were performed using Prism software (GraphPad) and significance was determined at P < 0.05. All statistical analyses were two-tailed. For two-group comparisons with equal variance as determined by the F-test, an unpaired Student’s t test was used. Welch’s correction was used for unequal variance. P values are indicated in the figures. No statistical methods were used to predetermine the sample size, but our sample sizes are similar to those generally employed in the field. No randomization was used. Data collection and analysis were not performed blind to the conditions of the experiments.

Results

Mosaic analysis reveals SHH-GLI2 signaling is critical for maintaining GCPs in an undifferentiated proliferative state and promoting their survival

Our previous studies demonstrated that loss of the majority of HH-signaling in the entire CB at mid-gestation (Nes-Gli2 conditional knockout or CKO - Nestin-Cre; Gli2<sup>flox/flox</sup> mice) results in an almost complete lack of GCPs at birth and a very diminished CB in adults [20]. Since HH-signaling is required after birth in NEPs for their expansion and production of late born interneurons and astrocytes in the CB [13], it is possible that part
of the phenotype observed in Nes-Gli2 CKOs was due to loss of HH-signaling in Non-GCP cells. We therefore took two approaches to test the cell autonomous requirement for HH signaling in GCPs. First we used the R26\textsuperscript{MERTK-GFP} MASTR allele (R26\textsuperscript{MASTR}) [40] and an Atoh1-\textsuperscript{FlpoER} transgene to knock out Gli2 in scattered GCPs at ~P3 by administering Tamoxifen (Tm) at P2 and analyzed the percentage of undifferentiated GFP+ GCPs (GFP+ cells in the proliferating outer EGL/total GFP+ cells – proliferating and post mitotic) at both P4 and P8 (Fig. 1a-c). We did indeed observe a significant decrease in the percentage of GFP+ cells that were GCPs in the medial CB (vermis) of P8 Atoh1-M-Gli2 CKOs (R26\textsuperscript{MASTR/}; Atoh1-\textsuperscript{FlpoER/+}; Gli2\textsuperscript{floxed/floxed} mice; n = 3) compared to Atoh1-M-Gli2 heterozygous (het) controls (R26\textsuperscript{MASTR/}; Atoh1-\textsuperscript{FlpoER/+}; Gli2\textsuperscript{floxed/floxed} mice; n = 3) (29.79% compared to 67.09%) (Fig. 1d). Using a 1 h pulse of EdU, we found that the proliferation index (#EdU+/GFP+ cells in the outer EGL/total GFP+ cells in the outer EGL) of Atoh1-M-Gli2 CKO GCPs was significantly decreased compared to controls (n = 3; 14.39% compared to 29.06%) (Fig. 1e). At P4, there was no significant difference in the percentage of undifferentiated GFP+ GCPs between Atoh1-M-Gli2 CKOs and controls (p = 0.162) (Fig. 1d). However, we observed a significant decrease in the proliferation index in P4 Atoh1-M-Gli2 CKO cerebella (CKO vs control, p = 0.015) (Fig. 1e). At P4, only 2 days after Tm injection, the number of GFP+ cells in the oEGL was not significantly reduced (CKO vs control, p = 0.081) (Additional file 1: Figure S1a). Nevertheless, TUNEL staining revealed a significant increase in cell death in the entire EGL at P4 (69.44 ± 7.76/section in mutants vs 37.67 ± 5.1 in controls, p = 0.027). We performed the same analysis in the lateral CB (hemispheres) and found a similar outcome (Additional file 1: Figure S1b-d). These results reveal that HH-signaling through Gli2 plays an important role in maintaining GCPs in an undifferentiated state, and also promotes their proliferation and survival.

