Cerebellar neuronal progenitors undergo a series of divisions before irreversibly exiting the cell cycle and differentiating into neurons. Dysfunction of this process underlies many neurological diseases including ataxia and the most common pediatric brain tumor, medulloblastoma. To better define the pathways controlling the most abundant neuronal cells in the mammalian cerebellum, cerebellar granule cell progenitors (GCPs), we performed RNA-sequencing of GCPs exiting the cell cycle. Time-series modeling of GCP cell cycle exit identified downregulation of activity of the epigenetic reader protein Brd4. Brd4 binding to the Gli1 locus is controlled by Casein Kinase 1δ (CK1δ)-dependent phosphorylation during GCP proliferation, and decreases during GCP cell cycle exit. Importantly, conditional deletion of Brd4 in vivo in the developing cerebellum induces cerebellar morphological deficits and ataxia. These studies define an essential role for Brd4 in cerebellar granule cell neurogenesis and are critical for designing clinical trials utilizing Brd4 inhibitors in neurological indications.
During postnatal mammalian development, granule cell progenitors (GCPs) undergo symmetric divisions in the external germinal layer (EGL) of the brain and exit the cell cycle within a narrow time frame, resulting in rapid cellular expansion and differentiation. However, the fundamental mechanisms controlling irreversible GCP cell-cycle exit have not been elucidated. It is essential to discover these mechanisms to understand cerebellar development, as defects in GCP expansion have been linked to cerebellar ataxia and the most common pediatric brain tumor, medulloblastoma. Epigenetic modifiers control gene expression without changing DNA sequence. These modifiers include histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetyltransferases attach acetyl groups to lysine residues on histone proteins, while HDACs remove those modifications. Histones normally bind DNA molecules via their positively charged lysine and arginine tails. Histone–DNA binding initiates DNA compaction and transcriptional silencing. Histone tail acetylation reduces this positive charge, attenuates DNA binding and compaction and allows transcription. Histone deacetylation has the opposite effect, thus allowing histone–DNA binding and reducing transcription. Working in combination with HATs (writers) and HDACs (erasers) are histone-reader proteins, which bind to acetylated lysines on histones, recruit transcriptional complexes, and mediate gene transcription. Among the "reader" proteins are bromodomain and extra-terminal domain (BET) proteins. BET proteins include Brd2, Brd3, Brd4, and BrdT. Tissue expression studies suggest that Brd4 is heavily expressed in the brain, making it a likely candidate for modulating neurogenesis in the nervous system. Recent sequencing studies have implicated many epigenetic regulators in medulloblastoma. The epigenetic reader protein Brd4 has been implicated in various cancers, including medulloblastoma. Brd4 controls expression of the medulloblastoma essential gene MYC in G3 medulloblastomas, which have poor prognosis as well as GLI1 and GLI2 levels in Sonic hedgehog (SHH)-driven medulloblastomas, which have intermediate prognosis. Highly selective Brd4 inhibitors have been developed that reduce MYC, GLI1, and GLI2 levels. These inhibitors have gone into clinical trials for multiple cancer indications, and one Brd4 inhibitor has received fast-track designation from the FDA for myelofibrosis. However, it is unclear whether these inhibitors can be given to children suffering from medulloblastoma, as we do not fully understand the role of Brd4 during normal development. To address this issue, we deleted Brd4 in the developing cerebellum in mice and find that it is essential for cerebellar growth. Brd4 knockout leads to cerebellar ataxia that is linked to defects in cerebellar development starting at postnatal day 3. These studies suggest that Brd4 inhibitors may need to be given during a short developmental window in children to reduce potential negative effects on cerebellar development.

