Published in final edited form as:

Acta Neuropathol. 2012 April ; 123(4): 573–586. doi:10.1007/s00401-012-0946-z.

An essential role for p38 MAPK in cerebellar granule neuron precursor proliferation

Cemile G. Guldal^{1,3}, Adiba Ahmad³, Andrey Korshunov⁴, Massimo Squatrito³, Aashir Awan¹, Lori A. Mainwaring², Bipin Bhatia³, Susana R. Parathath³, Zaher Nahle¹, Stefan Pfister⁴, and Anna M. Kenney^{1,†}

¹Departments of Neurological Surgery and Cancer Biology, Vanderbilt University, Nashville, TN USA 37212

²Biochemistry, Cell, and Molecular Biology Program, Weill Medical College of Cornell University, New York NY 10021 USA

³Department of Cancer Biology and Genetics, Memorial Sloan-Kettering Cancer Center, New York, NY 10065 USA

⁴German Research Center, Heidelberg University Hospital, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

Abstract

Development of the cerebellum occurs postnatally and is marked by a rapid proliferation of cerebellar granule neuron precursors (CGNPs). CGNPs are the cells-of-origin for SHH-driven medulloblastoma, the most common malignant brain tumor in children. Here, we investigated the role of ERK, JNK, and p38 mitogen-activated protein kinases (MAPKs) in CGNP proliferation. We found high levels of p38 α in proliferating CGNPs. Concomitantly, members of the p38 pathway, such as ASK1, MKK3 and ATF-2, were also elevated. Inhibition of the Shh pathway or CGNP proliferation blunts p38 α levels, irrespective of Shh treatment. Strikingly, p38 α levels were high *in vivo* in the external granule layer (EGL) of the postnatal cerebellum, Shh-dependent mouse medulloblastomas and human medulloblastomas of the SHH subtype. Finally, knocking down p38 α by short hairpin RNA-carrying lentiviruses as well as the pharmacologically inhibiting of its kinase activity caused a marked decrease in CGNP proliferation, underscoring its requirement for Shh-dependent proliferation in CGNPs. The inhibition of p38 α also caused a decrease in *Gli1* and *N-myc* transcript levels, consistent with reduced proliferation. These findings suggest p38 inhibition as a potential way to increase the efficacy of treatments available for malignancies associated with deregulated SHH signaling, such as basal cell carcinoma and medulloblastoma.

Keywords

Sonic hedgehog; p38 MAPK; proliferation; neural precursor; cerebellum; medulloblastoma

[†]Corresponding author: Anna Marie Kenney, PhD, anna.kenney@vanderbilt.edu, (615) 936-3863 (tel), (615) 343-8104 (fax).

Conflict of interest The authors declare no conflict of interest.

INTRODUCTION

The cerebellum is a foliated dorsal brain structure that regulates coordination, fine movements, and posture. In its distinctly layered architecture there are several subtypes of neurons, including granule neurons. In humans and in mouse models, cerebellar granule neuron precursors (CGNPs) are proposed to be the cell of origin for medulloblastomas marked by aberrant activation of the Shh signaling pathway [51,44,53,62]. Understanding the regulation of CGNP proliferation by mitogenic Shh signaling in the normal developing cerebellum will lead to greater insight as to how aberrant Shh pathway activity contributes to cancers, such as medulloblastoma and basal cell carcinoma [48].

During development of the cerebellum in mice and humans, CGNPs undergo a rapid post-natal proliferation phase in the external granule layer (EGL). After several rounds of rapid cell division, CGNPs migrate through the underlying layer of Purkinje neurons, the source of Shh, to form the internal granule layer (IGL), where they terminally differentiate into mature interneurons [8,51,66,11]. Post-natal expansion of CGNPs in the EGL requires Shh signaling and the loss of Shh leads to reduced proliferation and cerebellar defects in neonatal mice [33,55,59]. Shh is a mitogen that, by interacting with the transmembrane protein Patched (Ptch), relieves the inhibition of Smoothened (Smo) [for detailed review of Shh signaling in the CNS, see 34]. Activation of Smo ultimately leads to transcriptional activity of the Gli transcription factors, which themselves regulate a gene expression program resulting in proliferation and survival [22,23,25,54]. In addition to the Gli factors, Shh activity also induces expression of oncogenes N-Myc, E2F1, YAP, and the microRNA miR 17/92, which are required for CGNP proliferation [4,16,28,42,56]. An additional feed-forward loop wherein N-Myc up-regulates eIF4E to ultimately inhibit S6 Kinase, stabilizing IRS1 also drives N-Myc-dependent CGNP proliferation [36]. Indicating conservation between Shh-induced CGNP proliferation and medulloblastomagenesis, N-Myc, YAP, and miR 17/92 are also amplified or over-expressed in mouse and human Shh-associated medulloblastomas [16,42].

In addition to Shh, CGNPs also require IGF signaling for their proliferation. Both IGF1 and IGF2 activate the IGF1 receptor, and both are expressed in the neonatal cerebellum. The activated IGF1 receptor recruits IRS1, which serves as a scaffolding platform for downstream kinases, ultimately leading to the activation of Akt. Akt activity inhibits GSK3 β , which normally phosphorylates and promotes degradation of N-Myc [28,31,45,20]. As a result, Shh and IGF pathways both impinge on N-Myc to ensure CGNP proliferation. Akt activity also leads to mTOR activation, leading to increased mRNA translation through dis-inhibition of eIF4E. In mouse models for Shh-mediated medulloblastoma, mTOR inhibition blocks tumor cell proliferation [5].