As an alternative approach to a mosaic mutant analysis, we deleted Gli2 in the vast majority of GCPs (Atoh1-Cre/+; Gli2\textsuperscript{floxed/floxed} or Atoh1-Gli2 CKOs). Consistent with previous studies using whole cerebellum Cre transgenes and our mosaic analysis, at P0 the anterior vermis of Atoh1-Gli2 CKOs (n = 5) appeared consistently smaller than controls (Gli2\textsuperscript{floxed/floxed}) and the EGL was greatly diminished (Fig. 1g-j). SHH-Gli2 signaling loss was confirmed by the lack of Gli1 expression in the EGL of Atoh1-Gli2 CKO cerebella (Fig. 1g-h). Moreover, proliferation (Ki67) in the outer EGL and differentiation (P27 marking post mitotic cells in the inner EGL) were disrupted in the mutant EGL since two distinct EGL layers were not present (Fig. 1i-j). Interestingly, we observed an apparent increase of Gli1 expression in the Purkinje cell layer (PCL) suggesting that deletion of Gli2 in the EGL induced a cell non-autonomous up-regulation of HH-signaling in this layer (star in Fig. 1g-h). The lack of a phenotype in the posterior vermis is likely explained by low expression of Cre [44] in this region and thus low recombination [45] (Additional file 2: Figure S2).

All together, these results confirm a major role played by SHH-signaling through GLI2 to promote the expansion of the EGL and thus ensure the generation of the correct number of GCPs.

The size of the Atoh1-Gli2 KO cerebellum progressively recovers after birth

We have recently shown that the size of the CB can recover to ~80% its normal size after postnatal injury (irradiation) to the EGL [25]. To test whether genetic ablation of Gli2 in the EGL can trigger a similar recovery mechanism, we analyzed the phenotype of adult (P30) Atoh1-Gli2 CKO cerebella. The area of mid-sagittal sections of P30 animals was quantified, and revealed only a 21.7 ± 12.0% reduction (n = 6) in Atoh-Gli2 CKOs compared to littermate controls (Fig. 2a-d). Unlike normal mice, we observed a large variability in the area of the midline cerebellum and degree to which the IGL was depleted in mutants, indicating some mutant cerebella recovered the EGL better than others (compare Fig. 2b and c). We then measured the area of mid-sagittal cerebellar sections from P4, P8 and P12 mice to determine the growth trajectory of mutant mice compared to controls (Fig. 2e-j). The size of Atoh1-Gli2 CKOs cerebella was significantly reduced by ~20% at all time points (Fig. 2k-l). Since our mosaic mutant results showed a similar behavior of Gli2 CKO GCPs in the hemispheres and vermis, we analyzed the phenotype of Atoh1-Gli2 CKOs in the hemisphere. Curiously, unlike the vermis we did not observe a significant decrease in the size of the mutant hemispheres at P30 compared to controls (p = 0.152) (Fig. 3a-c). Analysis of hemispheric sagittal sections from P4 (n = 3), P8 (n = 3) and P12 (n = 3) revealed how the cytoarchitecture recovered (Fig. 3d-i). Interestingly, whereas the vermis of Atoh1-Gli2 CKOs mice at P8 showed a clear hypoplasia phenotype, the hemispheres exhibited extra folia (arrow in Fig. 3g), suggesting different compensation responses in the two locations of the CB in Atoh1-Gli2 CKOs.

In summary, we found that depletion of the EGL at P0 by removing Gli2 from embryonic GCPs is not sufficient to induce consistent major hypoplasia of the vermis at P30. This raised the possibility of a compensation mechanism that allows partial recovery of the developing CB after genetic depletion of the EGL.

Wild type cells replenish the anterior EGL of Atoh1-Gli2 CKOs

Since the final size of the CB is largely dependent on the expansion of the EGL, we analyzed Atoh1-Gli2 CKO...
Fig. 1 (See legend on next page.)
to our previous study using irradiation at P1 [25], the cerebella at P8 when the EGL is normally thick. Similar expression of Gli1 at P0 (Fig. 1h), the EGL of P8 with Gli1 mRNA on P0 mid-sagittal cerebellar sections of Gli2lox/lox (control, black) and Atoh1-Gli2 CKO, red) mice. Black arrowhead indicates the loss of Gli1 expression in the anterior mutant EGL, black arrow Gli1 remaining in the posterior EGL and yellow arrowheads indicate Gli1 expression in Bergmann glia in the Purkinje Cell Layer (PCL). j FIHC detection of the indicated proteins and dapi on P0 mid-sagittal cerebellar sections of Gli2lox/lox (control, i) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, j) mice. High power images are shown of the areas indicated by white rectangles and the thickness of the EGL is indicated by yellow bracket. Scale bars represent 100 µm (b-c) and 500 µm (g-j).