Results
Brd4 phosphorylation decreases during cell-cycle exit of granule cell progenitors. GCPs undergo cell-cycle exit and differentiation when plated on poly-D-lysine/laminin coated plates. We utilized an in vitro system to isolate purified GCPs at various times during differentiation and performed RNA sequencing on purified cell populations from postnatal day (P) 6 mice to determine their characteristics during the exit process (Fig. 1). We found that GCPs exited the cell cycle within 24 h of plating as judged by PI-FACS analysis (Fig. 1a) and EdU incorporation (Fig. 1b). The mRNA expression of several proliferation markers decreased during this time period while those of differentiation markers increased (Fig. 1c). For example, the levels of the cell-cycle regulator, Cyclin b1, and the bHLH transcription factor important for maintaining GCPs in a proliferative state, Atoh1, decreased by 24 h post plating. By contrast, neuron-specific class III beta-tubulin (Tuj1) and the axonal growth marker, Gap43, increased at 24 h. To determine the exact timing of changes in cellular pathways during cell-cycle exit, we performed short-time-series modeling with gene ontology clustering analysis of all mRNAs expressed at 0, 2, 4, 6, 12, 24, and 48 h after plating (Fig. 1d). Interestingly, downregulation of cell proliferation pathways (Cluster #5, Fig. 1d) occurred at the same time as upregulation of neuronal development pathways (Cluster #77, Fig. 1d; Supplementary Fig. 1, Supplementary Data 1), suggesting that the two processes may be temporally and mechanistically linked (Fig. 1d, Supplementary Fig. 1). Importantly, the SHH pathway, which is a major regulator of GCP expansion, is downregulated beginning at 2 h of GCP cell-cycle exit, with the SHH effectors GLI1 and GLI2 decreasing basal levels by 24 h, which is recapitulated in vivo (Supplementary Figs. 1, 2).

Our prior studies demonstrated that the epigenetic reader protein Brd4 regulates GLI levels in mouse embryonic fibroblasts (MEFs) by directly binding the GLI1 locus. Brd4 is part of a family of bromodomain and extraterminal domain proteins (BETs) that bind to acetylated lysines on histones and recruit transcriptional complexes to induce transcription of various genes involved in cell proliferation, signaling, and inflammation. To test whether Brd4-dependent regulation of GLI changes during GCP cell-cycle exit, we performed Brd4 chromatin immunoprecipitation (ChIP) analysis in GCPs exiting the cell cycle. Brd4 is expressed in the developing cerebellum during P6-9 when GCPs are proliferating and exiting the cell cycle (Supplementary Fig. 3). As observed in Fig. 2a, Brd4 binding to the GLI1 locus decreased dramatically within the first 2 h of GCP cell-cycle exit. Brd4 activity has been shown to be regulated by phosphorylation and therefore, we tested whether Brd4 phosphorylation decreases during cell-cycle exit. Brd4 phosphorylation decreases with the same kinetics as Brd4 binding to the GLI1 locus during cell-cycle exit (Fig. 2b, c; Supplementary Fig. 1).

Casein Kinase 1δ inhibition or deletion reduces Brd4 binding to the GLI1 locus. We have previously demonstrated that Casein Kinase 1 delta (CK1δ) is required for GCP proliferation, and its protein levels decrease with the same kinetics as Brd4 phosphorylation during cell-cycle exit. In vitro phosphorylation assays using purified Brd4 and CK1δ demonstrated that Brd4 is a CK1δ substrate in vitro. Earlier studies have shown that Brd4 phosphorylation on serines 492/494 relieves Brd4 autoinhibition, thereby allowing it to bind to chromatin. We found that serines 492 and 494 are necessary for CK1δ-mediated phosphorylation of Brd4 as mutating both to alanine abrogated the ability of CK1δ to phosphorylate Brd4 in vitro (Fig. 2d-f). To determine whether CK1δ controls Brd4 activity in GCPs, we incubated proliferating GCPs with the selective CK1δ inhibitor, SR-1277, or vehicle in the presence of SHH and measured Brd4 phosphorylation on serines 492/494 at 24 h (Fig. 3a, b). SR-1277 treatment decreased phospho-S492/494 Brd4 levels relative to the total Brd4 (Fig. 3a, b), which correlated with decreased Brd4 binding to the GLI1 locus as measured by Brd4 ChIP analysis (Fig. 3c). Importantly, CK1δ inhibition reduced Brd4 association with the GLI1 locus to a similar extent as treatment with the selective Brd4 inhibitor, I-BET1512, suggesting that CK1δ activity is required for maintaining Brd4 in an active state (Fig. 3c). In agreement with this, conditional deletion of CK1δ in GCPs in vivo reduced Brd4...
phosphorylation on serines 492/494 (Fig. 3d), and binding to the Gli1 locus (Fig. 3e). Consistent with a direct effect on Brd4, CK1δ inhibition in Suppressor of Fused17 deleted (Sufu−/−) cells that contain active SHH signaling independent of the membrane receptor Smoothened also reduced Gli1 levels and Brd4 binding to the Gli1 locus (Supplementary Fig. 4). Collectively, these studies suggest that CK1δ-mediated phosphorylation of Brd4 on serines 492/494 potentiates Brd4 localization to the Gli1 locus.
during GCP proliferation. By contrast, during GCP cell-cycle exit, rapid downregulation of CK1δ activity is associated with reduced Brd4 phosphorylation on serines 492/494, decreased Brd4 binding to the Gli1 locus, and attenuated Gli1 expression.