As continued proliferation and failure to commence differentiation is thought to be essential for the progression of Shh-dependent medulloblastomas, it is essential to understand the signaling pathways involved in regulation of CGNP proliferation by Shh. This will also be critical for developing new targeted therapies to treat medulloblastoma patients that will both increase their survival rate and ameliorate the life-long, debilitating side effects that occur as a result of the current standard of care (surgical resection, craniospinal radiation,

chemotherapy). Mitogen-Activated Protein Kinases (MAPKs) play a prominent role in regulating proliferation in all mammalian cells and Shh has been noted to act very much like a classic mitogen [27,46,59]. Moreover, classic MAP/Erk kinase pathway activity is known to be required for long-term CGNP survival, albeit dispensable for short-term CGNP proliferation [6,27]. We therefore investigated whether the Shh pathway interacts with other MAPK pathways to regulate proliferation.

There are three major classic MAPK families in mammalian cells: ERK family, p38 family, and SAPK/JNK family. The former is usually associated with mitogenic growth signals and the latter two are classic responders to stress-related stimuli, such as inflammation, oxidative stress, and DNA damage [9,24,30]. Evidence has also accumulated showing that p38 and JNK can have non-classical roles in proliferation and survival programs [29,57,58]. We found that p38 α MAPK protein is elevated and active in CGNPs exposed to Shh, and that both p38 α and phosphorylated (activated) p38 α localized to the neonatal mouse EGL. Indeed, we found a striking upregulation in the whole pathway, including upstream kinases MKK3 and ASK1 and downstream effector ATF-2. Furthermore, p38 α levels and activity were increased in mouse medulloblastomas compared to adjacent non-tumor cerebellum. Analysis of biopsies of human medulloblastomas revealed high levels of active p38 α in the SHH-subtype tumors. Interestingly, Smo inhibition in CGNPs was associated with a decrease in p38 activity, and p38 pathway inhibition caused a marked decrease in proliferation of cultured CGNPs. We also found that *Gli1* and *N-myc* expression levels decrease when p38 is inhibited, which could underlie the reduced CGNP proliferation.

Taken together, our findings that Shh signaling is associated with induction of p38 α activity in normal CGNPs and in mouse and human medulloblastomas, and blocking its activity reduces Shh-mediated proliferation suggest that the p38 pathway may be a viable therapeutic target in SHH-associated medulloblastoma. This is especially important in light of recent studies showing that use of anti-SHH drugs targeting SMO result in drug resistance and tumor relapse [7,14,63]. Moreover, many SHH-associated tumors already have amplification of SMO downstream targets, suggesting that SMO inhibition itself may be fruitless and a more useful approach may be to target pathways impinging on the expression of stability of SMO effectors.

MATERIALS AND METHODS

Animal studies

Preparation of cerebella and tumor tissue was carried out in compliance with the Memorial Sloan-Kettering Institutional Animal Care and Use Committee guidelines. *NeuroD2-SmoA1* mice were purchased from Jackson Labs (008831).

CGNP and cell cultures

Cerebella were isolated from postnatal day (PN) 4-5 Swiss-Webster or *NeuroD2-Smo A1* mice and primary cultures were prepared as described [45]. Pzp53med cells, generously provided by Matt Scott (Stanford), were grown in DMEM/1% FCS with antibiotics. Other compounds used were: Shh (R&D Systems, 3 μ g/mL), Cyclopamine (1 μ g/mL, gift of Dale

Gardner, USDA), bFGF (20 ng/mL, Peprotech), SANT-2 (100 nM, Enzo Life Sciences), SB203580 (559398, EMD Biosciences), BrdU (10 μ M, Sigma).

Lentivirus production and CGNP infection

293T packaging cells (ATCC) were transfected as described before [36,45] with Mission shRNA lentiviral plasmids (Sigma; p38-1, TRCN0000023120; p38-2, TRCN0000055225) predicted to target p38, that also expressed GFP for detection. GFP shRNA was used as a control (shGFP, Sigma).

RNA extraction and real-time PCR

Total RNA from CGNPs and tissue was extracted either with TRIzol reagent (Invitrogen) or RNeasy Kit (Qiagen) according to the manufacturers' directions. cDNA was prepared from 1 μ g of total RNA using iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays with Step One Plus (Applied Biosystems).

Protein preparation and immunoblotting

For immunoblot analysis, cells were washed once in PBS and scraped in lysis buffer for protein extracts as previously described [27]. 50-70 μ g of each sample was separated on 7-12% polyacrylamide gels and transferred in 20% methanol buffer to Immobilon-P membranes (Millipore). Antibodies used were: Cyclin D2 (Santa Cruz Biotechnology, sc-593), p38 and phospho-p38 (Cell Signaling Technology [CST], 9211, 9212, and 4631), IRS1 (CST, 2382), cleaved caspase 3 (CST, 9661), MKK3 (CST, 9238), ASK1 (CST, 3762), JNK and phospho-JNK (CST, 9251 and 9252), phospho-c-Jun (CST, 2361), phospho-ATF-2 (CST, 9221 and 9225), cleaved PARP (CST, 9544), phospho-ERK (CST, 9101), BrdU (BD Biosciences, 347580), GFP (Invitrogen, A11122), Ki67 (Vector Laboratories, VP-RM04), and β -tubulin (Sigma, T5201). Secondary antibodies were donkey anti-mouse (Jackson Research Laboratories, 715-036-150) and goat anti-rabbit (Thermo Scientific, 31460). Peroxidase activity was detected using Luminata (Millipore).