Fig. 1 The size of the cerebellum partially recovers in Atoh-Gli2 CKOs over time. a-c Mid-sagittal sections of P30 Gli2lox/lox (control, a) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, b-c) cerebella stained with Hematoxilin and Eosin (H&E). d Graph of the area of mid-sagittal CB sections of P30 Gli2lox/lox (control, black) (n = 6); Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, red) (n = 6) mice. e-j Mid-sagittal CB sections of P4 (e, f); P6 (g-h) and P12 (i-j) Gli2lox/lox (control, b, g and i) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, f, h and j) mice stained with H&E. k Graph of the area of 3 mid-sagittal sections of Gli2lox/lox (control, P0: n = 3, P4: n = 3; P8: n = 3, P12: n = 3 and P30: n = 6) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, P0: n = 3, P4: n = 3, P8: n = 4, P12: n = 6 and P30: n = 6) cerebella. I Graph showing the decrease in area of 3 mid-sagittal sections of Atoh1-Gli2 CKO cerebella as a percentage of controls during development. All graphical data are presented as means ± SEM and significance determined using two-tailed T-test. Scale bars represent 1 mm.
outer EGL (oEGL) by seemingly delaying their differentiation compared to controls as revealed by the increase [oEGL/EGL area] ratio \( (n = 4; 68.91 \pm 2.44\% \text{ vs } 53.85 \pm 0.94\% ) \) (Fig. 4f). Analysis of Cre expression revealed that only a small subset of cells in the anterior EGL expressed Cre in the anterior CB compared to controls \((\text{Atoh1-Cre/++; Gli2 flox/+ or Atoh1-Gli2 het})\) (Fig. 4g-h). Consistent with the presence of wild type (WT) cells in the EGL, GLI2 protein was detected in most cells in the EGL (Fig. 4i-i'). Interestingly, GCPs in the partially rescued anterior EGL expressed a low level of the stem cell marker SOX2 at P8, something that was never observed in control animals (Fig. 4j-k). We thus hypothesized that WT NEPs (SOX2+) are able to change their fate to become GCPs and replenish the EGL as part of a compensation mechanism in Atoh1-Gli2 CKOs. Using a Nestin-FlpoER transgene \([40]\) and a Flippase (Flp)-dependent R26 reporter allele that expresses TDTom, we performed genetic inducible fate mapping (GIFM) of NEPs in the Atoh1-Gli2 CKO cerebella. In contrast to P30 controls \((\text{Nestin-FlpoER/+; R26 frt-STOP-frt-TDTom/+ or Nes-TDTom mice administered Tm at P0})\), we observed many TDTom+ cells in the EGL of Atoh1-Gli2 CKOs at P0 stimulates a compensation process that leads to replenishment of the GCPs by WT cells.

**NEPs switch their fate to become GCPs and produce GCs in Atoh1-Gli2 CKO cerebella**

Our previous study demonstrated that SOX2+ NEPs can migrate to the EGL after injury and then turn off SOX2 and turn on Atoh1 \([25]\), and our present results revealed that some cells in the rescued EGL in P8 Atoh1-Gli2 CKO cerebella express a low level of SOX2. We thus hypothesized that WT NEPs (SOX2+) are able to change their fate to become GCPs and replenish the EGL as part of a compensation mechanism in Atoh1-Gli2 CKOs. Using a Nestin-FlpoER transgene \([40]\) and a Flippase (Flp)-dependent R26 reporter allele that expresses TDTom, we performed genetic inducible fate mapping (GIFM) of NEPs in the Atoh1-Gli2 CKO cerebella. In contrast to P30 controls \((\text{Nestin-FlpoER/+; R26 frt-STOP-frt-TDTom/+ or Nes-TDTom mice administered Tm at P0})\), we observed many TDTom+ cells in the EGL of Atoh1-Gli2 CKOs at P0 stimulates a compensation process that leads to replenishment of the GCPs by WT cells.

