Aspm sustains postnatal cerebellar neurogenesis and medulloblastoma growth in mice

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ABSTRACT

Alterations in genes that regulate brain size may contribute to both microcephaly and brain tumor formation. Here, we report that Aspm, a gene that is mutated in familial microcephaly, regulates postnatal neurogenesis in the cerebellum and supports the growth of medulloblastoma, the most common malignant pediatric brain tumor. Cerebellar granule neuron progenitors (CGNPs) express Aspm when maintained in a proliferative state by sonic hedgehog (Shh) signaling, and Aspm is expressed in Shh-driven medulloblastoma in mice. Genetic deletion of Aspm reduces cerebellar growth, while paradoxically increasing the mitotic rate of CGNPs. Aspm-deficient CGNPs show impaired mitotic progression, altered patterns of division orientation and differentiation, and increased DNA damage, which causes progenitor attrition through apoptosis. Deletion of Aspm and either of the apoptosis regulators Bax or Trp53 (also known as p53) rescues the survival of neural progenitors and reduces the growth restriction imposed by Aspm deletion. Our data show that Aspm functions to regulate mitosis and to mitigate DNA damage during CGNP cell division, causes microcephaly through progenitor apoptosis when mutated, and sustains tumor growth in medulloblastoma.

KEY WORDS: CGNPs, Cerebellar neurogenesis, Medulloblastoma, Microcephaly, Mitosis, Division orientation

INTRODUCTION

Microcephaly syndromes may provide clues for identifying genetic targets for brain tumor therapy. During brain development, proliferation of transient-amplifying cells generates appropriately sized populations of different types of neurons. In primary microcephaly, impaired progenitor amplification produces a developmental outcome whereby the overall brain size is markedly reduced, but the diversity of cell types is preserved (Woods et al., 2005). Mutations found in microcephaly genes may indicate genes required by transient-amplifying cells. Because specific brain tumors grow as transient-amplifying cells, disrupting microcephaly genes may produce a clinically useful anti-tumor effect.

The microtubule-associated centrosomal protein ASPM (abnormal spindle microcephaly-associated) has been implicated in both primary microcephaly and cancer. Mutations in human ASPM occur in familial microcephaly (Bond et al., 2002, 2003; Woods et al., 2005), and studies in model organisms have shown that ASPM is expressed by neural stem cells and promotes brain growth (Bond et al., 2002; Pulvers et al., 2010; Marinaro et al., 2011; Rujano et al., 2013). ASPM has also been implicated in the growth of diverse cancers, including medulloblastomas (Vulcani-Freitas et al., 2011), gliomas (Horvath et al., 2006; Bixeye et al., 2010), hepatocellular carcinomas (Lin et al., 2008), ovarian cancers (Brüning-Richardson et al., 2011) and pancreatic cancers (Wang et al., 2013). Thus, loss of Aspm function is associated with growth failure, and aberrant ASPM is associated with excessive growth.

Various functions of ASPM have been identified that may support proliferation. In Drosophila, asp maintains mitotic spindle orientation during both mitosis and meiosis (Zhong et al., 2005; van der Voet et al., 2009; Kaindl et al., 2010; Rujano et al., 2013). In mice, Asmp is required to maintain spindle organization and positioning and acts as a microtubule-organizing center in telencephalic neuroepithelial progenitors (Fish et al., 2006; Higgins et al., 2010). Asp and Aspm are expressed at centrosomes and spindle midbody microtubules, and their loss causes defects in spindle assembly and mitotic progression (Saunders et al., 1997; Wakefield et al., 2001; Fish et al., 2006; van der Voet et al., 2009; Pulvers et al., 2010; Novorol et al., 2013). Aspm might also play a role in DNA double-strand break repair through non-homologous end joining (Kato et al., 2011). The net effect of these diverse functions is promotion of proliferation, but it remains unclear how these functions converge.

The postnatal cerebellum presents an opportunity to study Aspm function in both developmental brain growth and tumorigenesis. Cerebellar growth is typically reduced in primary microcephaly (Adachi et al., 2014), whereas medulloblastoma, the most common malignant pediatric brain tumor, is a neoplastic overgrowth of the cerebellum. Postnatal CGNP proliferation drives cerebellar growth. This proliferation lasts through the first 2 weeks of life in mice or first year of life in humans, generating the largest population of neurons in the brain. During this period, CGNPs proliferate in the external granule layer (EGL) of the cerebellum. Actively cycling CGNPs are PCNA+ and typically reside in the outer zone (oEGL), whereas postmitotic CGNPs express Cdkn1b (p27) and accumulate in the inner zone (iEGL). From the iEGL, postmitotic CGNPs migrate through the molecular layer (ML) into the internal granule layer (IGL) where they complete differentiation to become granule...
neurons (GNs) (Hatten et al., 1982; Roussel and Hatten, 2011; Hibi and Shimizu, 2012).

Postnatal CGNP proliferation depends on mitogenic signaling from locally secreted Shh (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000). Shh pathway activation is found in 25% of medulloblastoma patients including 65% of patients younger than 2 or older than 16 years (Zurawel et al., 2000a,b; Ellison et al., 2011; Northcott et al., 2011). In genetically engineered mice, Shh pathway hyperactivation in CGNPs produces malignant cerebellar tumors that resemble human SHH subtype medulloblastomas, providing in vivo models of medulloblastoma tumorigenesis (Zurawel et al., 2000a,b; Hallahan et al., 2004; Schüller et al., 2008; Yang et al., 2008) and metastasis (Hatton et al., 2008).

Here, we show that Aspm plays an essential role in CGNP proliferation, differentiation and survival in mice. Genetic deletion of Aspm reveals important functions in mitotic progression, maintenance of CGNP position and orientation during mitosis, and mitigation of proliferation-dependent DNA damage. Deletion of Aspm increases DNA damage and induces progenitor apoptosis that depends on Bax and p53. Aspm expression persists in Shh-driven medulloblastomas in transgenic mice, where these functions support tumor growth.

RESULTS
Aspm is induced by Shh and upregulated during cerebellar neurogenesis
Aspm is expressed at major sites of postnatal neurogenesis in the brain. At postnatal day (P) 7, the peak of CGNP proliferation, Aspm mRNA localized predominantly to the oEGL (Fig. 1A). We mapped Aspm expression and postnatal neurogenesis by injecting P6 Aspm-GFP reporter mice with 5-ethynyl-2′-deoxyuridine (EdU) and harvesting brains 24 h later. GFP reporter fluorescence waned by P16 (Fig. 1F), following the temporal pattern of CGNP proliferation (Fujita, 1967). The relationship between Aspm expression and CGNP proliferation was further demonstrated by real-time, quantitative PCR (RT-qPCR). Aspm expression was relatively high at the peak of neurogenesis (P7) and waned by P16 (Fig. 1F), mirroring the expression of cyclin D2 (Ccn2), a previously identified marker of CGNP proliferation (Kenney and Rowitch, 2000).

We examined the relationship between Shh signaling and Aspm expression in isolated, freshly explanted CGNPs. Shh-supplemented CGNPs in vitro expressed >4-fold more Aspm mRNA compared with Shh-deprived CGNPs (Fig. 1G). Shh signaling is transduced by Smoothened (Smo) and can be blocked by the Smo inhibitor vismodegib (Rubin and de Sauvage, 2006). CGNPs treated with Shh+vismodegib expressed Aspm similarly to Shh-deprived CGNPs (Fig. 1G). CGNPs from Aspm-GFP mice expressed robust GFP when cultured with Shh and downregulated GFP when Shh-deprived (Fig. 1H). These findings show that Aspm expression is integral to the Shh-driven CGNP proliferation program.