Immunofluorescence

CGNPs were grown on poly-DL-ornithine coated glass cover slips as described previously [45]. The cells were fixed with 4% paraformaldehyde for 20 minutes, washed in PBS, blocked for 45 minutes in blocking buffer with goat serum (Sigma), and incubated with primary and secondary antibodies according to manufacturer's instructions. All antibodies were used at 1:100 dilution except for phospho-p38, which were used at 1:50. Secondary antibodies (Invitrogen, A11070, A11001, A21430, and A21425) were used at 1:2000 dilution.

Immunohistochemistry and tissue microarray analysis

Paraffin-embedded sections from PN 3, 5, and 7 mouse brains were stained with the p38 antibody at 1:80 dilution and phospho-p38 antibody (CST, 4631) at 1:40 dilution with extended antigen retrieval step for PN 3 to enhance the signal. The human pediatric medulloblastoma tissue microarray (TMA) was obtained according to regulations and

previously characterized as described in Remke *et al.* [50]. Phospho-p38 antibody (CST, 4631) was used at a 1:50 dilution for staining the TMA slides. Immunohistochemistry staining was done using the Ventana Medical Systems Discovery XT Staining Module. The biopsies were scored blindly from 0 to 3 for amount of phospho-p38 staining intensity and the total area of each biopsy sample that was positive. Biopsies that scored 0 and 1 were categorized as low, and 2 and 3 as high. Then samples were assigned subtype information. Chi-squared test was applied to determine if there was an overrepresentation of the high or low category within a subtype.

Image capturing and quantification

Staining of primary cells and tissue sections was visualized and images were collected as described before [36,45]. In experiments where CGNPs were seeded at low numbers to avoid the formation of large clusters, individual cells were counted and Ki67-positive cell numbers were normalized to the total number of cells determined by DAPI staining using Volocity software (Perkin Elmer). In other experiments, where CGNPs formed large clusters, the Ki67-positive area in each image was normalized to the DAPI-positive area using ImageJ software. (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011).

RESULTS

p38 MAPK pathway is active in proliferating CGNPs

In order to gain new insights into the role of MAP kinases in CGNP proliferation, we first examined the status of p38 MAPK, since published data indicated that ERK and JNK might not be involved in this process [17,27]. After CGNPs were cultured *in vitro* from postnatal day (PN) 5 pups in the presence or absence of Shh, we examined the protein levels of p38 pathway members by western blot analysis (Fig. 1a). CGNPs cultured without Shh exit the cell cycle and start differentiating whereas those cultured with Shh continue proliferating and retain their precursor properties, and elevated levels of cyclin D2 is an indicator of ongoing proliferation [43,45]. Interestingly, we observed increased levels of both total and phosphorylated (activated) p38 α MAPK in CGNPs cultured in the presence of Shh compared to cells cultured without Shh (Fig. 1a). What was even more surprising was the striking manner in which the whole pathway was elevated; total levels of ASK1, MKK3, and p38 were increased CGNPs with Shh.

We then confirmed previous findings showing that ERK kinases p42/44 are not involved in CGNP proliferation. Proliferating CGNPs do not have altered levels of activated ERK, nor are these levels affected by the addition of cyclopamine (Supp. Fig. ESM 1a). We also investigated the levels of active JNK and its established signaling target, c-Jun, in cultured CGNPs by western blot analysis. CGNPs cultured with or without Shh showed no difference in phospho-JNK and phospho-c-Jun levels (Fig. 1b). This finding confirms the previously reported result that, similar to ERK, JNK activation is not a part of the mitogenic Shh response in CGNPs [17]. Our findings with all three major MAPK pathways show that p38 pathway is the only one likely to be involved in Shh-dependent CGNP proliferation, though

active JNK and ERK may play Shh-independent roles in CGNP biology at this or later stages.

To investigate whether the high p38 protein levels in proliferating CGNPs was a result of direct Shh regulation of p38 transcription, we performed a quantitative PCR experiment, where total RNA from CGNPs cultured with or without Shh was assayed for *p38/MAPK14* expression levels at 3, 6, 12, 24, and 48 hours in culture (Supp. Fig. ESM 1b). Transcripts, such as *Gli1*, that are direct targets of Gli2/3 downstream of Shh have been shown to increase rapidly at these early time points and remain elevated throughout the 48 hours of culture [46]. In contrast, we did not observe any significant changes in p38 mRNA levels with or without Shh. We repeatedly assayed mRNA levels at the end point (48hrs) of our CGNP cultures, where we see a marked difference in p38 protein levels, yet no significant difference in mRNA levels was observed, leading to the conclusion that p38 is under post-transcriptional regulation during CGNP proliferation.

In order to determine the relationship between elevated p38 protein/activation and CGNP proliferation at the cellular level, we cultured CGNPs from PN 5 mice on cover slips with or without Shh, pulsed them with BrdU for 2 hours to label those that were actively going through the cell cycle, and performed immunofluorescence analysis. A significant increase in CGNPs marked with BrdU staining was found in Shh-treated cultures (Fig. 1c). These proliferative regions also exhibited high levels of p38 and phospho-p38. Quantification of the overall proliferation and levels of p38 and phospho-p38 in the cultures revealed significant increases in proliferating CGNPs (Fig. 1d). At the cellular level, all CGNPs that were labeled with BrdU and hence were proliferating also had higher levels of p38 and phospho-p38 (Supp. Fig. ESM 1c). Very few CGNPs were proliferating in cultures without Shh after 2 days, as expected. Our immunofluorescence results underline a strict correlation between elevated p38 levels and the proliferative state of CGNPs.