**Brd4 Inhibition reduces granule cell progenitor proliferation in vitro, ex vivo, and in vivo.** Downregulation of Brd4 phosphorylation and binding during GCP cell-cycle exit suggests that it is an essential regulator of SHH signaling in GCPs, and that disrupting Brd4 activity may limit GCP proliferation. To test this, we measured the effect of BET inhibition on GCP proliferation via the Brd4 inhibitor I-BET151. I-BET151 treatment reduced GCP proliferation and Gli1 expression in vitro (Fig. 4a, b; Supplementary Fig. 5). Further, I-BET151 treatment of cerebellar slices ex vivo reduced EdU incorporation in the EGL (Fig. 4c, d). Finally, treatment of P8 pups with a brain penetrant BET inhibitor, JQ1,23 also reduced cerebellar GCP proliferation in vivo (Fig. 4e, f). Collectively, these studies suggest that pharmacological inhibition of Brd4 reduces GCP expansion in the developing cerebellum.

**Brd4 deletion reduces GCP proliferation and induces ataxia.** To determine whether genetic disruption of Brd4 affects cerebellar development, we conditionally deleted Brd4 in GCPs by breeding Brd4<sup>fl/fl</sup> mice to Tg (Atoh1-Cre) mice, which express Cre under the Atoh1 promoter after embryonic day 13.5.24 (Fig. 5a). Tg
Gli1 and Gli2 were correlated with lower levels of the positive effectors of (Fig. 5b). Furthermore, the level of the cell-cycle protein Gli3 (Fig. 6b; Supplementary Figs. 7, 8). Importantly, reduced cerebellar size correlated with behavioral deficits in Tg (Atoh1-Cre−);Brd4fl/fl mice, which exhibited symptoms of cerebellar ataxia not evident in Tg (Atoh1-Cre−);Brd4fl/fl mice (Fig. 6c; Supplementary Fig. 6). Taken together, these studies demonstrate that Brd4 is an essential regulator of GCP proliferation and cerebellar development in vivo.

Discussion

We report a novel in vivo function for the epigenetic-reader protein Brd4. Brd4 controls granule cell progenitor expansion in the developing cerebellum. Brd4 deletion leads to defects in cerebellar morphology, which leads to ataxia. Brd4 activity is temporally regulated during cerebellar granule cell development, as both CK1δ-dependent phosphorylation of Brd4 on serines 492/494 and Brd4 binding to the Gli loci is required for Brd4 function and that I-BET115 or SR-1277 was able to displace it from the Gli locus.