Fig. 1. Aspm expression in the postnatal cerebellum. (A,A′) P7 in situ hybridization demonstrates Aspm mRNA primarily in the oEGL. PC, Purkinje cell. (B-B″) Sagittal section of P7 Aspm-GFP mouse brain injected at P6 with EdU shows GFP expression in EdU+ zones of postnatal neurogenesis, the cerebellum (CB) and SVZ. (C-E) GFP fluorescence in Aspm-GFP cerebella at P7 (C, high magnification in D) and P16 (E). (F) RT-qPCR quantification of Aspm and Ccn2, normalized to Gapdh, during (P7) and after (P16) CGNP proliferation. (G) Shh induced a 4- to 5-fold increase in Aspm and Ccn2 mRNAs in vitro that was blocked by vismodegib. (H) Isolated CGNPs from Aspm-GFP mice express GFP only when cultured with Shh. (I,J) Sagittal cerebellar section of a N2;SmoA1; Aspm-GFP mouse shows continued expression of GFP at P16, in contrast to the wild-type Aspm-GFP cerebellum shown in E. White arrowheads indicate the persistent EGL resulting from sustained Shh activity. (J,K) Medulloblastomas in N2;SmoA1;Aspm-GFP mice express GFP specifically in tumor cells. Scale bars: 100 μm (A,D,H,K); 1 mm (B,J); 500 μm (C,E,I).
To determine whether Shh hyperactivation induces Aspm in vivo, we crossed Aspm-GFP reporter mice with the transgenic, medulloblastoma-prone ND2:SmoA1 mouse line, which expresses a constitutively active allele of Smo under control of the NeuroD2 promoter. CGNPs of ND2:SmoA1 mice show cell-autonomous Shh-pathway activation, prolonged CGNP proliferation, and predisposition to medulloblastoma (Hallahan et al., 2004). In Aspm-GFP;ND2:SmoA1 mice, CGNPs proliferated beyond the typical 15-day period, and expressed Aspm-GFP uniformly throughout the aberrantly persistent EGL (Fig. 1I). In spontaneous medulloblastomas, GFP was consistently and homogeneously expressed (n>15; Fig. 1J,K). Although not specifically implicating components of the Shh pathway as transcriptional regulators of Aspm, these data show that Shh signaling through Smo was necessary to maintain Aspm expression in vivo and is sufficient to drive Aspm expression in vivo, as an integral part of the Shh-driven proliferative program in both persistent CGNPs and Shh-driven medulloblastomas in ND2:SmoA1 mice.

To examine the fate of Aspm-expressing postnatal progenitors, we generated Aspm-CreER;Rosa26-Lox-STOP-Lox-tdTomato mice, which express a tamoxifen-inducible red fluorescent reporter controlled by the Aspm promoter (Marinaro et al., 2011). Mice were harvested at P11 following tamoxifen injection at P4 and P7. Red fluorescence was observed in neurogenic regions throughout the brain (Fig. 2A), including neural progenitors in the rostral migratory stream (Fig. 2B), hippocampus (Fig. 2C; Fig. S1D,E) and EGL (Fig. 2D,E). TdTomato also labeled differentiated neurons in the cerebellum, including interneurons (Fig. 2E and Fig. S1B, yellow arrowheads) and GNs, identified by their position in the IGL and colocalization with NeuN (Rbfox3 – Mouse Genome Informatics) (Fig. 2F; Fig. S1A,B). In the ML, tdtTomato and NeuN co-labeled differentiating CGNPs migrating to the IGL (Fig. 2G and Fig. S1A, red arrowheads). In addition, tdtTomato tubular structures throughout the brain expressed the endothelial marker CD31 (Pecam1 – Mouse Genome Informatics), identifying them as capillaries (Fig. 2E and Fig. S1A”C, blue arrowheads). No tdtTomato cells expressed the glial marker GFAP, which labels Bergmann glia and astrocytes (Fig. 2H; Fig. S2F). Thus, Aspm progenitors in the postnatal brain gave rise to CGNPs (identified by their position in the EGL), differentiated neurons, and capillary endothelial cells, with minimal glial progeny.

**Aspm is required for cerebellar growth**

To determine whether Aspm function is required for CGNP proliferation, we generated and analyzed AspmSA/SA mice, which harbor a splice-acceptor and lacZ-neomycin cassette into the intron between exons 6 and 7 (see supplementary Materials and Methods; Fig. S2A). We verified Aspm deficiency in AspmSA/SA mice by RT-qPCR, which demonstrated an absence of full-length Aspm mRNA in AspmSA/SA cerebella, compared with AspmSA+ littermates (Fig. 3A). AspmSA/SA males were sterile, like the Aspm-deficient males previously described (Pulvers et al., 2010). Because of the complex geometry of the brain, we used brain weight to detect changes in size. Whole brains and dissected cerebella of AspmSA/SA mice were significantly smaller than brains and cerebella of AspmSA+ or Aspm++ littermates (Fig. 3B,C). These results show that the AspmSA/SA mouse line phenocopies previously reported Aspm mutant mice (Pulvers et al., 2010), and that Aspm deficiency reduced the size of the cerebellum along with the rest of the brain.

To determine whether Aspm is specifically required by CGNPs, we generated an Aspm conditional allele (Aspm′; Fig. S2B) by crossing AspmSA/SA and Rosa26-FLPe (Farley et al., 2000) mouse lines. We then used Cre-mediated recombination to delete Aspm conditionally in the Math1 (Atoh1 – Mouse Genome Informatics) (Fig. 3A). Because of the restricted localization of the Aspm conditional allele (Aspmf; Fig. S2B) by crossing AspmSA/SA and Rosa26-FLPe (Farley et al., 2000) mouse lines. We then used Cre-mediated recombination to delete Aspm conditionally in the Math1 (Atoh1 – Mouse Genome Informatics) lineage (Fig. 3A,B,C), which in the cerebellum is restricted to CGNPs (Helms et al., 2000; Machold and Fishell, 2005; Mati et al., 2005). The resulting Math1-Cre;AspmET mice (Aspm cKO), expressed less Aspm mRNA in cerebella, compared with AspmET littermates without Cre (Fig. 3A). Although overall brain weight was preserved in Aspm cKO mice owing to limited expression of...
Math1-Cre outside the cerebellum (Fig. 3D), cerebellum weight was significantly reduced compared with littermate controls (Fig. 3E). We also measured the volume of intact, unprocessed brains using magnetic resonance imaging (MRI), and used manual segmentation to determine the volume of the cerebellum (Fig. 3F-I; Movies 1, 2). Consistent with weight measurements, MRIs showed reduced brain and cerebellum volume in AspmSA/SA mutants, and reduced cerebellar volume in AspmcKOs. The smaller cerebellum in the cKO demonstrates a cell-autonomous requirement for Aspm in CGNPs to maintain normal cerebellar growth.

We next sought to determine the onset of growth restriction observed in Aspm cKOs. The EGL typically thins between P7 and P15 as proliferation wanes, whereas the IGL grows as GNs populate it. Measurements of EGL and IGL area at P7 and P11 showed that both regions were significantly smaller in Aspm cKOs, with the most significant difference between genotypes occurring in the IGL at P11 (Fig. 3J). Thus, in Aspm cKOs, the thinning of the EGL was accelerated and the growth of the IGL reduced.

The organization of the EGL was largely preserved in cerebella of Aspm cKOs, with the PCNA+ oEGL and p27+ iEGL present at similar ratios as in controls (Fig. 3K,L). High magnification of boxed areas are shown on the right. (M) Ratio of PCNA+/p27+ areas is not significantly different in Aspm cKOs at P7 and P11. (N) Thinning of the p27+ iEGL was statistically significant at P11. Scale bars: 1 mm (F); 250 µm (K,L); 50 µm (K,L insets).

Aspm cKO CGNPs show hyperproliferation and mitotic defects

To determine whether Aspm deficiency decreased the mitogenic response of CGNPs to Shh, we compared proliferation in CGNPs with the PCNA+ oEGL and post-mitotic, p27+ iEGL present at similar ratios as in controls (Fig. 3K,L). In littermate controls at P7, the ratio of the area of the PCNA+ region to the p27+ region was 1.031±0.032 (mean±s.e.m.), decreasing to 0.838±0.043 by P11 as proliferation wanes. In Aspm cKOs from P7 to P11, the PCNA:p27 ratio decreased more rapidly, from 1.135±0.12 to 0.771±0.054 (Fig. 3M). Comparison of the thickness of the oEGL (PCNA+) and iEGL (p27+) layers between cKOs and controls showed that each layer grew progressively thinner in cKOs, with the most significant difference occurring in the iEGL at P11 (Fig. 3N). These data suggest a defect in the differentiating population of CGNPs in the Aspm cKOs that becomes progressively more evident over time.

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isolated from either Aspm cKO mice or Aspm<sup>ΔT</sup> littermates, cultured with or without Shh for 48 h by counting mitotic figures, identified by immunofluorescence labeling for phospho-histone H3 (pHH3). This comparison showed that although Shh increased the mitotic rate of both genotypes, cKO CGNPs demonstrated significantly more mitoses (Fig. 4A,B). Thus, in vitro, Shh-induced proliferation was increased, rather than decreased, by Aspm deficiency.