Inhibition of the Shh Pathway and CGNP proliferation inhibits p38

In order to further establish that p38 pathway activity was dependent on CGNP proliferation, we cultured CGNPs with the Shh antagonist, SANT-2. In the presence of Shh, SANT-2 successfully inhibited the Shh pathway and CGNP proliferation and by 24 hours IRS1 and cyclin D2 levels were down to basal levels observed in CGNPs cultured without Shh (Fig. 1e and Supp. Fig. ESM 1d). In line with decreased proliferation, a marked decrease in total and active p38 α levels was observed in CGNPs treated with SANT-2; however, this effect was most prominent at the 48-hour time point, indicating that p38 is unlikely to be a direct target of Smoothened signaling and is more likely regulated by a distal downstream effector of Shh.

Basic FGF (bFGF) has been shown to cause CGNPs to exit the cell cycle and differentiate, even in the presence of Shh [17,36]. We used bFGF to force CGNPs to cease proliferation. As shown in Figure 1f, the longer CGNPs are exposed to bFGF, the less active the Shh proliferative response is, as judged by cyclin D2 levels. Consistent with a role for p38 in Shh-mediated proliferation, a decrease in p38 α levels was also observed. This result is consistent with our SANT-2 results, supporting a role for a downstream proliferation-associated effector of Smoothened in driving p38 MAPK pathway activity.

p38 is active in the EGL of the developing cerebellum

We next investigated whether the levels of p38 in the developing postnatal cerebellum *in vivo* parallel our observations *in vitro*. It is known that the prominent p38 isoform in the adult cerebellum is p38 β (MAPK11) [2]. p38 α (MAPK14) seems to be important in earlier development, as lack of p38 α causes embryonic lethality due to placental and vascular defects [1]. As seen in immunohistochemistry images in Figure 2a, p38 α was highly expressed in the EGL of the cerebellum during the first seven postnatal days during the time when Shh-induced proliferation increases and peaks at day 7. Indeed, the level of active p38 α was markedly increased by PN 5 and peaked at day 7, correlating with Shh-dependent proliferation of CGNPs (Fig. 2b). After this highly proliferative stage, as the EGL diminishes and CGNPs migrate to form the IGL, p38 activity diminishes. At PN 21, some cells of the IGL express p-p38 while others don't, but this expression level is not as intense as at PN 5 and PN 7 (data not shown). Our *in vivo* findings confirm that high levels of p38 α strictly correlate with CGNP proliferation.

p38 pathway is upregulated in Shh-driven medulloblastoma

Since high p38 α levels and CGNP proliferation are strictly correlated, we postulated that the p38 pathway might be upregulated in medulloblastomas. To investigate this possibility, we utilized the *NeuroD2-SmoA1* mouse model that carries a constitutively active Smo receptor driven by *NeuroD2* promoter. The *NeuroD2-SmoA1* transgenic mice develop medulloblastomas at 2-6 months of age with many of the hallmarks of human medulloblastomas [19,21]. Since *NeuroD2-SmoA1* transgenic mice are of C57BL/6 genetic background, which has some reported genetic differences from the Swiss Webster background that could interact with the p38 MAPK pathway [61,60], we examined the Shh-dependent state of the p38 MAPK pathway in CGNPs from wild type C57BL/6 mice. Protein levels of the whole pathway were elevated in C57BL/6 CGNPs similar to what we had observed in Swiss Webster CGNPs (Supp. Fig. ESM 2). Analysis of SmoA1 tumor lysates for active p38 α protein levels by western blot revealed that the tumors indeed had elevated levels compared to adjacent non-tumor cerebellum tissue, in correlation with higher levels of proliferation as determined by cyclin D2 protein levels (Fig. 3a). The tumor tissue had higher levels of apoptosis compared to non-tumor tissue as reported before in this mouse model as well as in human medulloblastoma [10,40,47], as determined by cleaved PARP levels, indicating good separation between tumor and non-tumor tissue.

We also investigated the state of p38 α expression in the tumors by immunostaining tumor and adjacent normal tissue for Ki67 and phospho-p38 α (Fig. 3b and Supp. Fig. ESM 3). As expected, Ki67 levels were very high in the tumor while the normal IGL and molecular layer of the adult cerebellum had no Ki67 positive cells. As previously published, the normal adult cerebellum does not have high levels of p38 α in the IGL or molecular layer regions [2,32]; however, in the tumor, Ki67 positive cells expressed high levels of phospho-p38 α , confirming our findings in CGNPs (Fig. 3b). Thus, we again observe a strict correlation between CGNP proliferation and p38 α activity in the cerebellum.

In order to determine if p38 was upregulated in Shh-driven human medulloblastoma, we analyzed 109 pediatric medulloblastoma biopsies for phospho-p38 α levels. These biopsies

have been previously characterized and assigned medulloblastoma subgroups [50]. 31 samples belonged to the SHH subgroup, 5 to the WNT subgroup, and the remaining 73 to the C and D subgroups (Supp. Tab. ESM 4). Importantly, the distribution of the samples analyzed did not represent the frequency of subtypes in human disease [38], but rather resulted from what was available to us at the time of the study. 3 out of 109 biopsies were completely negative for phospho-p38 α . We scored phospho-p38 α staining of the biopsy samples as described in Materials and Methods and categorized them as phospho-p38-high and phospho-p38-low. We determined that 28 out of 31 SHH-subgroup samples were in the high category (Fig. 3c). The C and D subtypes had a wider range of staining for phospho-p38, where 18 out of 27 C-subtype samples and 21 out of 46 D-subtype samples were high for phospho-p38 α . 4 out of 5 WNT-subtype samples also scored high in our system; however, with such few samples it is not possible to make any conclusions about p-p38 in the WNT subtype. When we analyzed these results statistically, the C subgroup did not exhibit an over-representation of phospho-p38-high samples ($\chi^2 = 0.077$). The D subgroup showed a significant over-representation of phospho-p38-low category ($\chi^2 = 7.753$, $p < 0.05$) and there was a significant over-representation of phospho-p38-high samples within the SHH-subtype ($\chi^2 = 9.343$, $p < 0.05$). These findings support the idea that under conditions of high Shh activity, normal and malignant CGNP proliferation is tightly correlated with p38 activity in mice and in humans. Further studies are needed to determine the relationship of p38 activity and the proliferative state of the human medulloblastomas as well as any possible potential role for p38 activity as a prognostic indicator. Interestingly, similar to our findings with p-p38 in human medulloblastoma samples, MIB-1 proliferation index is high in over 50% of human medulloblastomas and does not indicate a correlation with any histological subtype or survival [13,41,52]. It remains to be seen whether p-p38 high and low levels correlate with MIB-1 or other proliferative indices in the same samples. It should be noted that, similar to published findings for MIB-1, highest levels of p-p38 were observed outside desmoplastic nodules compared to inside the nodules (data not shown), which are known to be less proliferative [13,52].