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that also expressed the GC marker NeuN in the IGL of Atoh1-Gli2 CKO mutants (n = 6) (Fig. 5a-f). Interestingly, the vermis of P30 Atoh1-Gli2 CKOs that recovered well showed an apparently greater contribution of NEP-derived TDTom+ cells in the IGL than those mice that recovered poorly (compare Fig. 5c-d to e-f). Similar results were obtained when analyzing the hemispheres (Additional file 4: Figure S4). However, and consistent with our analysis of CB size, there appeared to be less variability in the percentage of TDTom+ cells ...

Fig. 4  Loss of Gli2 mutant GCPs at P0 is compensated by wild type (WT) cells at P8. a b In situ RNA hybridization analysis of Gli1 on P8 midsagittal cerebellar sections of Gli2lox/lox (control, a) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, b) mice. Red arrowhead indicates the strong Gli1 expression in the mutant EGL and red asterisks indicate normal Gli1 expression in Bergmann glia in the Purkinje Cell Layer (PCL). c-d FIHC detection of the indicated proteins and dapi on P8 mid-sagittal cerebellar sections of Gli2lox/lox (control, c) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, b) mice. High power images are shown of the areas indicated by white rectangles in (c and d) with the thickness of the outer EGL (c) indicated by yellow brackets. The white arrow in (b) indicates the proliferating EGL. e and f Graphs of the thickness proportion of the EGL at P8 (n = 4) (e) and the proportion of oEGL area at P8 (% [oEGL] area of total [EGL] area) (n = 4) (f) in Gli2lox/lox (CTL, black) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, red) mice. All of the analyses were performed on 3 midline sections per brain. All graphical data are presented as means ± SEM and significance determined using two-tailed T-test. g-h In situ hybridization of Cre RNA on P8 midsagittal cerebellar sections of Gli2lox/lox (control, g) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, h) mice. Black arrows indicate the loss of Cre expression in the partially rescued EGL. i-k FIHC detection of the indicated proteins and dapi on P8 mid-sagittal cerebellar sections of Gli2lox/lox (control, j) and Atoh1-Cre/+; Gli2lox/lox (Atoh1-Gli2 CKO, i and k) mice. EGL is indicated by the yellow dotted lines and yellow asterisk indicates low level of SOX2 expression in the mutant EGL. Scale bars represent 100 μm...
observed in the hemispheres compared to the vermis. Analysis of the vermis at P8 showed a significant increase in the number of Nestin-derived TdTom+ cells in the EGL compared to controls ($n = 3$; $3049 \pm 713.6$ vs $308.8 \pm 121$ Tdtom+ cells per mm$^2$ of EGL; $p = 0.0193$) (Fig. 5g-h). Furthermore, TdTom+ cells in the P8 EGL already expressed the GCP marker Atoh1 (as shown by Atoh1-GFP staining) ($n = 3$; $80.92 \pm 3.69$ vs $1.74 \pm 1.11\%$ of TdTom+ Atoh1-GFP+/total TdTom+ cells in the EGL; $p < 0.0001$) (Fig. 5g-h).

**A subset of proliferating PCL NEP-derived cells migrate to the IGL in Atoh1-Gli2 CKO cerebella**

We next analyzed the behavior of NEPs using a Nes-CFP reporter line [43]. Consistent with our GIFM experiment and unlike control animals (Nes-CFP), the EGL of Atoh1-Gli2 CKOs expressed a high level of CFP (Fig. 6a-b). Surprisingly, streams of CFP+ cells were seen in lobule 3 spanning between the IGL and EGL that were not present in controls (Fig. 6a-b) or in irradiated mice [25]. Interestingly, some cells in the streams expressed the proliferation marker Ki67 as well as the GCP/GC marker PAX6 (Fig. 6c-d) suggesting that a subset of NEP-derived cells were not able to stay in the EGL and thus migrated back to the cerebellar cortex. To test this idea we performed live imaging of P8 Nes-CFP cerebellar slice cultures from both control and Atoh1-Gli2 CKOs (Atoh1-Gli2 CKO; Nes-CFP). Strikingly, by tracking the movement of individual cells during ~6 h of imaging we observed Nes-CFP+ cells actively migrating from the PCL to the EGL in slices from Atoh1-Gli2 CKOs.
CKO cerebella but not control mice at P8 (Fig. 6e-f and Additional file 5: Video S1 and Additional file 6: Video S2). The CFP+ layer of cells also appeared thicker in the mutants, indicating the NEPs expanded in number. Interestingly, in the areas containing streams of CFP+ cells the majority of cells that were tracked moved in the opposite direction from the EGL to the IGL (Fig. 6g and Additional file 7: Video S3). Our live imaging experiments thus provide evidence that NEPs located in the PCL expand and then migrate to replenish the EGL in response to GCP-specific loss of Gli2. Furthermore, a subset of NEP-derived cells is not able to integrate into the EGL and migrate back down to toward the cerebellar cortex.