To compare the proliferation rate of CGNPs in vivo, we counted mitoses along the entire length of the EGL in midline sagittal sections of P5 Aspm cKO mice and Aspm<sup>ΔT</sup> littermate controls (<i>n</i>=6 per genotype). We identified mitotic cells by co-immunofluorescence for pHH3, which marks prophase-early anaphase, and survivin (Birc5 – Mouse Genome Informatics), which localizes to the midbody region and cleavage furrow during anaphase-telophase (Caldas et al., 2005; Williams et al., 2011). To control for differences in EGL thickness between controls and cKOs, we normalized mitotic cell counts to EGL area to determine the mitotic rate across the entire EGL population. Using these criteria, we noted a significant increase in overall mitotic rates in the Aspm cKO EGL (Fig. 4C,D), consistent with our in vitro data and with previous observations in zebrafish aspm mutants (Kim et al., 2011; Novorol et al., 2013).

The spatial pattern of mitoses was disrupted by Aspm deficiency. Because all mitotic cells are expected to be PCNA/Ki67+, we used spatial criteria, rather than PCNA and p27 (as in Fig. 3) to define EGL layers. We considered the superficial EGL (EGL<sub>S</sub>) to extend from the pial surface inwards to 50% of EGL depth, and the deep EGL (EGL<sub>D</sub>) to extend from the midway point to the inner EGL margin. At the ages examined (P5-P7), this demarcation approximates but does not exactly correspond with the border of the oEGL, defined by expression of PCNA or Ki67 (Mki67 – Mouse Genome Informatics), both of which extend over half the thickness of the EGL (Fig. S3F,G).

We measured the position of all pHH3<sup>+</sup> cells in the EGL at P5 relative to the pia mater (Fig. S3A,B). Mitotic figures tended to occur further from the pia in cKOs compared with Aspm<sup>ΔT</sup> littermates (Fig. S3C; <i>n</i>&gt;3000 cells/genotype). In controls, ~80% of mitoses occurred in the EGL<sub>S</sub>, consistent with previous observations (79.6%) in rat EGL (Zagon and McLaughlin, 1987). By contrast, in cKOs, the proportion of mitoses occurring in the

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**Fig. 4. Aspm-deficient CGNPs show mitotic defects.** (A,B) pHH3 immunofluorescence shows Shh-induced proliferation of cKO and control CGNPs in vitro. (C,D) In situ, the density of both early stage pHH3<sup>+</sup> and late-stage survivin<sup>+</sup> mitotic cells in the EGL was significantly elevated in Aspm cKOs. (E) Histogram showing frequency of mitoses by position within the EGL at P5, binned by 10% increments of EGL thickness. Compared with controls, a greater proportion of mitoses occurred farther from the pia in the EGL<sub>S</sub> in Aspm cKOs. Dashed line demarcates the midpoint of the EGL; arrows indicate the bins (50-60%, 60-70%) with the greatest increase in mitotic figures in cKOs. Blue and red figures indicate the percentage of mitoses falling within each bin. (F) Quantification of pHH3 density in superficial (EGL<sub>S</sub>) and deep (EGL<sub>D</sub>) regions showing an increase in supernumerary mitoses specifically within the EGL<sub>D</sub> in Aspm cKOs. (G) pHH3 and survivin identify CGNPs at specific phases of mitosis. Prophase cells are weakly pHH3<sup>+</sup> and are survivin<sup>-</sup>; prometaphase and metaphase cells are strongly pH3<sup>+</sup> with survivin<sup>+</sup> on kinetochores; metaphase DNA shows a characteristic bar shape; anaphase cells are weakly pH3<sup>−</sup> and are survivin<sup>+</sup> in the spindle midbody; telophase cells are pH3<sup>−</sup> with intense punctate survivin staining around the cleavage furrow. (H) A significant reduction in prophase and increase in prometaphase cells is observed in Aspm cKOs. (I-L) Anaphase chromosome morphology. (I) Representative images of Aspm<sup>ΔT</sup> (top) and cKO (bottom) anaphase cells, showing where the DNA aspect ratio (AR) and inter-chromosome distance (d) were measured. (J) Control anaphase chromosome morphology. (K) Control anaphase chromosomes were significantly rounder (lower AR) than wild-type counterparts, which adopt a characteristic bar shape. Dot plot and box-and-whisker plots (K) and histogram (L) of inter-chromosome distance. Aspm cKOs showed a Spike at intermediate distances, suggesting a delay in anaphase progression. Scale bars: 100 µm (A); 10 µm (G,J). P values determined by two-tailed Student’s t-tests except those in E (<i>χ</i><sup>2</sup>) and K (Mann–Whitney).
EGL$_S$ was reduced to ~67% (Fig. 4E). Normalizing cell counts to either EGL area (Fig. 4F) or length (Fig. S3D,E), we found similar mitotic frequency in the EGL$_S$ of both genotypes, but significantly increased mitotic frequency in the EGL$_D$ in cKOs. Co-labeling with pHH3$^+$ and either Ki67 or PCNA to mark the proliferative oEGL, showed that >90% of mitoses occurred within the Ki67/PCNA$^+$ domains, with no statistically significant difference between genotypes (Fig. S3F-H). Importantly, all mitotic cells outside of these domains (Fig. S3F,G, arrowheads) were PCNA$^-$ or Ki67$^-$ as expected. Thus, spatial measurement revealed differences in the position of mitoses that could not be determined by analysis of marker expression. In the independent cohort used for co-labeling with Ki67 or PCNA, we again observed a significant increase in pHH3$^+$ figures in the cKO compared with controls (Fig. S3I), predominantly localized to the EGL$_D$ (Fig. 4F; Fig. S3J). As shown in Fig. 4E (arrows), the greatest increase in mitotic figures in cKOs was observed in the 50th-70th percentile of EGL thickness near the EGL$_S$/EGL$_D$ boundary.

Because Asp/Aspm have been reported to play a role in mitotic spindle assembly and function (Wakefield et al., 2001; Riparbelli et al., 2002; Paramasivam et al., 2007), we used chromosome morphology and pHH3/survivin co-immunofluorescence to determine the distribution of mitotic stages found in wild-type and Aspm cKO CGNPs (Fig. 4G). We noted a decrease in the proportion of prophase cells and an increase in the proportion of prometaphase cells in cKOs relative to Aspm$^{+/+}$ controls (Fig. 4H). This altered distribution at early mitosis suggests that Aspm loss impairs mitotic progression at the time the spindle is forming. We also noted that Aspm-deficient CGNPs displayed abnormal rounded DNA during anaphase, suggestive of defects in organizing the spindle poles, whereas Aspm$^{+/+}$ control CGNPs showed a more typical bar shape (Fig. 4I,J). The median distance between anaphase chromosome pairs was also significantly shorter in Aspm cKOs compared with wild-type controls (Fig. 4K; Mann–Whitney test, $P=0.0157$). Moreover, whereas wild-type cells showed a Gaussian distribution of inter-chromosome distances (Shapiro–Wilk normality test, $P=0.6824$), this distribution was not maintained in cKOs ($P=0.0241$). A disproportionately high percentage of Aspm cKO chromosome pairs were separated by 7.5-8.0 µm (Fig. 4L; n=21/46 cells examined compared with 9/37 controls). This altered distribution suggests a delay in the transition between anaphase-A, during which chromosome separation is driven by destruction of sister-chromatid adhesion complexes, and anaphase-B, during which the mitotic spindle is elongated as the spindle poles pull the chromosomes apart. Overall, these data support a role for Aspm in promoting mitotic spindle assembly and progression through metaphase into anaphase.

Aspm loss leads to alterations in division orientation and cell cycle exit kinetics

Aspm (Asp) has been implicated in the maintenance of cleavage plane orientation in Drosophila neural progenitors (Rujano et al., 2013) though an analogous role for Aspm in mouse is controversial (Fish et al., 2006; Pulvers et al., 2010). In the developing cerebellum, CGNPs adopt different cleavage plane orientations depending on their position within the EGL (Zagon and McLaughlin, 1987). In the EGL$_S$, CGNPs more frequently orient their spindle perpendicular to the pial surface, whereas CGNPs dividing in the EGL$_D$ more often divide either parallel or transverse to the pial surface (Zagon and McLaughlin, 1987). Conditional deletion of Aspm produced significant changes in the orientation and distribution of divisions within the EGL. The incidence of transverse divisions in Aspm cKO CGNPs was increased at the expense of perpendicular divisions (Fig. 5A,B; $\chi^2$ test, $P<0.0001$). Analysis of division orientation relative to EGL depth demonstrated a bias in control cerebella towards perpendicular divisions in the EGL$_S$ (56.1%, n=301), with roughly equal frequencies of perpendicular, transverse and parallel divisions in the EGL$_D$ (n=149), consistent with prior studies (Zagon and McLaughlin, 1987). This orientation bias was lost in Aspm cKOs, in which CGNP division orientation appeared randomized (Fig. 5C). These data provide the first evidence that division orientation in the cerebellum is under molecular control, regulated at least in part by Aspm.