p38 pathway activity is required for CGNP proliferation

Having established that CGNP proliferation is associated with high p38 α levels and activity, we next investigated whether p38 pathway activity is required for proliferation. In order to examine the consequence of p38 α inactivation, we utilized short hairpin RNAs (shRNAs) to knock down p38 α in CGNPs *in vitro*. We infected CGNPs with lentiviruses carrying two different shRNAs predicted to target p38 α . The shRNA plasmids also expressed eGFP to track infection efficiency. As a control for infection and general shRNA-mediated inhibition, we used lentiviruses with an shRNA against GFP, which we have shown before not to affect CGNP biology significantly [45]. As predicted, the control shRNA also did not affect p38 α , phospho-p38 α , or IRS1 levels, as expected (Figure 4a). An shRNA predicted to target p38 α , but which did not successfully knock down p38 α (p38-2), also did not affect Shh pathway as judged by IRS1 levels. Since p38-2 had a slight effect on proliferation, though not via p38 reduction, we left this shRNA from further experiments. On the other hand, an shRNA that successfully knocked down p38 α (p38-1) reduced CGNP proliferation along with Shh pathway activity, as judged by cyclin D2 and IRS1 levels. This finding shows that p38 α is necessary for CGNP proliferation and may have an effect on overall Shh pathway activity.

In order to quantify the effect of p38 α knockdown on CGNP proliferation, we cultured CGNPs on cover slips and infected them with lentiviruses containing either the control shRNA targeting GFP or the p38 α shRNA that effectively knocks down p38 α in CGNPs (p38-1) and immunostained for Ki67 and GFP (Fig. 4b and Supp. Fig. ESM 5). As expected, CGNPs cultured without Shh showed low levels of proliferation compared with those cultured with Shh. CGNPs with the control shRNA targeting GFP had similar levels of proliferation as CGNPs cultured with Shh without any infection, confirming our findings in Figure 4a and previously published results. In CGNPs with the p38-1 shRNA plasmid, p38 α knockdown significantly decreased CGNP proliferation. On an individual cell level, none of the GFP-positive CGNPs expressed Ki67 (Supp. Fig. ESM 5, bottom panels), indicating that cells with reduced levels of p38 α could not proliferate. This was true even for those cells inside the proliferative clumps, surrounded by Ki67-positive, proliferating CGNPs.

In order to confirm by another means the essential requirement for p38 activity in Shh-mediated CGNP proliferation, we used the p38 pharmacological inhibitor SB203580 at 5 and 10 μ M concentrations in CGNPs cultured with Shh. 10 μ M SB203580 has previously been shown to be effective in inhibiting p38 α in CGNPs [18]. At these concentrations, SB203580 is thought to be specific to inhibiting p38 activity by competing with ATP for the ATP-binding pocket. Western blot analysis of protein lysates revealed that the p38 inhibitor caused a significant decrease in cyclin D2 levels, indicating a decrease in CGNP proliferation (Fig. 4c). Cleaved PARP levels, on the other hand, remained the same as controls, suggesting that, though SB203580 inhibited CGNP proliferation, it did not cause a marked increase in cell death at these concentrations. Similarly, JNK and phospho-JNK levels were unchanged, confirming that the p38 inhibitor at these concentrations did not affect the JNK pathway in CGNPs. Interestingly, we observed a decrease in p38 α and phospho-p38 α levels with SB203580, which indicates that the reduction in proliferation in turn might cause a decrease in p38 α levels, as this drug is not known to directly affect p38 α protein stability or gene expression. As expected, higher levels of SB203580, at 20 μ M, also showed a marked reduction in p38, cyclin D2, and phospho-ATF-2 levels (Supp. Fig. ESM 6).

We next cultured CGNPs with increasing amounts of SB203580 and determined the amount of proliferation with Ki67 immunostaining (Fig. 4d). Ki67 labeling was clearly reduced with increasing amounts of p38 inhibitor. In the absence of drug, 33.3% ($\pm$ 3.8%) of the CGNPs stained positive for Ki67, and increasing the dose of the p38 inhibitor caused a decrease in proliferation to 7.3% ($\pm$ 4.3%) with highest dose of 20 μ M, which was similar to the proliferation of CGNPs cultured without Shh (Vehicle, 11.1 $\pm$ 2.5%).

Considering that p38 inhibition can be a useful addition to existing Smo inhibitors for combinatorial therapy, we were interested in the apoptotic potential of SB203580. We observed that in wild type CGNPs only a high dose of SB203580 (20 μ M) caused increased level of apoptosis (Supp. Fig. ESM 7). Interestingly, in the Pzp53med mouse medulloblastoma cell line, which was derived from a mouse Ptc^{+/-}/p53^{-/-} medulloblastoma [3], we found that much lower doses of inhibitor (5 μ M) caused high levels of apoptosis (Supp. Fig. ESM 7). This preliminary result indicated that it may be worthwhile to test the value of inhibition of p38 in addition to targeting Smo for therapy, not only because p38

inhibition reduces CGNP proliferation, but also because low doses of p38 inhibitor can cause apoptosis in cancer cells while sparing wild type cells.