c Conditional deletion of CK1δ in GCPs in vivo reduces phosphorylation of Brd4 at S492/494. CK1δ was deleted in GCPs by breeding CK1δfl/fl mice to Tg (Atoh1-Cre) mice and the extent of Brd4 phosphorylation levels determined via western analysis. The total Brd4 and Gli3 were included as loading controls. Note that CK1δ is efficiently deleted in Tg (Atoh1-Cre−);CK1δfl/fl mice and that phosphorylation at serines 492/494 was lower in these animals relative to Tg (Atoh1-Cre);CK1δfl/fl mice. e CK1δ knockout reduces Brd4 binding to the Gli locus. GCPs were purified from Tg (Atoh1-Cre−); CK1δfl/fl mice or Tg (Atoh1-Cre−);CK1δfl/fl mice, and the amount of Brd4 bound to the Gli locus was measured. Note that CK1δ deletion reduced Brd4 binding to the Gli locus. The results are shown as the average values of three independent experiments and are represented as the mean ± SEM. A paired t test was performed (∗p < 0.05). Source data can be found in source data graphs under tabs for b, c, e and in source data Fig. 3 and Fig. 4.
demonstrate that Brd4 is required for cerebellar growth. Brd4 is required for GCP proliferation by controlling the SHH pathway effectors, Gli1 and Gli2. Brd4 inhibition or deletion reduces GCP proliferation and responsiveness to SHH signaling. Brd4 deletion decreases cerebellum size and induces symptoms of cerebellar ataxia either as a direct effect of neuron loss or indirectly through the aberrant cerebellum morphology (Fig. 6). Brd4 deletion reduces levels of Gli2, an essential gene required for vertebrate development. Therefore, Brd4’s essential role in cerebellar granule cell development is likely to be related to Gli2 activity required for proper GCP expansion. Brd4, like Gli2, is a target in developmental diseases and cancer, and Brd4 inhibitors may be useful in clinical settings in children and in cerebellum-associated developmental disorders and pediatric cerebellar tumors such as medulloblastoma. However, cerebellum-associated developmental disorders and pediatric cerebellar tumors such as medulloblastoma are associated with deficits in procedural learning, which could be exacerbated with prolonged Brd4 inhibitor usage. Our findings suggest that therapeutic use of Brd4 inhibitors may need to be given during a temporal window, as Brd4 may be required for proper cerebellar development in humans.

Methods
Animal husbandry. All mice were housed in an American Association of Laboratory Animal Care–accredited facility at the University of Miami and were maintained in accordance with NIH guidelines. Animal use was approved by the Institutional Animal Care and Use Committee of the University of Miami.

GCP isolation and compound treatment. GCPs were purified from cerebellar cortex of P6-8 CD1 and Tg(Atoh1-Cre+);CK1δfl/fl mice by using Percoll gradient.
Fig. 5 Conditional deletion of Brd4 in GCPs inhibits the sonic hedgehog pathway and SHH mediated proliferation. a Strategy utilized for deleting Brd4 conditionally in the cerebellum. Brd4tm1a(EUCOMM)Wtsi heterozygous mice were obtained through the Knockout Mouse Project Repository at Baylor University. These mice were bred to Tg (ACTFLPe) mice to create Brd4fl/+ mice. Brd4fl/+ mice were bred to Tg (Atoh1-cre) mice to obtain conditional cre expression in the granule cell lineage. Tg (Atoh1-cre) +/-;Brd4fl/+ mice were then backcrossed to generate Tg (Atoh1-cre+);Brd4fl/+ and Tg (Atoh1-cre-);Brd4fl/+ litters. RNA was extracted from isolated GCPs from P8 mice, and qRT-PCR was performed and normalized to Brd4 expression in the granule cell lineage. Tg (Atoh1-cre+);Brd4fl/+ and Tg (Atoh1-cre-);Brd4fl/+ littersates were resolved by SDS-PAGE and western blot analysis. GCPs were then used as a loading control. b GCPs from Tg (Atoh1-cre+);Brd4fl/+ mice have less total Brd4, phospho-Brd4 S492/494, and Cyclin D1 than Tg (Atoh1-cre-);Brd4fl/+ mice. GCPs from P8 mice were isolated, and nuclear extracts or whole cell lysates were resolved by SDS-PAGE and western blot analysis. Gapdh was used as a loading control. c GCPs from Tg (Atoh1-cre+);Brd4fl/+ mice proliferate less than Tg (Atoh1-cre-);Brd4fl/+ GCPs. Following purification, GCPs were plated for 48 h with SHH, incubated with Edu and with or without SHH for 2 h, then plated with fresh media on poly-D-lysine/laminin coated coverslips for 3 h. Cells were fixed for Edu detection, reaggregates were imaged with a confocal laser-scanning microscope, and Edu positive cells were quantified with ImageJ. The results are the average of three independent experiments and are represented as the mean ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison testing (5B) or a two-way ANOVA followed by Bonferroni’s multiple comparison testing (5D) was performed (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, N.S. no significance). Source data can be found in source data graphs under tabs for Fig. 5b, d and in source data Fig. 5c.

