Aspm sustains postnatal cerebellar neurogenesis and medulloblastoma growth in mice

Scott E. Williams¹,²,§, Idoia García³,+,⁴,§, Andrew J. Crowther³,⁴, Shiyi Li³, Alyssa Stewart³, Hedi Liu³, Kendall J. Lough¹, Sean O’Neill³, Katherine Veleta³,⁴, Esteban A. Oyarzabal³,⁴, Joseph R. Merrill⁵, Yan-Yu Ian Shih¹,⁴,⁵,⁶ and Timothy R. Gershon²,³,⁴,¶

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 in mice with Smo-induced medulloblastoma reduces tumor growth and increases DNA damage. Co-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 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; Marinario 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; Bikeye 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, Asp 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 expression corresponded with sites of proliferation indicated by EdU, including the EGL and subventricular zone (SVZ; Fig. 1B). Widefield (Fig. 1C) and confocal (Fig. 1D) microscopy demonstrated GFP throughout the EGL in P7 Aspm-GFP mice, including differentiating iEGL cells. In light of our in situ hybridization data, we attribute the persistent GFP signal in the iEGL to stability of the GFP protein beyond its period of transcription. Like Aspm mRNA, GFP reporter fluorescence waned by P16 (Fig. 1E), 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 (Ccnd2), 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 Ccnd2 mRNAs in vitro that was blocked by vismodegib. (G) Isolated CGNPs from Aspm-GFP mice express GFP only when cultured with Shh. (H) Sagittal cerebellar section of a ND2: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 ND2: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 vitro 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 reporter mice with the transgenic, 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 EGL 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, tdTomato 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 tdTomato 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 Aspm<sup>SA/SA</sup> 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 Aspm<sup>SA/SA</sup> mice by RT-qPCR, which demonstrated an absence of full-length Aspm mRNA in Aspm<sup>SA/SA</sup> cerebella, compared with Aspm<sup>SA/SA+</sup> littermates (Fig. 3A). Aspm<sup>SA/SA</sup> 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 Aspm<sup>SA/SA</sup> mice were significantly smaller than brains and cerebella of Aspm<sup>SA/SA+</sup> or Aspm<sup>SA+<sub>p</sub></sup> littermates (Fig. 3B,C). These results show that the Aspm<sup>SA/SA</sup> 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<sup>2B</sup>; Fig. S2B) by crossing Aspm<sup>SA/SA</sup> 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. S2B,C), which in the cerebellum is restricted to CGNPs (Helms et al., 2000; Machold and Fishell, 2005; Matei et al., 2005). The resulting Math1-Cre;Aspm<sup>F</sup> mice (Aspm<sup>cKO</sup>) expressed less Aspm mRNA in cerebella, compared with Aspm<sup>F</sup> littermates without Cre (Fig. 3A). Although overall brain weight was preserved in Aspm<sup>cKO</sup> 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 Aspm SA/SA mutants, and reduced cerebellar volume in Aspm cKOs. 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 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). In littermate controls at P7, the ratio of the area of the PCNAI region to the p27I area 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 (PCNAI) and iEGL (p27I) 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.

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
isolated from either Aspm cKO mice or Aspm<sup>fl/fl</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>fl/fl</sup> littermate controls (n=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+<sup>-</sup>, 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 EoG, 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+ 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>fl/fl</sup> littersmates (Fig. S3C; n>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
EGLS 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 EGLS of both genotypes, but significantly increased mitotic frequency in the EGLD in cKOs. Co-labeling with pH3³ 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 pH3³ figures in the cKO compared with controls (Fig. S3I), predominantly localized to the EGLD (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 EGLS/EGLD 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 pH3⁵/survivin co-immunofluorescence to determine the distribution of mitotic stages found in wild-type and Aspm cKO CGNs (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 Aspm⁵⁻ 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 CGNs displayed abnormal rounded DNA during anaphase, suggestive of defects in organizing the spindle poles, whereas Aspm⁵⁻ control CGNs 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 to 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, CGNs adopt different cleavage plane orientations depending on their position within the EGL (Zagon and McLaughlin, 1987). In the EGLS, CGNs are more frequently oriented to the pial surface and divide in the EGLD 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 CGNs was increased at the expense of perpendicular divisions (Fig. 5A,B; χ² test, P<0.0001). Analysis of division orientation relative to EGL depth demonstrated a bias in control cerebella towards perpendicular divisions in the EGLS (56.1%, n=301), with roughly equal frequencies of perpendicular, transverse and parallel divisions in the EGLD (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 CGNs to re-enter or to exit the cell cycle (e.g. self-renew or differentiate), we examined the fate of CGNs 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). CGNs in the EGL were labeled with BrdU with similar frequency at the 24 h time point in Aspm⁵⁻ control and cKOs (Fig. 5L), suggesting that CGNs in both genotypes enter S phase with similar frequency. However, there was a tendency towards fewer actively cycling (p27⁻) and more differentiating (p27⁺) BrdU⁺ CGNs in the cKOs compared with controls (Fig. 5L). Further analysis showed that a greater proportion of p27⁺ CGNs in the iEGL were labeled with BrdU in cKOs compared with controls (Fig. 5M). We quantified the cell cycle exit fraction (proportion of BrdU⁺ CGNs 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⁺ CGNs in the EGL at 48 h (Fig. 5P). Together, these data demonstrate that Aspm cKO CGNs prematurely exit the cell cycle but fail to populate the IGL as effectively as normal CGNs.