Since Gli1 and N-Myc are two transcription factors that are directly involved in CGNP proliferation and are highly induced by Shh, we asked whether inhibiting p38 lowers *Gli1* or *N-myc* expression. We performed quantitative PCR to measure *Gli1* and *N-myc* expression levels in CGNPs cultured for 48 hours with or without 10 μ M SB203580 for the last 24 hours (Fig. 4e). While both transcript levels were high with Shh at the end of 48 hours, treatment with SB203580 resulted in a significant decrease in both, similar to that seen with cyclopamine [26,45]. Since Gli1 and N-Myc are two of the major CGNP proliferation drivers, the observed reduction in their expression levels likely explains why inhibition of p38 results in reduced CGNP proliferation. Exactly how inhibition of p38 leads to a reduction in *Gli1* and *N-myc* expression is currently under investigation.

DISCUSSION

In this study, we report the first known connection between the Shh pathway and a MAP kinase in the context of CGNP proliferation and postnatal cerebellum development. Previous studies that probed a possible role for MAP kinases in CGNP proliferation found that neither the ERK1/2 nor JNK MAP kinases were involved in CGNP proliferation [17,27]. Here we show that the third major MAP kinase pathway in mammalian cells, the p38 MAPK pathway, is activated in response to Shh treatment of CGNPs *in vitro* and is required for their proliferation. Moreover, p38 α and phosphorylated (activated) p38 α are present in the cerebellar EGL *in vivo* during the period of Shh-dependent CGNP proliferation. Most importantly, active p38 α is present in mouse and human Shh-associated medulloblastomas. Our results indicate that p38 α -dependent induction/maintenance of *N-myc* and *Gli1* expression downstream of Shh may underlie the reduced proliferation of CGNPs under conditions of p38 inhibition, and suggest that targeting p38 MAPK signaling could be explored as a future therapeutic approach to medulloblastoma, the most common malignant pediatric brain tumor.

p38 activity and CGNP proliferation

Although a previous study reported that the pharmacological inhibition of p38 reduces CGNP proliferation of PN 6-8 CGNPs [15], a link between p38 α activity and the Shh pathway in CGNPs was not described. Most studies concerning the role of p38 in neural precursors have focused on its role in driving apoptosis in response to environmental cues and assaults known to induce neuron and precursor cell death. In recent years, however, evidence of involvement of p38 in proliferative programs has been accumulating. Indeed, p38 α is now known to be required for the proliferation of hematopoietic cells, vascular smooth muscle cells, and fibroblasts [12,35,37,49,58,65]. In addition to normal development, proliferation via p38 α activation has been shown in some cancers, including breast, colon, and ovarian cancer [39,64,67].

We show here that CGNPs cultured with Shh have high levels of total and active p38 α , compared to CGNPs cultured without Shh, which exit the cell cycle and differentiate. Our findings highlight an important involvement of p38 pathway activity in the Shh-dependent

early proliferation of these precursor cells *in vivo* and *in vitro*. Importantly, inhibiting Shh in proliferating CGNPs ablates the high levels of p38 pathway. Although the relationship between Shh and p38 α could be direct, we have not found any evidence for a direct interaction, e.g. activation of p38 α intracellularly by Smo or another Shh pathway component, or regulation of p38 α expression by Gli or N-Myc (data not shown). Indeed, we found that p38 α is not transcriptionally regulated by Shh; increased levels of total p38 α suggest that p38 α is regulated post-transcriptionally as well as post-translationally. In addition, activation of kinases upstream of p38 α suggests that interactions between a Shh proliferative signal and p38 MAPK take place upstream of p38 α . Future studies will determine the precise mechanism of p38 MAPK activation downstream of Smo activity, including the possibility that the p38 pathway is activated by a by-product of the proliferation process itself, such as alterations of metabolic states or activities of cell cycle kinases towards upstream regulators of p38 α .

Furthermore, we have shown that inhibition of p38 causes a decrease in CGNP proliferation and a concomitant decrease in p38 α levels and active p38 α . These results indicate that p38 activity is required for the full mitogenic response of CGNPs to Shh. Our results also suggest that maintenance of a proliferative state is required for p38 α protein elevation, although we cannot rule out that p38 signaling also regulates its own mRNA translation or protein stabilization as part of a built-in feed-forward loop.

We have shown that inhibition of p38 causes a decrease in the levels of *Gli1* and *N-myc* expression, which are genes required for CGNP proliferation. The mechanism through which p38 inhibition results in *Gli1* and *N-myc* down-regulation remains to be determined. It is well-established that *Gli1* transcription is regulated by Gli2 and Gli1 transcription factors. It is not clear whether *N-myc* is a Gli1 transcriptional target; indeed, knock-down of IRS1 in CGNPs reduces proliferation and *N-myc* expression, but has no effect on *Gli1* expression, indicating that *N-myc* may not be a Gli1 transcriptional target [45]. p38 has many substrates, including transcription factors that may directly affect *N-myc* and *Gli1* levels, as well as other kinases, which could interact with the Shh pathway to affect transcriptional levels downstream.