To determine whether alterations in division orientation and mitotic progression caused by Aspm mutation affected the tendency of CGNPs to re-enter or to exit the cell cycle (e.g. self-renew or differentiate), we examined the fate of CGNPs 24 h or 48 h after injection of 5-bromo-2′-deoxyuridine (BrdU) at P5. We used co-labeling with BrdU and the differentiation marker p27 to determine the proportions of BrdU-labeled cells that either continued or exited the cell cycle (Fig. 5D-K). CGNPs in the EGL were labeled with BrdU with similar frequency at the 24 h time point in Aspm$^{+/+}$ control and cKOs (Fig. 5L), suggesting that CGNPs in both genotypes enter S phase with similar frequency. However, there was a tendency towards fewer actively cycling (p27$^-$) and more differentiating (p27$^+$) BrdU$^+$ CGNPs in the cKOs compared with controls (Fig. 5L). Further analysis showed that a greater proportion of p27$^+$ CGNPs in the iEGL were labeled with BrdU in cKOs compared with controls (Fig. 5M). We quantified the cell cycle exit fraction (proportion of BrdU$^+$ CGNPs in the EGL that were p27$^+$) for both genotypes and found that a greater proportion of BrdU$^+$ cells had exited the cell cycle in Aspm cKOs at both the 24 h and 48 h time points (Fig. 5N). In addition, the differentiation index, a measure of the number of p27$^+$ cells produced per p27$^+$ CGNP, was significantly higher in Aspm cKOs (Fig. 5O). Finally, we noted a consistent decrease in terminally differentiated p27$^+$/BrdU$^+$ GNs in the IGL in Aspm cKOs, and an increase in p27$^+$/BrdU$^+$ CGNPs in the EGL at 48 h (Fig. 5P). Together, these data demonstrate that Aspm cKO CGNPs prematurely exit the cell cycle but fail to populate the IGL as effectively as normal CGNPs.

Aspm deficiency increases progenitor DNA damage and apoptosis

The overproduction of p27$^+$ CGNPs in the EGL coupled with underproduction of mature GNs in the IGL suggests that Aspm cKO CGNPs exhibit either delayed migration or impaired survival. We found no evidence of migration defects as similar numbers of BrdU$^+$/p27$^+$ neurons were found in the molecular layer in both genotypes at both time points (Fig. 5P). Therefore, we analyzed cell death in the EGL of cKOs and controls by immunohistochemistry for cleaved caspase-3 (cC3) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Apoptosis is rarely detected in the EGL of wild-type mice (Garcia et al., 2012). By contrast, cC3$^+$ cells were significantly increased in the Aspm$^{+/+}$ and Aspm cKO EGL (Fig. 6A-C) and were proportional to proliferation, i.e. highest at P7 and lower at P11. Similar results were obtained with TUNEL (Fig. 6D). Although apoptotic cells were rare in controls, they were most frequently observed in the EGL$_S$ (86.7±1.2%), whereas in Aspm cKOs the density of cC3$^+$ or TUNEL$^+$ apoptotic cells in the EGL$_S$ was increased ~5- to 7-fold over controls (Fig. 6E,F). Thus, apoptosis increased in Aspm cKOs in both the proliferating and differentiating populations of CGNPs, with the largest relative increase in the differentiating population. This increase in apoptosis specifically in the EGL$_D$ could explain why fewer mature GNs are produced in Aspm cKOs despite the increased production of p27$^+$ CGNPs.
CGNPs are highly sensitive to apoptosis in response to genotoxic stress (Chong et al., 2000; Lee et al., 2012). The mitotic abnormalities in Aspm-deficient CGNPs, and prior studies demonstrating a role for Aspm in mitotic spindle dynamics (Zhong et al., 2005; Fish et al., 2006; Higgins et al., 2010) and DNA repair (Kato et al., 2011) suggest that increased CGNP apoptosis in Aspm mutants might be caused by increased DNA damage. Immunolabeling for the DNA damage marker γH2AX demonstrated small, relatively faint γH2AX+ foci in the EGL of all genotypes, and larger foci that were more intense and less frequent. Co-labeling with γH2AX and BrdU, 2 h after BrdU injection, demonstrated that DNA damage was enriched in CGNPs undergoing DNA replication (Fig. 6G). In both Aspm<sup>S/A</sup> and Aspm cKO mice, γH2AX+ CGNPs were more frequent compared with Aspm-intact controls (Fig. 6H-J). These findings implicate DNA damage as a potential mechanism of increased progenitor apoptosis in Aspm-deficient mice.

Deletion of key regulators of apoptosis rescues Aspm-induced microcephaly

To determine the contribution of genotoxic stress and progenitor death to growth restriction in Aspm-deficient mice, we investigated whether genetic deletions that interrupt the link between DNA damage and apoptosis could rescue the growth of Aspm-deficient cerebella. In neural progenitors, DNA damage activates p53, which triggers Bax-dependent apoptosis (Vousden and Lu, 2002; Fridman and Lowe, 2003; Lee and McKinnon, 2007; Crowther et al., 2013). We examined how co-deletion of either Bax or Trp53 affected the Aspm <sup>cKO</sup> phenotype. Because p53 may exert gene dosage-related effects, we selected Math1-Cre;Aspm<sup>f/f</sup>;Trp53<sup>f/f</sup> (<sup>cKO</sup> Aspm) and Math1-Cre;Aspm<sup>f/f</sup>;Trp53<sup>+/+</sup> (<sup>cKO</sup> Aspm) littermates for comparison. For Bax studies, we compared littermates with Math1-Cre;Aspm<sup>f/f</sup> without Cre. Double mutants and controls were harvested at P30 for comparison of cerebellar weight, or at P7 for comparison of mitosis, DNA damage and apoptosis.

Compared with deletion of Aspm alone, cerebellum weight was significantly increased by co-deletion of either Bax or Trp53 (Fig. 7A-D). Volumetric MRI confirmed that Aspm/Bax dKO cerebella did not exhibit the growth restriction observed in Aspm single mutants, and were similar in volume to Aspm-intact controls (Fig. 7E). Histological examination, however, showed focal areas of thinning in the IGL in each of the three Aspm/Bax dKO cerebella.
examined (Fig. 7A,B), though quantification of mean cell density over a broad area of the IGL in replicate sections did not show a statistically significant difference (Fig. 7F). These focal areas of IGL thinning were not observed in Aspm cKOs, (data not shown) or in our prior studies of Bax single mutants (Garcia et al., 2012). As in Aspm single mutants, we noted an increase in mitotic rate across the EGL in each of the double mutants (Fig. 7G). Like Aspm single mutants, Aspm;Bax and Aspm;Trp53 mutants showed an elevated percentage of cells at prometaphase and a reduction in prophase (Fig. 7H), demonstrating impaired cell cycle progression. These

Fig. 6. Aspm disruption increases CGNP apoptosis and DNA damage. (A, B) Representative P7 sagittal sections from Aspm+/+ (A) and AspmSA/SA (B) cerebella stained for cC3 (black arrowheads). (C) Quantification of cC3+ cells in the EGL of Aspm+/+, Aspm cKOs, and control littermates demonstrates statistically significant increase in cell death with Aspm deficiency. (D) Increased apoptotic cell density (cC3+ or TUNEL+ cells/mm of EGL) in Aspm mutants. (E,F) The most significant increase in cell death in Aspm mutants, detected by cC3 (E) or TUNEL (F) occurs in differentiating cells of the EGL. (G) In control P7 mice, γH2AX+ cells occur primarily in the oEGL and largely overlap with the BrdU+ proliferating population. (H,I) Immunofluorescence for γH2AX in P7 Aspm cKOs. Red arrowheads indicate intense γH2AX+ cells and arrows indicate the presence of γH2AX+ foci. (J) Statistically significant increase in γH2AX+ cells in cKOs at P7. Scale bars: 500 µm (A,B,H,I); 50 µm (A,B,H,I, insets); 50 µm (G).