Cerebellar organotypic slice culture and treatment. Cerebella were isolated from P8 CD1 mice. Sagittal slices (250 μm) of cerebellar cortex were generated using a Leica VT1000S vibratome, and slices were plated on Millipore culture inserts in six-well culture dishes containing 1.5 ml of serum-free medium (Basal Medium Eagle (Gibco), 0.45% D(+)-glucose solution (Sigma), 1× ITS supplement (Sigma), 2 mM L-glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco). The slices were then submerged in 2.5 ml of medium containing DMSO or I-BET151 (1 μM) for 24 h, after which 1 ml was removed so that the slices were no longer submerged, and the medium was replaced.

RNA sequencing. Extracted RNA was sent to the John P. Hussman Institute for Human Genomics for sequencing. RNA quality was tested by ThermoScientific NanoDrop or Agilent Bioanalyzer and confirmed to have RIN numbers > 8.5. Sequencing was performed on Illumina HiSeq2000 with three samples per lane, generating an average of 95 M × 100 bp reads per sample. After quality filtering and trimming with FastQC remaining RNA-seq reads were aligned to the Ensemble mouse genome (v.87) using TopHat (v.2.1). For all samples, 89–90% of reads aligned successfully. Differential expression analysis was performed by CuffDiff 2.2 using the “classic-fpkm” parameter for the normalization method, and the “pooled” parameter for the dispersion method. Sequencing results from this study have been deposited in under accession number SRP146235.

Functional annotation analysis. For each STEM profile, we performed functional annotation analysis using DAVID41 against the Level 5 Gene Ontology Biological Processes terms (GOTERM_BP_5). To adjust for the false discovery rate, we only considered terms with a Benjamini–Hochberg adjusted p-value of 0.05.

Flow cytometry. For flow-cytometric analysis, isolated GCPs were removed from dishes, washed with PBS and 1% BSA, and fixed with 10% ethanol in PBS overnight at 4 °C. GCPs were then stained with 69 μM propidium iodide in 38 μM sodium citrate buffer and 1 μM RNAse A at 37 °C for 30 min. The number of cells in G1, S, or G2 phases was determined using a fluorescence-activated cell-sorting device (LSRII, Becton Dickinson) and analyzed by FlowJo software.
Fig. 6 Conditional deletion of Brd4 in the developing cerebellum induces cerebellar deficits and ataxia. **Tg (Atoh1-Cre+);Brd4fl/fl** mice have disrupted cerebellar morphology relative to **Tg (Atoh1-Cre–);Brd4fl/fl** mice. Representative images are from P8 cerebellum mid-sagittal sections of approximately the same location in vermis. **a** Cresyl violet stain shows reduced cerebellum size in **Tg (Atoh1-Cre+);Brd4fl/fl** mice versus **Tg (Atoh1-Cre–);Brd4fl/fl** mice. Cerebellar lobes are indicated I–X. Dotted line in **Tg (Atoh1-Cre+);Brd4fl/fl** divides the anterior and poster cerebellum to emphasize changes to proliferation and structure and proliferation, respectively, in each region. Scale = 500 µm. **b** Brd4 loss in GCPs disrupts Purkinje cell development. Confocal images are from the posterior lobe IX and anterior lobe II. Tag1 is a marker for migrating GCPs, Calbindin is a marker for Purkinje cells, and DRAQ5 is a DNA marker. Scale = 50 µM. EGL = external granule layer, ML = molecular layer, PL = Purkinje cell layer, IGL = internal granule layer. **c** **Tg (Atoh1-Cre+);Brd4fl/fl** mice exhibit symptoms of cerebellar ataxia. Adult (≥ P28) **Tg (Atoh1-Cre+);Brd4fl/fl** mice or **Tg (Atoh1-Cre–);Brd4fl/fl** mice were assessed for ataxia symptoms using multiple rank composite tests. Scores from each test were pooled with higher scores indicating symptom severity. Rotarod testing was performed, and latency to fall and speed at fall were recorded for each group after 5 days of training. The results are the average of three independent experiments, and are represented as the mean ± SEM. For ataxia, rank scores a Mann–Whitney U test and for rotarod testing an unpaired t test were performed (*p < 0.05, **p < 0.01). Source data can be found in source data graphs under tab for Fig. 6c.