**Gli2 KO in NEPs inhibits the recovery of the EGL in Atoh1-Gli2 CKOs**

Since we have shown previously that SHH signaling (Smo) is necessary in NEPs for CB recovery following irradiation, we tested whether Gli2 plays a role in this process. We generated littermates of 4 different genotypes that were administered Tm at P0, and analyzed each genotype (n = 3) at P8, P12 and P21: Gli2flox/flox WT (control) mice, Nestin-FlpoER/+; R26 MASTR/+; Gli2flox/flox single (Nes-Gli2 CKOs mutants lacking Gli2 in NEPs), Atoh1-Cre/+; Gli2flox/flox double (Atoh1-Nes-Gli2 CKOs lacking Gli2 in NEPs and GCPs) mutants. We did not observe any obvious phenotype in the Nes-Gli2 CKOs at all stages compared to controls (compare Fig. 7c-d to a-b, Additional file 7: Video S3). However, H&E staining revealed that the anterior CB appeared reduced in the double mutants (Atoh1-Nes-Gli2 CKOs) compared to Atoh1-Gli2 CKOs at both P8 and P12 (compare Fig. 7e to g and Additional file 8: Figure S5e to g). Analysis of proliferating GCPs in the outer EGL (Ki67+)
and post mitotic GCs in the inner EGL and IGL (P27+) at both stages showed that the anterior EGL appeared more depleted in Atoh1-Nes-Gli2 CKO compared to Atoh1-Gli2 CKO cerebella and that Atoh1-Nes-Gli2 CKO animals failed to form a proper P27+ inner EGL and IGL (compare Fig. 7f to h and Additional file 8: Figure S5f to h). Analysis of GFP+ Nestin-expressing cells in P21 Atoh1-Nes-Gli2 CKOs using a Flp-mediated R26 reporter allele that expresses nuclear β-Galactosidase (Bgal) (R26frt-stop-frt-lacZ/+ ) revealed that unlike Atoh1-Gli2 CKOs in which Nestin-derived GCs populated the IGL (Figs. 5a-f and 8c), the Gli2 mutant Nestin-derived cells populated the IGL poorly (Fig. 8f). The few GFP+ cells in the IGL of the double mutants were likely interneurons or astrocytes.

These results demonstrate that SHH-signaling through GLI2 plays a crucial role during NEP-mediated cerebellar recovery from loss of GCPs.

**Discussion**

In this study we developed a conditional mutant strategy to delete Gli2 (the gene encoding the major effector of SHH signaling) in most GCPs in the anterior cerebellum using an Atoh1-Cre transgene used in many studies (e.g. [41, 45–52]). Although we show using a mosaic analysis that SHH-GLI2 signaling is crucial for generating the correct pool of GCs by promoting GCP viability and proliferation, deletion of Gli2 in the EGL-expressing cells inhibits the compensation mechanism when Gli2 is removed in GCPs (g compared to e, b, d, f and h). High magnifications (as shown by yellow squares in a, c, e and g) of anterior vermis of P12 Gli2flox/flox (b), Nes-FlpoER/+; R26MASTR/+; Gli2flox/flox (Nes-Gli2 CKO, d), Atoh1-Cre/+; Gli2flox/flox (Atoh1-Gli2 CKO, f), and Atoh1-Cre/+; Nes-FlpoER/+; R26MASTR/+; Gli2flox/flox (Atoh1-Nes-Gli2 CKO, h) cerebella stained with the indicated proteins and dapi. White arrowhead and white asterisk indicate the loss of EGL and IGL respectively in Atoh1-Nes-Gli2 CKO. Scale bars represent 1 mm (a, c, e and g) and 100 μm (b, d, f and h).
mechanism is that the depletion of the EGL results in a change in Purkinje cell signaling, possibly because of a reduction in excitatory input since less granule cells are generated. In turn, SHH might preferentially accumulate in the cell bodies of Purkinje cells, which lie close to the NEPs, thus increasing HH signaling to NEPs [25]. Consistent with a role for GCs in regulating NEP behaviors, the Atoh1-Cre transgene is first expressed in GCPs at E13.5 [45], but the replenishment of the EGL in Atoh1-Gli2 CKOs only occurs several days after the EGL is depleted and when the IGL normally first becomes apparent (P3-P4). Thus, a possible involvement of NEPs in a compensation process should be considered for conditional mutants that alter not only GCP proliferation and survival, but also genes involved in differentiation of GCs.