Fig. 7. Co-deletion of Bax or Trp53 with Aspm restored cerebellar growth and reduced apoptosis. (A,B) P30 H&E stains show that Aspm;Bax dKO cerebellar anatomy is similar to Aspm+/+;Bax++ controls, but with focal areas of abnormally thin IGL (inset). (C,D) Quantification of cerebellum weight in Aspm;Bax and Aspm;Trp53 dKOs, showing a normalization to control levels in the double mutants. (E,F) Cerebellar size, measured by volumetric MRI (E) and IGL cell density, determined by automated counting of nuclei (F) are similar in Aspm;Bax dKOs and wild-type controls. (G) Quantification of pHH3+ cells shows that mitotic activity is increased in Bax;Aspm and Trp53;Aspm double mutants, as in Aspm cKOs (Fig. 4E,F). (H) Both double mutants at P7 show a decrease in prophase and increase in prometaphase cells compared with controls, as in Aspm cKOs. (I,J) Reduced apoptosis in P7 Aspm;Bax double mutants (compare with Fig. 6A,B). (K,L) DNA damage (γH2AX) persists or is increased in both double mutants. Scale bars: 500 µm; 50 µm (insets).
abnormalities might underlie the failure of Bax co-deletion to completely normalize the cerebellum.

At P7, in both Aspm;Bax and Aspm;Trp53 dKO mice, apoptosis was significantly reduced compared with Aspm cKOs (Fig. 7I,J), although DNA damage persisted (Fig. 7K,L). Thus, loss of either Bax or Trp53 led to increased tolerance of DNA damage and reduced cell death, significantly restoring cerebellar growth. The lack of cell death in Aspm;Bax dKO mice confirms that the cell death induced by Aspm deletion is apoptotic. Focal hypoplasia within the IGL of Aspm;Bax dKOs, however, demonstrates that blocking apoptosis did not completely normalize the phenotype of Aspm mutation.

Aspm deficiency slows the growth of medulloblastoma

Because Aspm is upregulated in medulloblastoma in both ND2: SmoA1 mice (Fig. 11) and patient-derived samples (Vulcani-Freitas et al., 2011), we examined whether Aspm function is essential for tumor growth. We bred Aspmf/f mice into the rapidly tumorigenic medulloblastoma model, hGFAP-Cre;SmoM2 (G-Smo). G-Smo mice express a constitutively active allele of the Shh receptor component Smo (Mao et al., 2006) in stem cells that give rise to the neurons and glia of the cerebrum and cerebellum excluding the Purkinje cells (Zhuo et al., 2001; Kuang et al., 2012), and develop medulloblastoma with 100% incidence, dying of tumor progression by P20 (Schüller et al., 2008). We investigated the effect of Aspm deletion on tumor growth by comparing G-Smo mice with Aspmf/f (G-Smo;AspmKo) and Aspmf1/2 (G-Smo;Aspmhet) genotypes. G-Smo;AspmKo medulloblastomas grew significantly more slowly than G-Smo;Aspmhet tumors, although the rate of tumor formation was 100% in both genotypes. Tumors could be differentiated from normal brain in Hematoxylin & Eosin (H&E)-stained sections by their characteristic histology (supplemental Materials and Methods). To compare tumor size, we measured tumor cross-sectional area, normalized to the cross-sectional area of non-tumor cerebellum. At P3, tumor size appeared to be similar in G-Smo;AspmKo (n=4) or G-Smo;Aspmhet (n=3) mice, and none of the mice showed hydrocephalus (Fig. 8A,B). At P13, however, tumors in G-Smo;Aspmhet mice had expanded, producing obstructive hydrocephalus, whereas tumors in G-Smo;AspmKo mice (n=4 both genotypes) were significantly smaller and did not develop hydrocephalus (Fig. 8C-E).

Within tumors of both genotypes, γH2AX+ cells were too numerous and densely packed to be counted. Using western blot, however, we consistently found significantly increased γH2AX+ cells in Aspm-deleted tumors at P3 (Fig. 8F). By P13, γH2AX+ abundance was equivalent (data not shown), suggesting that tumors had developed a mechanism to compensate for Aspm deletion. Despite decreased tumor growth and absence of hydrocephalus, cerebellar anatomy and function was severely disrupted in G-Smo;AspmKo mice. These mice demonstrated severe motor deficits and could not transition from nursing to autonomous feeding, failed to maintain weight on weaning, and required euthanasia to prevent animal suffering. Although G-Smo;AspmKo mice did not seem to die from tumor expansion, because of the motor deficits, G-Smo;AspmKo mice did not survive longer than G-Smo;Aspmhet controls (Fig. 8G). Although we could not detect a survival benefit in this model, Aspm deletion effectively impaired tumor growth.

DISCUSSION

Our studies demonstrate that Aspm plays an essential role in postnatal neurogenesis, and that this role is co-opted in malignant proliferation in medulloblastoma. Aspm is integral to the proliferative program induced in CGNPs by Shh signaling and remains upregulated in Shh-driven medulloblastomas. Conditional deletion of Aspm in CGNPs is sufficient to impair cerebellar growth. This growth impairment cannot be attributed to decreased proliferation, as the frequency of CGNP mitosis is actually increased in Aspm cKOs. Rather, we show that growth impairment results from a combination of mitotic defects, precocious differentiation, increased DNA damage and increased apoptosis. The increase in proliferation rates in Aspm mutants might represent compensatory proliferation, a phenomenon that has been recently described in Drosophila centrosomal mutants, which also show spindle assembly defects, elevated DNA damage and increased apoptosis (Poulton et al., 2014).
Apoptosis of \textit{Aspm}-deleted CGNPs was prevented by co-deletion of \textit{Bax} or \textit{Trp53}, which significantly but incompletely restored cerebellar growth. The normalization of cerebellar weight and size in mice with co-deletion of \textit{Bax} and either \textit{Bax} or \textit{Trp53} demonstrates that preventing apoptosis can largely rescue \textit{Aspm}-dependent growth restriction. The persistent focal abnormalities in the IGL of \textit{Aspm};\textit{Bax} dKO mice, however, show that apoptosis is not the only process determining growth failure.

Along with increased DNA damage and apoptosis, we note impaired mitotic progression, changes in the radial position and orientation of mitotic cells, and abnormal patterns of differentiation in \textit{Aspm} cKO mice. This raises the intriguing question of whether the heterogeneity in division orientation we and others (Zagon and McLaughlin, 1987) observe in the EGL reflects different modes of division. Recent work has shown that both self-renewing CGNPs and differentiating CGNPs predominantly divide symmetrically (Espinosa and Luo, 2008; Nakashima et al., 2015). Division orientation might influence whether symmetric divisions result in self-renewal or differentiation, such that perpendicular divisions, which are most common in the EGL, may promote continuous proliferation, whereas transverse orientations, which are elevated in the \textit{Aspm} cKO may promote cell cycle exit.

Human and mouse studies have shown that diverse mutations including \textit{Nde1}, \textit{Cdk5rap2} and \textit{Magoh} induce microcephaly through a combination of loss of progenitor self-renewal, premature differentiation, and progenitor apoptosis (Pawlisz et al., 2008; Lizzaraga et al., 2010; Silver et al., 2010; Houlihan and Feng, 2014). Mutations of both \textit{Nde1} and \textit{Cep192} have been shown to induce microcephaly through p53-dependent apoptosis (Bazzi and Anderson, 2014; Houlihan and Feng, 2014). In all of these examples, the phenotype produced in mice is severe brain malformation. In both our studies and prior investigations, however, \textit{Aspm} deletion produced a phenotype in mice that is much less severe than the orthologous phenotype in humans (Pulvers et al., 2012). Of interest, the microcephaly genes \textit{ASPM} and \textit{CDK5RAP2} have been found to undergo positive selection in primates that correlates with increased brain size (Montgomery and Mundy, 2014); thus, \textit{Aspm} appears to be particularly important in large-brained animals. We propose that \textit{Aspm} loss impedes brain growth by impairing transient-amplifying cells in a manner that is proportional to the requirement for amplification. Accordingly, \textit{Aspm} deletion is more deleterious in humans, in which brain size is larger and the period of neurogenesis is more prolonged. We have previously shown that a very low rate of apoptosis can exert a significant impact on cerebellar growth, detectable only by comparing growth in apoptosis-deficient, \textit{Bax}-deleted mice (Garcia et al., 2012). A small increase in the rate of progenitor apoptosis may thus produce a significant reduction in brain growth that becomes more pronounced as the proliferation period is extended.