**Table 1 Accession numbers for genes used in quantitative PCR**

| Gene | TaqMan® Gene Expression Master Mix (Applied Biosystems) accession number |
|------|------------------------------------------------------------------------|
| Cyclin B1 | Mm01322149_g1 |
| Atoh1 | Mm00476035_s1 |
| Tuj1 | Mm00727586_s1 |
| Gap43 | Mm0500404d_m1 |
| Gapdh | Mm99999915_g1 |
| Brd4, exon 4-5 | Mm01348074_m1 |
| Brd4, exon 5-6 | Mm00480939_m1 |
| Brd4, exon 7-8 | Mm01271717_g1 |
| Brd3 | Mm00466733_m1 |
| Gli1 | Mm00494654_m1 |
| Gli2 | Mm01293117_m1 |
| Gli3 | Mm00492337_m1 |
| Cyclin A1 | Mm00432337_m1 |
| Cyclin D1 | Mm00432359_m1 |

**3H-thymidine assay.** GCPs were plated (3 × 10^5 cells/well) in 96-well dishes. DMEM or 1-BET151 was added to the medium, and cells were maintained in culture for 24 h, in the presence of 8μCi [3H] thymidine (Amersham) was added to each well, and cells were harvested 22 h later and analyzed using TopCount (Perkin–Elmer).

**EdU incorporation assay.** For the in vitro study, P8 CD1 mice were injected intraperitoneally twice with 10, 25, or 50 mg/kg of JQ1 during 24 h. Two hours after the last injection, 15 μg/ml of EdU was added to the culture medium and incubated for 4 h. After fixation, cells were permeablized and stained using the Click-IT® EdU Alexa Fluor® 594 Imaging Kit (Invitrogen). Samples were imaged using the z-stack of a confocal laser-scanning microscope (Olympus, FV1000) and the images were analyzed using Fiji software (ImageJ).

**Immunohistochemistry.** Mice were perfused with 4% PFA, and cerebella were extracted. Cerebella were fixed in 4% PFA for 2 h, embedded in 30% sucrose in PBS, and cut into 20 μm sections with a cryostat (Leica). Sections were then permeablized and blocked in 0.5% Triton X-100, 5% fetal bovine serum for 1 h at room temperature and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-Brd4 (1/1000, Bethyl Laboratories Inc.), mouse or rabbit anti-cyclin B1 (C-8) (1/1000, Santa Cruz Biotechnology), goat anti-Brd4 (1/2000), and rabbit anti-Flag (1/5000, A8592, Sigma). The sections were then washed with PBS, plated at 20 μg/ml in PBS, and incubated for 4 h at room temperature with the following secondary antibodies as appropriate: Alexa Fluor® 488 goat anti-rabbit IgG, Alexa Fluor® 594 goat anti-mouse IgG or Alexa Fluor® 594 goat anti-mouse IgM (all 1/500, Invitrogen). Sections were then washed with PBS and incubated with Hoechst stain (Invitrogen) or DRAQ5 (AbCam) before mounting using Fluoromount G mounting medium (Southern Biotech). Confocal images were acquired with a confocal laser-scanning microscope and were further analyzed with Fiji software (ImageJ).