It might be expected that Atoh1-Smo CKOs would have a similar phenotype to Atoh1-Gli2 CKOs, given that both genes are required for SHH signaling. However, Atoh1-CreER/+; SmoloxPΔ mice in which one allele of the Smoothened gene is deleted in the germline and deletion of the floxed allele is dependent on tamoxifen (Tm) administration have a severe cerebellum hypoplasia [13]. A possible explanation for the phenotype in such mutants is that reprogramming of NEPs in Smo heterozygous mutants is partially compromised since HH signaling is crucial for the expansion of PCL NEPs and their migration to the EGL [25]. In addition, since Tm diminishes cerebellum recovery after EGL depletion [25], the combination of a lower level of SMO protein in NEPs and administration of Tm to Atoh1-Cre/+; SmoloxPΔ mice might blunt the response of NEPs to Smo-dependent depletion of the EGL leading to severe hypoplasia.

We observed a large variability in the vermal hypoplasia of Atoh1-Gli2 CKO adults, suggesting that only some mutant cerebella can efficiently recover. The variability in recovery is likely because the degree to which the Atoh1-Cre transgene is turned on in newly formed WT GCPs varies between mice. Since loss of Gli2 leads to cell death, the TUNEL staining observed in the EGL of P8 Atoh1-Gli2 CKO mice injected with Tm at P0. IGL is indicated by the yellow dotted line. Scale bars represent 500 μm (a, b, d and e) and 100 μm (c and f).
menses signals through CXCR4 to maintain GCPs in the outer EGL and to enhance SHH-dependent proliferation [53]. Furthermore, SHH-GLI1 signaling induces the transcription of Cxcr4 and Cxcr7 [54]. We propose that in Atoh1-Gli2 CKOs a subset of PCL-derived NEPs express CRE after entering the EGL, and the subsequent loss of GLI2 protein reduces CXCR4, leading to migration of proliferating GCP-like cells back into the cerebellar cortex. Thus, the variable extent of growth of the cerebellum in Atoh1-Gli2 CKOs likely results from the amount of Atoh1-Cre induced after P4 in GCPs, and the resulting balance of cell death and premature migration from the EGL.

The cerebellum is broadly divided along the medio-lateral axis into a central vermis and two lateral hemispheres [19]. Although recovery from depletion of the EGL at P0 occurs in both regions of Atoh1-Gli2 CKOs, the recovery is more robust in the hemispheres. Curiously, the hemispheres of Atoh1-Gli2 CKOs exhibit extra folds at P8 (arrow in Fig. 3g), suggesting a differential recovery response along the medio-lateral axis. The vermis and hemispheric GCPs have a higher sensitivity to recovery phenomena occur in other regions of the brain, and depending on the transgene used could complicate interpretation of mutant phenotypes.

**Conclusion**

In this study, we show that the ability of NEPs to compensate for postnatal cerebellar damage must be considered in the interpretation of any mutant phenotype where genes involved in EGL cell proliferation/differentiation and survival have been disrupted. This is particularly the case if the Atoh1-Cre transgene utilized in this study [41] is employed to generate conditional mutants. Compensation for a loss of GCPs is most likely to occur for genes that are required after birth, once NEPs are present. Finally, our findings raise the question of whether similar recovery phenomena occur in other regions of the brain, and depending on the transgene used could complicate interpretation of mutant phenotypes.