The restriction of tumor growth in \textit{Aspm}-deleted medulloblastoma is consistent with our model in which the effect of \textit{Aspm} loss is proportional to the extent of proliferation. In both cerebellar development and medulloblastoma formation, \textit{Aspm} deletion increased DNA damage. In tumors, however, where proliferation is more extensive than in normal tissues, DNA damage and growth restriction with \textit{Aspm} loss were most pronounced. Importantly, previous investigators have demonstrated that \textit{Aspm} knockdown impairs DNA repair after ionizing radiation \textit{in vitro} (Kato et al., 2011). Our findings that medulloblastomas require \textit{Aspm} for typical growth suggest that \textit{Aspm} could be therapeutically targeted to increase the efficacy of radiation and chemotherapy for medulloblastoma. Primary mouse medulloblastoma with \textit{Aspm} deletion provides an ideal model in which to test this possibility in preclinical studies.

**Materials and Methods**

**Mice**

\textit{Aspm-GFP} reporter mice [Tg(Aspm-EGFP)1H113Gsat/Mmucd] and \textit{Aspm-CreER} mice are previously described (Gong et al., 2010; Marinaro et al., 2011). For lineage tracing, \textit{Aspm-CreER} mice were crossed with Rosa26-Lox-STOP-Lox-TetOmp (Madisen et al., 2010) reporter mice (strain#7914; Jackson Laboratories). Tamoxifen (275 µg in 25 µl sunflower oil) was injected intraperitoneally into \textit{Aspm-CreER};\textit{TetOmp} mice and \textit{TetOmp} littermate controls lacking \textit{Aspm-CreER}, at the indicated ages.

\textit{Aspm}\textsubscript{SA/SA} mice were generated from \textit{Aspm}-targeted embryonic stem cells (line EPD0320\_1\_A06, Aspm\textsubscript{Sam1KOMP}Wtsi; KOMP Repository, Davis, CA, USA). The targeting vector is described in Fig. S2. Generation of \textit{Aspm}\textsubscript{SA/SA}, \textit{Aspm} cKO and hGFAP-Cre;\textit{SmoM2};\textit{Aspm}\textsubscript{−}\textsubscript{−} mice are detailed in supplementary Materials and Methods.

Medulloblastoma-prone \textit{NeuroD2};\textit{SmoA1} mice are previously described (Hallahan et al., 2004). \textit{Bax} floxed mice were derived from the \textit{Bax}\textsuperscript{Frt}\textsuperscript{−}\textsuperscript{−} strain (Takeuchi et al., 2005) crossed with C57BL/6 to recover wild-type \textit{Bak}. Conditional \textsuperscript{p53} (Jonkers et al., 2001) were provided by the NCI (strain#01XC2, Frederick, MD, USA). The \textit{SmoM2}, \textit{SmoA1}, \textit{Bax}\textsuperscript{Frt}, \textit{Trp53}\textsuperscript{Frt} and all Cre-expressing lines were crossed through at least four generations into a C57BL/6 genetic background. All animal handling and protocols were carried out in accordance with approved NIH practices and approved under UNC IACUC# 10-126.

**CGNP culture**

CGNPs were isolated and explanted as previously described (Kenney et al., 2003) and maintained in 0.5 µg/ml Shh (R&D Systems) or vehicle (0.5% BSA in PBS).

**RNA isolation and quantitative real-time PCR (RT-qPCR)**

Total RNA was isolated from cerebella or explanted CGNPs using the RNeasy kit (Qiagen), and cDNA synthesized from 1 µg total RNA using Superscript III (Invitrogen/Life Technologies). Gene expression was quantified on an ABI PRISM 7500 Sequence Detection System, using the \DeltaΔC\textsubscript{T} relative quantification method. All experiments included no template controls and were performed in triplicate and repeated twice independently. Primer sequences are listed in supplementary Materials and Methods.

**In vivo proliferation analysis**

Mice were injected intraperitoneally at the indicated ages with 50 µl Hanks’ Balanced Salt Solution containing EdU or BrdU (250 µM, Invitrogen). After the indicated interval, brains were dissected and fixed in 4% formaldehyde in PBS for 24 h at 4°C, then processed for histology.

**Histology, immunohistochemistry and quantification and MRI**

Mouse brains were processed and \textit{in situ} hybridization and immunohistochemistry were performed as previously described (Gershon et al., 2009, 2013; Garcia et al., 2012), using the \textit{Aspm} probe (Advanced Cell Diagnostics, #318711) or primary antibodies listed in supplementary Materials and Methods. Methods for quantitative analysis of mitosis, spindle orientation, BrdU/p27, c, γH2AX, tumor size and volumetric MRI are detailed in supplementary Materials and Methods. ITK-Snap software (www.itksnap.org) was used to isolate the cerebellar region in MR images (Yushkevich et al., 2006). TUNEL was performed using the Click-iT TUNEL Kit (Life Technologies, #C10245).

**Western blot analysis**

Whole cerebella were processed as previously described (Garcia et al., 2012) using primary antibodies to γH2AX and β-actin (Cell Signaling, #4970).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Experiments were conceived by I.G., S.E.W. and T.R.G. Experiments were performed by I.G., S.E.W., A.J.C., A.S., S.L., S.O., K.V., J.R.M. and H.L. Data were analyzed by S.E.W., I.G., K.J.L., S.O., E.A.O., Y.-Y.I.S., K.V. and T.R.G. Manuscript was written by I.G., S.E.W. and T.R.G., with input from all authors.

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References


SUPPLEMENTARY METHODS

Generation of mice with Aspm deletion

ES cells containing a targeted allele of Aspm were obtained from the Knockout Mouse Project (KOMP) Repository (Aspm<sup>Gt(KOMP)Wtsi</sup>; ES cell clone EPD0320_1_A06; here termed Aspm<sup>SA</sup>). ES cells were injected into blastocysts to generate chimeras, which were crossed with albino C57 Bl6 mice. The resulting black mice were genotyped by PCR to identify mice heterozygous for insertion of a splice-acceptor cassette between exons 6 and 7 of Aspm. The selected Aspm<sup>SA/+</sup> mice were then back-crossed into C57 Bl6 mice, then intercrossed to generate Aspm<sup>SA/SA</sup>, Aspm<sup>SA/+</sup>, and Aspm<sup>+/+</sup> littermates.

Aspm is located on the forward strand of Chromosome 1E4. The targeting cassette contains a ~4500bp 5' homology arm spanning the intron between exons 3 and 4 and into exon 6, and a ~4700bp 3' homology arm spanning exon 7 to the intron between exons 9 and 10. This "knockout-first" targeted Aspm<sup>SA</sup> allele inserts an FRT-flanked LacZ-neomycin (βgeo) cassette downstream of exon 6, and includes loxP sites flanking exon 7 (Fig. S2A). The βgeo cassette contains an Engrailed2 splice acceptor that leads to aberrant splicing of exon 6 to the βgeo cassette, followed by stop codon and polyadenylation signal. This is predicted to produce an Aspm transcript truncated at nucleotide 2482 (corresponding to amino acid 774), subsequently fused to LacZ. A similar gene trap line was generated by the Sanger Institute Gene Trap Resource (SIGTR, Aspm<sup>Gt(AA0137)Wtsi</sup>) and characterized by the Huttner lab, in which a βgeo cassette was inserted between exons 7 and 8, leading to truncation of Aspm at amino acid 794, which contains only the N-terminal microtubule domains and lacks the calponin homology domains and C-terminal calmodulin-binding isoleucine-glutamine (IQ) repeats (Pulvers et al., 2010). This gene-trap allele, Aspm<sup>exons1-7</sup>, was shown to produce a truncated mRNA, and an antibody directed against the peptide region transcribed by exon3 of Aspm or against βgeo showed that a truncated protein was produced (Pulvers et al., 2010). This truncated protein was shown to localize to spindle poles at metaphase, though at reduced levels compared to wild-type Aspm; but was
absent from the midbody at telophase, in contrast to wild-type Aspm (Pulvers et al., 2010). Thus, it is likely that both Aspm<sup>xons1-7</sup> and our very similar Aspm<sup>SA</sup> are hypomorphic alleles. In support of this, using qPCR primers specific to exons 24-25, we could not detect any full-length transcript in the homozygous Aspm<sup>SA/SA</sup> mutant (Fig. 3A), consistent with the fact that this transcript would be truncated at exon 6.