**Protein extract preparation, antibodies, and western blot analysis.** Cells were homogenized, and extracts were prepared using lysis buffer (NER buffer from the NER-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific), 1 μM protease inhibitor cocktail, 1 μM microcystin LR). The soluble fraction was recovered by centrifugation at 16,000 × g for 10 min at 4 °C. Protein concentration was measured with the BCA Protein Assay Kit (Pierce Biotechnology), and 30 μg of protein from each sample was resolved by SDS-PAGE. The resolved bands were transferred onto a nitrocellulose membrane and subjected to western blotting with the appropriate antibodies. The following primary antibodies were used: mouse anti-CK1δ (C-8) (1/1000, Santa Cruz Biotechnology), rabbit anti-Flag (1/500, sc-6479, Santa Cruz Biotechnology), mouse anti-CK1δ (C-8) (1/1000, sc-6479, Santa Cruz Biotechnology), rabbit anti-Flag (1/2000)19, rabbit anti-Brd4 (1/1000, Bethyl Laboratories Inc.), mouse anti-cyclin B1 (C-8) (1/1000, Santa Cruz Biotechnology), mouse-anti-Flag (1/5000, NA9340V, GE Healthcare). The following secondary antibodies were used: anti-goat IgG–HRP (1/1000, 7074, Cell Signaling), anti-mouse IgG–HRP (1/1000, NAX931, GE Healthcare) and anti-rabbit IgG–HRP (1/1000, NA9340V, GE Healthcare).

**RNA isolation and qRT-PCR.** Cells were lysed in 1 mL of TriZol Reagent (Invitrogen), and the RNA was purified with the RNeasy Mini Kit (Qiagen). RNA was then reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan probes were designed with the TaqMan Gene Expression Assay tool (Applied Biosystems). The qRT-PCR was performed using a TaqMan Gene Expression Master Mix (Applied Biosystems) in a CFX384 TouchTM Real-Time PCR Detection System (Bio-Rad). Fold change in gene expression was estimated using the computed topography comparative method and normalizing to the Gapdh computed topography values and relative to control samples (Table 1).

**In vitro phosphorylation of Brd4 with CK1δ.** Purified bacterial Brd4 phosphorylation-dependent interaction domain (PDID) proteins were used for this study (PDID wild-type, PDID S484A/488 A, PDID S492/494 A). In each reaction, 100 ng of PDID protein was incubated with 200 μM of ATP, 1000U of CSNK1D (NEB, P60305), and 1X NEBuffer for kinase proteins for 30 min at 30 °C. Laemmli sample buffer was added to terminate the reactions, and the samples were heated at 95 °C and resolved by SDS-PAGE. The resolved bands were transferred onto a nitrocellulose membrane and subjected to western blotting with the appropriate antibodies.

**Behavioral testing for ataxia rank composite scoring.** We followed the composite scoring method for ataxia outlined in Guyenet et al.42 with modifications. Briefly, blinded researchers scored Tg (Atoh1-cre−/−)Brd4fl/fl mice on the ledge test, on the hindlimb clasp test, and on the gait test. These three composite tests are used most frequently to establish ataxia severity. Composite scores were averaged and compared in Tg (Atoh1-cre−/−)Brd4fl/fl or Tg (Atoh1-cre−/−)Brd4fl/fl littermates (Table 3). Tg (Atoh1-cre−/−)Brd4fl/fl do not breed successfully, therefore, Tg (Atoh1-cre−/−)Brd4fl/fl were maintained for experiments.

**Generation of Brd4fl/fl mice.** Brd4fl/fl mice were obtained through the Knockout Mouse Project Repository at Baylor University from Dr. John Seavitt. These mice were bred to B6.Cg-Tg (ACTFLP920D55Y1) mouse (The Jackson Laboratory Stock #005703) to remove the neomycin resistance gene and LacZ reporter gene (forward primer, CCTGGCTGAGAGGAGCACTTCTC, reverse primer, AGTGGAGATGACAGGAGGAGGCTCG to create Brd4flomic heterozygous mice. In parallel, Brd4flomic heterozygous mice were bred to homozygosity (hereafter referred to as Brd4fl/fl) or to B6.Cg-Tg (Atoh1-cre−/−)Brd4fl/fl mouse (The Jackson Laboratory Stock #011104, hereafter referred to as Tg (Atoh1-cre−/−)Brd4fl/fl mice were crossed to generate Tg (Atoh1-cre−/−)Brd4fl/fl mice. We used a rotarod paradigm to examine mouse balance, coordination and muscle function simultaneously. Mice were trained with rotarod twice daily for 5 days in the accelerating mode (5–9 rotations per minute over 2.5 min), and the latency to fall and speed were recorded after the training period. Average latency times and speed for the testing interval were compared in Tg (Atoh1-cre−/−)Brd4fl/fl or Tg (Atoh1-cre−/−)Brd4fl/fl mice. We used a rotarod paradigm to examine mouse balance, coordination and muscle function simultaneously. Mice were trained with rotarod twice daily for 5 days in the accelerating mode (5–9 rotations per minute over 2.5 min), and the latency to fall and speed were recorded after the training period. Average latency times and speed for the testing interval were compared in Tg (Atoh1-cre−/−)Brd4fl/fl or Tg (Atoh1-cre−/−)Brd4fl/fl mice.
similar expression pattern to that from Supplementary Fig. 1. Furthermore, to quantify the overlap between the DEGs of the two data sets, we calculated the pairwise percentage of overlap among all time points, as shown in Supplementary Fig. 2. The percentage of overlap was defined as the number of DEGs that were common between the two data sets divided by the total number of DEGs in the corresponding RNA-seq Timeseries timepoint.