**Additional files**

**Additional file 1:** Figure S1. Similar to the in the vermis, SHH-Gli2 maintains GCP in an undifferentiated state and promotes their survival in the hemispheres. (a) Graphs of the number of GFP+ cells in the outer (d) EGL at P4 (n = 3) and P8 (n = 3) in both hemispheres and vermis of R26M+h2lox/+; Atoh1-FlpoERR+; Gli2flox/+ (control, black) and R26M+h2lox/+; Atoh1-FlpoERR+; Gli2flox/+ (Atoh1-M-Gli2 Cre, red) mice treated with Tm at P4. (b-d) Graphs of the proportion of GFP+ cells in the outer (c) EGL at P8 (n = 3) (b), the proliferation index at P8 (% [GFP+ EdU+] cells of all [GFP+] cells in the oEGL]) (n = 3) (c) and the number of TUNEL+ particles per section at P4 (n = 3) (d) in the hemispheres of R26M+h2lox/+; Atoh1-FlpoERR+; Gli2flox/+ (Atoh1-M-Gli2 Cre, black) and R26M+h2lox/+; Atoh1-FlpoERR+; Gli2flox/+ (Atoh1-M-Gli2 Cre, red) mice treated with Tm at P2. All of the analyses were performed on 3 sections per region and per brain. All graphical data are presented as means ± SEM and significance determined using two-tailed test. (d) Detection of native GFP fluorescence of P8 Atoh1-FlpoERR/+; Gli2lox/lox; Atoh1-GFP/+(Atoh1-Gli2 Cre, CB). All of the analyses were performed on 3 sections per region and per brain. All graphical data are presented as means ± SEM and significance determined using two-tailed test.

**Additional file 2:** Figure S2. GLI2 protein is lost in the P0 Atoh1-Gli2 CKO EGL. (a and d) In situ hybridization of Cre mRNA on P0 mid-sagittal cerebellar sections of Gli2flox/+ (control, a) and Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 CKO, d) mice. Black arrows indicate the lack of Cre expression in the most posterior part of the CB. (b-c and e-f) FIHC detection of GLI2 protein and dapi in the indicated regions (as shown by black squares in a and d) in P0 Gli2 CKO (control, b-c) and Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 CKO, e-f) CB. Yellow arrowhead in e and white arrow in f indicate respectively the absence and presence of GLI2 protein in the EGL. Scale bars represent 1 mm (a and d) and 100 μm (b-c and e-f). (JPG 1811 kb)

**Additional file 3:** Figure S3. Rescued EGL still exhibits an increase in cell death. (a-b) TUNEL and dapi detection on mid-sagittal sections of P8 Gli2flox/+ (Control) and Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 CKO) CB. White arrowheads indicate the presence of the EGL (b). (c) Graphs of the number of TUNEL+ particles per mm² of EGL (n = 4) in the vermis (boule I to V) of P8 Gli2flox/+ (Control, black) and Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 CKO, red) CB. All of the analyses were performed on 3 sections per region and per brain. All graphical data are presented as means ± SEM and significance determined using two-tailed test.

**Additional file 4:** Figure S4. Nestin-Expressing Progenitors (NEPs) differentiate into granule neurons in response to loss of GLI2 in the hemispheres. (a and c) H&E staining of hemispheric sagittal sections of P30 Nes-FlpoER/+; R26h2-lox/+; FLPeRcre; Gli2flox/+ (Nes-CFP, a) and Atoh1-Cre/+; Gli2flox/+ (Atoh1-Gli2 Cre, b) mice injected with Tm at P0. (b and d) FIHC detection of the indicated proteins and dapi on hemispheric sagittal cerebellar sections at P30. High power images are shown of the areas indicated by yellow rectangles in (a and c). GIL is indicated by the yellow dotted line. Scale bars represent 1 mm (a and c) and 100 μm (b and d). (JPG 1479 kb)