To generate a conditional allele of Aspm (Aspm<sup>f+</sup>, Fig. S2B), Aspm<sup>SA/SA</sup> mice were crossed with Rosa26-FLPe mice (Farley et al., 2000; strain 3946, Jackson Laboratories). Mice were screened by PCR for the excision of the FRT-flanked splice-acceptor cassette and the presence of loxP sites flanking exon 7. Aspm<sup>f+</sup> progeny were bred to wild-type C57 Bl6 mice, then bred to homozygosity of the Aspm<sup>f+</sup> allele.

To conditionally delete Aspm in CGNPs, Aspm<sup>f+</sup> and Math1-cre (Matei et al., 2005) lines were intercrossed, then back crossed with Aspm<sup>f+</sup>, generating the genotype Math1-cre;Aspm<sup>f+</sup> (Aspm cKO). Deletion of exon 7 leads to introduction of a frameshift between exons 6 and 8. The N-terminal portion of the protein would be transcribed correctly through I774, but the frameshift is predicted to mutate G775D, followed by a short peptide, SFWRTDPSGRQQ, and a premature termination codon (Fig. S3C). This is likely to lead to nonsense mediated degradation of the truncated transcript, and in fact we detected minimal full-length Aspm transcript in the cKO cerebellum using RT-PCR primers spanning exons 24-25 (Fig. 3A). We interpret the residual Aspm full-length mRNA detected in Aspm cKOs (Fig. 3A) to be due to the fact that RNA was isolated from whole cerebella, and Aspm is only deleted in the Math1 lineage. Thus, Aspm mRNA present in blood vessels or other cell types in the cerebellum that express Aspm could account for the ~20% we detected in the cKOs compared to Aspm<sup>f+</sup> controls.

To generate animals with Aspm deletion and medulloblastoma, Aspm<sup>f+</sup> mice were crossed with hGFAP-Cre (Zhuo et al., 2001) and SmoM2 (Mao et al., 2006) (Jackson Laboratories, strain 5130) mouse lines, and the resulting progeny were intercrossed to generate the genotypes hGFAP-cre;SmoM2;Aspm<sup>f+</sup> (G-Smo;Aspm<sup>KO</sup>) or hGFAP-cre;SmoM2;Aspm<sup>f+</sup> (G-Smo;Aspm<sup>het</sup>). hGFAP-cre mice express Cre during brain development in stem cells that give rise to diverse progeny, including the
entire cerebellum, excluding the Purkinje lineage (Zhuo et al., 2001; Kuang et al., 2012) and hGFAP-Cre;SmoM2 mice develop rapidly progressive medulloblastoma (Schüler et al., 2008).

**PCR Primers and Primary Antibodies**

For RT-qPCR: *Aspm* primers we used have been previously published (GCTTCATCACCTGCTCACCTAC and TAGATACCGCTCCGCTTTAC; Wu et al., 2008). Additional primer pairs were *CyclinD2* (GCGTGAGAAGGGCAGTCCA and CACTTTTGTCCCTCAGACCTCTCTAG), *GAPDH* (TGTGTCCGTCGTGGATCTGA and CCTGCTTCACCACCTTCTTGA).

For genotyping: *Aspm*SA mice primers were TTGTATACAGCAGCTTCCA, TACGAAGTTATGCTGAGATATCACTAG, and TTGTGACATCAATTTTCTAG, producing a ~300 bp band from the *Aspm*SA allele, and a ~400 bp band from the WT allele. Aspmfl primers were TTGTATACAGCAGCTGCAA and TTGTGACATCAATTTTCTAG, producing a ~500 bp band from the floxed allele and a ~400 bp band from the WT allele. Math1-cre primers were GCGGTCTGGCAGTAAAAACTATC and GTGAAACAGCATTGCTGTCACTT, producing a ~200 bp band. SmoM2 primers were AAGTTCATCTGCACCACCG and TCCTTGAAGAGATGTTGCC, producing a ~200 bp band.

Primary antibodies (all except as noted from Cell Signaling, Danvers, MA, USA): cC3 (cat#9661), PCNA (cat#2586), Survivin (cat#2808), pH3 (cat#9706), γH2AX (cat#9718), BrdU (Accurate Scientific, Westbury NY, cat#OB0030G), Ki67 (Leica Biosystems/Novocastra, cat#NCL-L-Ki67-MM1), and p27 (Dako, Carpinteria, CA, cat#M7203).

**Quantitative analysis of mitotic progression, spindle orientation, BrdU/p27, cC3, γH2AX and tumor cross sectional area**

For analyses of proliferation, cerebellar sections were imaged on a 4-channel Leica DM5500 confocal SPEII microscope using LAS acquisition software to perform high magnification (32x) tilescans with a 20x ACS Apochromat 20x/0.60 multi-immersion objective. For images of mitotic cells, an ACS
Apochromat 63x/1.30 oil objective was used to perform z-stacks that confirmed division orientation. Mitotic stages were identified by the combination of Survivin and pHH3 staining patterns (Fig. 4G), and were manually counted using the cell counting module in LAS software. Two independent mid-sagittal sections from 3-4 animals of each genotype were analyzed, typically including 200-400 mitotic cells/section. Due to breeding schemes for Aspm;Bax double mutant analyses, Aspm<sup>f/+</sup>;Bax<sup>f/+</sup>;Math1-Cre mice were used as controls, as their cell cycle profile was indistinguishable from Aspm<sup>f/f</sup> wild-type mice (compare blue bars in Fig. 4H and Fig. 7H). Mitotic cell counts were normalized to EGL area to control for differences in cerebellum size between controls and Aspm cKOs, and because individual cells in the EGL were too densely-packed to accurately count. EGL and IGL area were determined with ImageJ, using a thresholded binary image of the DAPI channel. The position of each mitotic cell was determined by measuring its distance from the pial surface and dividing this value by the width of the EGL at that position (Fig. S3A). Values of <0.5 were considered EGL<sub>S</sub>, and >0.5 EGL<sub>D</sub>, which corresponded well with both morphologic differences in cell shape between cells in the oEGL and iEGL, as well as the boundary between PCNA and p27 expression at these ages (P5-P7, see Figs 3K-M).

For anaphase metrics, the shape of daughter pronuclei was analyzed using ImageJ to determine the aspect ratio (AR), where a value of 1.0 corresponds to a circle and higher values indicate more elliptical shapes. Z-stacks were taken to confirm that only cells dividing within the plane of imaging were quantified, thus avoiding potential error in measurements due to oblique angle divisions. The distance between daughter pronuclei (d) was measured between centromeres using ImageJ. Division orientation was determined for both anaphase and telophase cells as shown in Fig. 5A. Perpendicular divisions were defined as those in which the angle bisecting the daughter pronuclei and the pial membrane was greater than 45°, and parallel were less than 45°. Transverse divisions were defined as those in which Survivin staining was evident, generally in a semi-circular pattern, but only one daughter pronucleus could be visualized beneath it. This orientation was confirmed through z-stacks, and ambiguous cells were excluded from the analyses. Data were collected and exported from ImageJ into Microsoft Excel, then binned, analyzed, and plotted using GraphPad Prism.
For BrdU incorporation, P5 littermate pups were injected with 50 mg/kg BrdU and sacrificed 24 or 48 hours later. Mid-sagittal brain sections were double labeled with antibodies to BrdU (Accurate Scientific, Westbury NY, cat#OBT0030G) and p27 (Dako, Carpinteria, CA, cat#M7203). For the 48 hour pulse, the fraction of p27+ cells in the iEGL, molecular layer (ML) and IGL that were BrdU+ was quantified using Metamorph’s multiwavelength cell scoring module (Fig. 5M). For the 48 hour pulse, analysis included 5 (control) or 7 (Aspm cKO) sections from 3 (control) or 4 (Aspm cKO) littermates; for the 24 hour pulse, 4 sections (1 from each littermate animal) were analyzed for each genotype. Typically 3000-5000 BrdU+ cells per section were present and counted in each region. BrdU+ cells which were p27+ and p27- were manually counted and scored for the data presented in Figs 5L,N,O,P.