Mouse embryonic fibroblast (MEF) cultures. MEF Sfnsf+/−/− cells11 were maintained in DMEM media with 10% neonatal calf serum and 1% penicillin/streptomycin. Overall, 1 × 10^5 cells per well were plated in a 12-well plate. In all, 100 nM of SR-653234 or SR-1277 CK1δ inhibitors were added into the media. DMSO was used as a vehicle control, 100 nM of GDC0449 as negative control, and 10 μM GANT-61 as positive control. Twenty-four hours later, RNA was extracted and SHH target gene expression was examined by Taqman probe-based qRT-PCR. Brd4 ChIP analysis was performed as described above.

siRNA transfections in GCP cultures. GCPs were isolated from P6 CD1 pups, and 7.5 × 10^5 cells per well were plated in suspension in a 48-well plate. Cells were transfected with 1 μM SMARTpool Accell Mouse Brd4 siRNA (57261, E-041945-00, Dharmacon GE Healthcare) or 1 μM SMARTpool Accell Mouse GFP Scramble siRNA (D-001950-01-05, Dharmacon GE Healthcare) in low-serum media (Accell 00, Dharmacon GE Healthcare) or 1 µM SMARTpool Accell Mouse GFP Scramble siRNA (D-001950-01-05, Dharmacon GE Healthcare), 1.5% glucose, 20 mM glutathione, 2% horse serum, 1% fetal bovine serum, 1% penicillin/streptomycin) with mouse recombinant SHH (0.25 ng/mL). Cells were collected 72 h post transfection, and RNA isolation and qRT-PCR were performed as described.

Statistical analysis. All experiments were conducted independently at least three times. Statistical analysis was performed with Prism software (Graphpad). Figures 1b, c, 2c, e, 4a, b, f one-way ANOVA followed by Bonferroni multiple comparison testing (p < 0.05). Figures 3b, e, 4d: paired t test (p < 0.05). Figure 3b: one-way ANOVA followed by Tukey’s multiple comparison testing (p < 0.05). Figure 5d: two-way ANOVA followed by Bonferroni’s multiple comparison testing (p < 0.05). Figure 6c: for ataxia rank scores Mann–Whitney test (p < 0.05) and for rotoradot testing unpaired t test (p < 0.05).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all transcriptional data supporting the findings of this study are freely available from the databases provided within this paper. Sequencing results from this study have been deposited in under accession number SRP146255. All other data supporting the findings of this study are available from the corresponding author, Dr. Nagi G. Ayad, upon request.

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Author contributions

C.P. designed research, performed experiments, developed methods, analyzed data, and wrote the paper. M.E.M. designed research, performed experiments, developed methods, analyzed data, and wrote the paper. V.S. designed research, performed experiments, developed methods, analyzed data, and wrote the paper. Y.F., J.L., J.M., J.R-B. and S.K.T. performed experiments. C.V. analyzed data. C.-M.C. provided reagents. J.K.L, D.L., D.J.R. and M.E.H. analyzed data and wrote the paper. J.C. designed research, analyzed data, and wrote the paper. N.G.A. designed research, analyzed data, and wrote the paper.

Additional information

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