**Additional file 5:** Video S1. P8 WT cerebellum shows no obvious movement of NEPs towards the EGL. Detection of native CFP fluorescence on sagittal slices of the vermis (boule 1/3) of P8 Nes-FlpoER/+ mice showing displacement of CFP+ cells. Image stacks were acquired every 5 min for 4 h. (MOV 3481 kb)

**Additional file 6:** Video S2. PCL NEPs migrate toward the EGL in Atoh1-Gli2 CKO CB at P8. Detection of native CFP fluorescence on sagittal slices of the vermis (boule 1/3) of P8 Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 Cre, b) mice showing displacement of CFP+ cells. Image stacks were acquired every 5 min for 4 h. (MOV 6450 kb)

**Additional file 7:** Video S3. A subset of NEP-derived cells migrate from the EGL towards the GCL in Atoh1-Gli2 CKO CB at P8. Detection of native CFP fluorescence on sagittal slices of the vermis (boule 1/3) of P8 Atoh1-Cre+/−; Gli2flox/+ (Atoh1-Gli2 Cre, b) mice showing displacement of CFP+ cells. Image stacks were acquired every 5 min for 4 h. (MOV 6450 kb)

**Additional file 8:** Figure S5. Inactivation of GLI2 in both Nestin and Atoh1-expressing cells inhibits the recovery of the CB. (a-c, e and d) H&E staining of sagittal sections of the cerebellar vermis of P8 Gli2flox/+ (Control, a), Nes-FlpoER/+; R26h2-lox/++; Gli2flox/+ (Nes-Gli2 Cre, b) and Atoh1-Cre/+; Gli2flox/+ (Atoh1-Gli2 Cre, c) mice injected with Tm at P0. Note that inactivation of GLI2 only in Nestin-expressing cells has no major effect at P8. However, inactivation of GLI2 in Nestin-expressing cells inhibits the compensation mechanism (g compared to e). (b, d, f and h) Close-up as shown by yellow squares in a, c, e and g of anterior vermis of P8 Gli2flox/+ (b), Nes-FlpoER/+; R26h2-lox/++; Gli2flox/+ (Nes-Gli2 Cre, d) and Atoh1-Cre/+; Gli2flox/+ (Atoh1-Gli2 Cre, f) mouse injected with Tm at P8. (i) Staining of sagittal sections of Nestin-Gli2 Cre and Atoh1-Cre-/−; Gli2flox/+ (Atoh1-Null Cre, g) and Atoh1-Cre+/−; Gli2flox/+ (Atoh1-M Cre, h) mice. White arrowhead and white asterisk indicate the loss of EGL and IGL, respectively. The Atoh1-Null Cre mice. Scale bars represent 1 mm (a, c and e) and 100 μm (b, d, f and h). (JPG 2032 kb)
Abbreviations

A: Activator; CB: Cerebellum; Cc: Cubitus interruptus; COO: Conditional knockout; Dhh: Desert hedgehog; EGL: External Granule Layer; GCP: Granule Cell Precursor; GFP: Green Fluorescent Protein; GfCAM: Genetically Inducible Fate Mapping; hct: Heterozygous; Hh: Hedgehog; IHC: immunohistochemistry; iInh: Indian hedgehog; ISH: In situ hybridization; MASTR: Mosaic mutant analysis with spatial and temporal control of recombination; ML: Molecular layer; NEP: Nestin-expressing progenitor; P: Postnatal day; PCL: Purkinje cell layer; TDTom: Tandem Dimeric derivative of DsRed; Tm: Tamoxifen; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; VZ: ventricular zone; WT: Wild type

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Availability of data and materials

All mouse lines are available from the Joyner lab or Jackson laboratories. All data generated or analyzed for this study are included in this published article.

Authors’ contributions

AW and ALJ conceived the project; AW, AKL and ALJ designed the research; AW, MM, AKL and A.LJ performed the experiments; AW, MM, AKL and A.LJ analyzed the data and all authors discussed the data; AW and ALJ wrote the manuscript with contributions from all authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were performed according to a protocol (07–001-001) approved by the Memorial Sloan Kettering Cancer Center’s Institutional Animal Care and Use Committee.

Competing interests

The authors declare that they have no competing interests.

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