To quantify cC3+, TUNEL and γH2AX positive cells in the cerebellum, nuclei were counterstained with hematoxylin or DAPI, slides were digitized on an Aperio Scanscope and analyzed using Aperio Software (Aperio, Vista, CA, USA) for chromogen-stained slides or Tissue Studio (Definiens, München, Germany) for fluorescence. The entire EGL region was manually annotated and used for quantifications, which were normalized to the total number of nucleated cells in the designated region. Positive cells were manually counted and classified as EGLS or EGLD based on their position within the EGL (< or > 50% of EGL depth, respectively). To control for differences in cerebellum size between genotypes, counts were normalized to EGL length, quantified by manually tracing the outer perimeter of the cerebellum in ImageJ.

In Figure 3, to quantify the area of the EGL and IGL, we used ImageJ to threshold the DAPI channel, and manually defined the region of interest (ROI) to be restricted to the EGL or IGL, respectively (Fig. 3J). For calculations of the PCNA+ area of the EGL (oEGL) and p27+ area of the EGL (iEGL) in Figs 3M,N, similar procedures were used to threshold the PCNA and p27 channels. The perimeter (length) of the EGL was traced and calculated in ImageJ, and oEGL/PCNA+ and iEGL/p27+ thickness was determined by dividing these areas by the EGL length (Fig. 3N). EGL perimeter was similarly used to normalize counts of apoptotic cells in Fig. 5L and Fig. 6D. In Figs 4C,D,F and Figs 7F,G, similar procedures were used to calculate EGL area, whereby the DAPI channel was used to generate a binary threshold image, whose area was calculated in ImageJ. This area measurement was
used to normalize counts of mitotic cells, given the differences in EGL area between genotypes, where
Aspm cKOs showed a ~20% reduction on average.

To compare tumor size between the G-Smo;AspmKO and G-Smo;Aspmhet genotypes, brains
sections were stained with H&E and the sections with the maximum cross-sectional diameter were
selected. In these sections, tumor was identified by hypercellular pathology, nuclear shape, and intense
hematoxylin staining. Normal cerebellum was identified by histologic features, including cellularity and
nuclear shape within in the relatively hypercellular IGL. Tumor and non-tumor areas were annotated
manually and measured using Aperio software. Tumor area was normalized to the area of normal
cerebellum in order to consider proportion of total cerebellum attributable to tumor.

Volumetric MRI studies

For volumetric MRI studies, P30 mice were perfused and brains were dissected free and fixed
for 48 hours in 4% formaldehyde in PBS. Brains were then washed in PBS and then transferred to
Fomblin Y04 solution (Kurt J. Lesker Company, Cat#:MFY06/6BB) 24 hours before scanning. Brains
were then imaged in pairs on a Bruker Biospec 9.4T horizontal bore scanner with a RARE 3D
sequence using TR = 3000 ms, T Eff = 60 ms, RARE factor = 8, field of view = 4.2x1.4x1 cm, matrix
size = 420x140x100, resulting in 100µm isotropic resolution. Whole brain volume was quantified using
Amira software (FEI Visualization Sciences Group, Bordeaux, France) with intraventricular space
excluded after tissue segmentation. For cerebellar measurements, images were analyzed using ITK-
SNAP software (www.itksnap.org) (Yushkevich et al., 2006). 3D scans were projected as sets of linked
slices in the sagittal, coronal and axial orientations and the cerebellar region was manually annotated in
each slice. The cerebellar volume was thus defined and measured within the software environment.
Measured volumes were grouped by genotype and the two-tailed students’ t-test was used to compare
volumes between genotypes.
**Supplementary Figures**

**Supplementary Figure 1.** Fate of *Aspm-CreER;Rosa26-TdTomato* descendants in cerebellum and hippocampus.

A-A’”) Confocal section of P7 cerebellum pulsed with tamoxifen at P4. Red arrowheads indicate migrating GNs in the molecular layer (ML). Note also TdTomato+/NeuN+ GNs in the IGL. B-B’”) Confocal section of P7 cerebellum stained as in (A) showing TdTomato+/NeuN+ GNs in the IGL (hollow red arrowheads) and migrating GN in the ML (solid red arrowhead). A TdTomato+ interneuron in the plexiform layer that is weakly NeuN+, likely a Lugaro cell, is indicated by the yellow arrowhead.

C) TdTomato+/CD31+ endothelial cells in P11 cerebellum, indicating that Aspm is also expressed in blood vessels.

D,E) Confocal sections of P7 dentate gyrus (DG) region of the hippocampus following a 3 day pulse with tamoxifen at low (D) and high (E) magnification. Progenitors reside in the NeuN-subgranular zone (SGZ) and show the characteristic bipolar radial glia-like morphology. One example of a differentiated TdTomato+/NeuN+ hippocampal DG neuron is indicated by the red arrowhead in (E’”).

F) Aspm-derived progenitors in the subventricular zone (SVZ) of the P11 cerebral cortex are not of glial (GFAP+) origin. Scale bars: 50 µm in (A,C,E); 25 µm in (B); 100 µm in (D).
**Supplementary Figure 2.** Description of *Aspm* targeting vectors and mutant alleles.

**A)** Diagram of *Aspm* gene locus on chromosome 1E4, with targeting vector used to create the “knockout first” *Aspm<sub>SA</sub>* allele shown (See Supplemental Methods for details). This targeting vector inserts an FRT-flanked LacZ-neomycin (βgeo) cassette downstream of exon 6, and includes loxP sites flanking exon 7. The predicted protein product of this insertion is a fusion of exon 6 to the βgeo cassette via the *Engrailed2* splice acceptor.

**B)** The *Aspm<sub>SA</sub>* allele can be reverted to a wild-type allele by excision of the FRT-flanked βgeo cassette using flipase. This was accomplished by mating with the *Rosa26-FLPe* line, expressing the enhanced FLP recombinase under expression of the ubiquitous *Rosa26* promoter. The resultant line *Aspm<sub>SA</sub>* retains loxP sites in the introns surrounding exon 7 but produces a wild-type transcript.

**C)** The *Aspm<sub>SA</sub>* allele can then be converted to a conditional predicted null by crossing with transgenic lines expressing Cre recombinase in a tissue-specific manner. Deletion of exon 7 is predicted to introduce a frameshift that leads to a premature stop codon early in exon 8. To generate CGNP conditional cKOs, we used the *Math1-Cre* line, while the *hGFAP-Cre* line was used for medulloblastoma models.
**Supplementary Figure 3.** Distribution of proliferating cells in the EGL in Aspm cKOs. A,B) Representative regions of cerebellum between lobes III and IV labeled for pH3 (green) and DAPI (gray). A) The depth of pH3+ cells is calculated by measuring the distance from the pia (yellow) and dividing by the thickness of the EGL (magenta), shown here for two cells. A') Depth measurements for all pH3+ cells in this section, as quantified using the metric in (A). The approximate boundary between the EGLS and EGLD is shown by the dotted line, equivalent to half the distance between the pia and boundary of the EGL and molecular layer. Cells located within the EGLS are labeled in white and those within the EGLD in red. A'') White arrowheads indicate cells within the EGLS and red arrowheads, those within the EGLD. B-B'') Additional cKO (B) and Aspmf/f control (B',B'') sections indicating position of mitotic cells within the EGLS (white arrowheads) and EGLD (red arrowheads), as in (A'). Note increase in pH3+ figures within the EGLD in Aspm cKOs. C) Box-and-whisker plots of the position of mitotic cells in control and Aspm cKOs, showing a tendency toward deeper divisions in the cKO. D,E) Quantification of mitotic cell density by pH3 (D) and Survivin (E) staining in the EGLS and EGLD, normalized to EGL length. F,G) Representative regions of lobe VII for P7 Aspmf/f control and Aspm cKO cerebellum labeled with pH3 and Ki67 (F) or PCNA (G) to label cycling cells in the oEGL. White arrowheads indicate rare mitotic cells located outside of the Ki67/PCNA zone. H) Quantification of the frequency in which pH3+ cells are found outside of the Ki67 or PCNA zone. I,J) In this data cohort, the frequency of mitotic cells was analyzed and again found to be higher in cKOs (compare Fig. S3I to Fig. 4C). The increased incidence of mitotic figures was located mainly in the EGLD (compare Fig. S3J to Fig. 4F). Scale bars: 50 µm.
**Supplementary Movies 1 and 2.** Representative animated, rotating 3-dimensional MRIs of the mouse brain and digitally isolated cerebellum, obtained from an Aspm<sup>SA/SA</sup> mouse.

Video clip shows the whole brain (1) or cerebellar region (2), rotated to reveal all sides.