**Aspm deficiency increases progenitor DNA damage and apoptosis**

The overproduction of p27⁺ CGNs in the EGL coupled with underproduction of mature GNs in the IGL suggests that Aspm cKO CGNs 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 Aspm⁵−/⁵− 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 EGLS (86.7±1.2%, whereas in Aspm cKOs the density of cC3− or TUNEL⁺ apoptotic cells in the EGLD 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 CGNs, with the largest relative increase in the differentiating population. This increase in apoptosis specifically in the EGLD could explain why fewer mature GNs are produced in Aspm cKOs despite the increased production of p27⁺ CGNs.
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 AspmSA/SA 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 cKO phenotype. Because p53 may exert gene dosage-related effects, we selected Math1-Cre;Aspm<sup>f/f</sup>;Trp53<sup>−/−</sup> (Aspm cKO) and Math1-Cre;Aspm<sup>f/f</sup>;Trp53<sup>+/−</sup> (Aspm dKO) littermates for comparison. For Bax studies, we compared littermates with Aspm<sup>f/f</sup> 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 c3 (black arrowheads). (C) Quantification of c3+ 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 (c3+ or TUNEL+ cells/mm of EGL) in Aspm mutants. (E,F) The most significant increase in cell death in Aspm mutants, detected by c3 (E) or TUNEL (F) occurs in differentiating cells of the EGLD. (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 mutant controls and 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 pH3+ 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. 1I) and patient-derived samples (Vulcani-Freitas et al., 2011), we examined whether Aspm function is essential for tumor growth. We bred Aspm\textsuperscript{floxed} 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 (Zhao 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 Aspm\textsuperscript{floxed} (G-Smo;Aspm\textsuperscript{KO}) and Aspm\textsuperscript{floxed} (G-Smo;Aspm\textsuperscript{het}) genotypes.

G-Smo;Aspm\textsuperscript{KO} medulloblastomas grew significantly more slowly than G-Smo;Aspm\textsuperscript{het} 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;Aspm\textsuperscript{KO} (n=4) or G-Smo;Aspm\textsuperscript{het} (n=3) mice, and none of the mice showed hydrocephalus (Fig. 8A,B). At P13, however, tumors in G-Smo;Aspm\textsuperscript{het} mice had expanded, producing obstructive hydrocephalus, whereas tumors in G-Smo;Aspm\textsuperscript{KO} mice (n=4 both genotypes) were significantly smaller and did not develop hydrocephalus (Fig. 8C-E). Within tumors of both genotypes, γH2A\textsuperscript{X} cells were too numerous and densely packed to be counted. Using western blot, however, we consistently found significantly increased γH2A\textsuperscript{X} cells in Aspm-deleted tumors at P3 (Fig. 8F). By P13, γH2A\textsuperscript{X} 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;Aspm\textsuperscript{KO} 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;Aspm\textsuperscript{KO} mice did not seem to die from tumor expansion, because of the motor deficits, G-Smo;Aspm\textsuperscript{KO} mice did not survive longer than G-Smo;Aspm\textsuperscript{het} 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 Aspm-deleted CGNPs was prevented by co-deletion of Bax or Trp53, which significantly but incompletely restored cerebellar growth. The normalization of cerebellar weight and size in mice with co-deletion of Aspm and either Bax or Trp53 demonstrates that preventing apoptosis can largely rescue Aspm-dependent growth restriction. The persistent focal abnormalities in the IGL of Aspm;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 Aspm cgKOs. 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 continued proliferation, whereas transverse orientations, which are elevated in the Aspm cKO may promote cell cycle exit.

Human and mouse studies have shown that diverse mutations including Nde1, Cdk5rap2 and Magoh induce microcephaly through a combination of loss of progenitor self-renewal, premature differentiation, and progenitor apoptosis (Pawlisz et al., 2008; Lizarraga et al., 2010; Silver et al., 2010; Houlihan and Feng, 2014). Mutations of both Nde1 and Cenpj 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, Aspm deletion produced a phenotype in mice that is much less severe than the orthologous phenotype in humans (Pullers et al., 2010). Of interest, the microcephaly genes ASPM and CDK5RAP2 have been found to undergo positive selection in primates that correlates with increased brain size (Montgomery and Mundy, 2014); thus, Aspm appears to be particularly important in large-brained animals. We propose that Aspm loss impedes brain growth by impairing transient-amplifying cells in a manner that is proportional to the requirement for amplification. Accordingly, 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, 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 Aspm-deleted medulloblastoma is consistent with our model in which the effect of Aspm loss is proportional to the extent of proliferation. In both cerebellar development and medulloblastoma formation, Aspm deletion increased DNA damage. In tumors, however, where proliferation is more extensive than in normal tissues, DNA damage and growth restriction with Aspm loss were most pronounced. Importantly, previous investigators have demonstrated that Aspm knockdown impairs DNA repair after ionizing radiation in vitro (Kato et al., 2011). Our findings that medulloblastomas require Aspm for typical growth suggest that Aspm could be therapeutically targeted to increase the efficacy of radiation and chemotherapy for medulloblastoma. Primary mouse medulloblastomas with Aspm deletion provides an ideal model in which to test this possibility in preclinical studies.
NeuroD2 and SmoA1 mice. We thank the UNC Small Animal Imaging and Tissue Pathology Laboratory Core facilities for providing the MR imaging and immunohistochemistry.

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.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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Supplementary information
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References
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Development