p38 and medulloblastoma

We have shown that the high levels of p38 α we observe in proliferating CGNPs also exists in tumors from a *NeuroD2-SmoA1* mouse medulloblastoma model. As with CGNPs, we were not able to detect a corresponding increase in *MAPK14* transcript levels in these tumors (data not shown). We have also shown that the levels of p38 α MAPK activity in human medulloblastomas of the SHH subtype is high, which confirms our findings with mouse tumors and CGNPs. p38 α activity seems to be present at varying degrees in all of the human medulloblastoma subtypes, which is not surprising if the p38 pathway is activated as a result of proliferation. As indicated in the Results section, medulloblastoma exhibits high levels of proliferation in many cases and proliferation has never been associated with a certain subtype, histological class, or survival, though large, thorough studies involving all subtypes are lacking. At first pass, biopsies that seem to have nodular and nucleated (classic-type looking) areas seem to have high levels of phospho-p38 α outside the nodular regions in

all subtypes, which is consistent with p38 α being tightly correlated with proliferation. Considering the variability in phospho-p38 α levels especially in the poorly characterized C and D subtypes, it would be interesting to determine if active p38 α level can be a useful prognostic marker.

Although high levels of p38 α are present in mouse and human SHH medulloblastoma, similar to CGNPs cultured with Shh, it is possible that p38 α is activated for additional reasons and serves a different function in the tumor setting than in CGNP proliferation during normal development. Future studies to determine the exact mechanism of p38 α induction and activation in both settings will give insight into p38 α 's developmental and tumorigenic roles, as will identification of p38 α targets carrying out its effector functions under conditions of SHH mitogenic and oncogenic activation.

Our preliminary finding that low levels of p38 inhibitor can induce apoptosis in the Pzp53med cell line supports the notion that p38 is a viable target for combinatorial therapy along with Smo inhibitors. Immediate future experiments to measure tumor incidence and tumor cell survival in *NeuroD2-SmoA1* mice with p38 inhibitors in combination with Smo inhibitors can prove useful in assessing the value of targeting p38 in medulloblastoma. In tumors that are resistant to Smo antagonists, *Gli2* amplification is one mechanism of resistance [7], hence it would be interesting to explore the effect of p38 inhibition at the level of *Gli2* using CGNPs and *NeuroD2-SmoA1* mice. Since p38 seems active in all subtypes of human pediatric medulloblastomas, it would be interesting to see if targeting p38 can benefit all subtypes similarly or differentially. Though the only established mouse model for human medulloblastoma is for the SHH-subtype, the very recent development of mouse models for the other subtypes [for in-depth review, please see 38] will prove an invaluable resource to study this question. It will also be beneficial to determine if our finding that the inhibition of p38 does not lead to some of the side effects associated with p38 inhibition in clinical trials, such as the consequent activation of the JNK pathway [64], in fact translate successfully to the clinical application of these inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Emilie Legue, Dr. Sandra Wilson, Rowena Turnbull, Dr. Africa Fernandez-Lopez, and Dr. Praveen Raju for their technical help and discussions. We also thank Dr. Alexandra Joyner for her comments on the manuscript and generous support. These studies were supported by grants to AMK from the NINDS (R01NS061070), Childhood Brain Tumor Foundation, and Alex's Lemonade Stand Foundation for Childhood Cancer Research.

REFERENCES

1. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell*. 2000; 6(1):109–116. [PubMed: 10949032]

2. Beardmore VA, Hinton HJ, Eftychi C, Apostolaki M, Armaka M, Darragh J, McIlrath J, Carr JM, Armit LJ, Clacher C, Malone L, Kollias G, Arthur JS. Generation and characterization of p38beta (MAPK11) gene-targeted mice. *Mol Cell Biol*. 2005; 25(23):10454–10464. [PubMed: 16287858]
3. Berman DM, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA. Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science*. 2002; 297(5586):1559–1561. [PubMed: 12202832]
4. Bhatia B, Hsieh M, Kenney AM, Nahle Z. Mitogenic Sonic hedgehog signaling drives E2F1-dependent lipogenesis in progenitor cells and medulloblastoma. *Oncogene*. 2011; 30(4):410–422. [PubMed: 20890301]
5. Bhatia B, Northcott PA, Hambardzumyan D, Govindarajan B, Brat DJ, Arbiser JL, Holland EC, Taylor MD, Kenney AM. Tuberous sclerosis complex suppression in cerebellar development and medulloblastoma: separate regulation of mammalian target of rapamycin activity and p27 Kip1 localization. *Cancer Res*. 2009; 69(18):7224–7234. [PubMed: 19738049]
6. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and - independent mechanisms. *Science*. 1999; 286(5443):1358–1362. [PubMed: 10558990]
7. Buonamici S, Williams J, Morrissey M, Wang A, Guo R, Vattay A, Hsiao K, Yuan J, Green J, Ospina B, Yu Q, Ostrom L, Fordjour P, Anderson DL, Monahan JE, Kelleher JF, Peukert S, Pan S, Wu X, Maira SM, Garcia-Echeverria C, Briggs KJ, Watkins DN, Yao YM, Lengauer C, Warmuth M, Sellers WR, Dorsch M. Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. *Sci Transl Med*. 2010; 2(51):51ra70.
8. Butts T, Chaplin N, Wingate RJ. Can clues from evolution unlock the molecular development of the cerebellum? *Mol Neurobiol*. 2010; 43(1):67–76. [PubMed: 21174175]
9. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev*. 2011; 75(1):50–83. [PubMed: 21372320]
10. Castellino RC, Barwick BG, Schniederjan M, Buss MC, Becher O, Hambardzumyan D, Macdonald TJ, Brat DJ, Durden DL. Heterozygosity for Pten promotes tumorigenesis in a mouse model of medulloblastoma. *PloS one*. 2010; 5(5):e10849. [PubMed: 20520772]
11. Corrales JD, Blaess S, Mahoney EM, Joyner AL. The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. *Development*. 2006; 133(9):1811–1821. [PubMed: 16571625]
12. Crawley JB, Rawlinson L, Lali FV, Page TH, Saklatvala J, Foxwell BM. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J Biol Chem*. 1997; 272(23):15023–15027. [PubMed: 9169478]
13. Das P, Puri T, Suri V, Sharma MC, Sharma BS, Sarkar C. Medulloblastomas: a correlative study of MIB-1 proliferation index along with expression of c-Myc, ERBB2, and anti-apoptotic proteins along with histological typing and clinical outcome. *Child's nervous system: ChNS: official journal of the International Society for Pediatric Neurosurgery*. 2009; 25(7):825–835.
14. Dijkgraaf GJ, Alicke B, Weinmann L, Januario T, West K, Modrusan Z, Burdick D, Goldsmith R, Robarge K, Sutherlin D, Scales SJ, Gould SE, Yauch RL, de Sauvage FJ. Small molecule inhibition of GDC-0449 refractory smoothened mutants and downstream mechanisms of drug resistance. *Cancer Res*. 2011; 71(2):435–444. [PubMed: 21123452]
15. Dougherty JD, Garcia AD, Nakano I, Livingstone M, Norris B, Polakiewicz R, Wexler EM, Sofroniew MV, Kornblum HI, Geschwind DH. PBK/TOPK, a proliferating neural progenitor-specific mitogen-activated protein kinase kinase. *J Neurosci*. 2005; 25(46):10773–10785. [PubMed: 16291951]
16. Fernandez LA, Northcott PA, Dalton J, Fraga C, Ellison D, Angers S, Taylor MD, Kenney AM. YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. *Genes Dev*. 2009; 23(23):2729–2741. [PubMed: 19952108]
17. Fogarty MP, Emmenegger BA, Gräsfeder LL, Oliver TG, Wechsler-Reya RJ. Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. *Proc Natl Acad Sci U S A*. 2007; 104(8):2973–2978. [PubMed: 17299056]

18. Giordano G, White CC, McConnachie LA, Fernandez C, Kavanagh TJ, Costa LG. Neurotoxicity of domoic Acid in cerebellar granule neurons in a genetic model of glutathione deficiency. *Mol Pharmacol.* 2006; 70(6):2116–2126. [PubMed: 17000861]
19. Hallahan AR, Pritchard JI, Hansen S, Benson M, Stoeck J, Hatton BA, Russell TL, Ellenbogen RG, Bernstein ID, Beachy PA, Olson JM. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer Res.* 2004; 64(21):7794–7800. [PubMed: 15520185]
20. Hartmann W, Koch A, Brune H, Waha A, Schuller U, Dani I, Denkhaus D, Langmann W, Bode U, Wiestler OD, Schilling K, Pietsch T. Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. *Am J Pathol.* 2005; 166(4):1153–1162. [PubMed: 15793295]
21. Hatton BA, Villavicencio EH, Tsuchiya KD, Pritchard JI, Ditzler S, Pullar B, Hansen S, Knoblauch SE, Lee D, Eberhart CG, Hallahan AR, Olson JM. The Smo/Smo model: hedgehog-induced medulloblastoma with 90% incidence and leptomeningeal spread. *Cancer Res.* 2008; 68(6):1768–1776. [PubMed: 18339857]
22. High A, Zedan W. Basal cell nevus syndrome. *Curr Opin Oncol.* 2005; 17(2):160–166. [PubMed: 15725922]
23. Jenkins D. Hedgehog signalling: emerging evidence for non-canonical pathways. *Cell Signal.* 2009; 21(7):1023–1034. [PubMed: 19399989]
24. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science.* 2002; 298(5600):1911–1912. [PubMed: 12471242]
25. Katoh Y, Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr Mol Med.* 2009; 9(7):873–886. [PubMed: 19860666]
26. Kenney AM, Cole MD, Rowitch DH. Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development.* 2003; 130(1):15–28. [PubMed: 12441288]
27. Kenney AM, Rowitch DH. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol Cell Biol.* 2000; 20(23):9055–9067. [PubMed: 11074003]
28. Kenney AM, Widlund HR, Rowitch DH. Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. *Development.* 2004; 131(1):217–228. [PubMed: 14660435]
29. Keshet Y, Seger R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol Biol.* 2010; 661:3–38. [PubMed: 20811974]
30. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta.* 2010; 1802(4):396–405. [PubMed: 20079433]
31. Knoepfler PS, Kenney AM. Neural precursor cycling at sonic speed: N-Myc pedals, GSK-3 brakes. *Cell Cycle.* 2006; 5(1):47–52. [PubMed: 16322694]
32. Lee SH, Park J, Che Y, Han PL, Lee JK. Constitutive activity and differential localization of p38alpha and p38beta MAPKs in adult mouse brain. *J Neurosci Res.* 2000; 60(5):623–631. [PubMed: 10820433]
33. Lewis PM, Gritli-Linde A, Smeyne R, Kottmann A, McMahon AP. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol.* 2004; 270(2):393–410. [PubMed: 15183722]
34. Louvi A, Grove EA. Cilia in the CNS: the quiet organelle claims center stage. *Neuron.* 2011; 69(6):1046–1060. [PubMed: 21435552]
35. Maher P. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem.* 1999; 274(25):17491–17498. [PubMed: 10364180]
36. Mainwaring LA, Kenney AM. Divergent functions for eIF4E and S6 kinase by sonic hedgehog mitogenic signaling in the developing cerebellum. *Oncogene.* 2011; 30(15):1784–1797. [PubMed: 21339731]

37. Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenberg ME. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science*. 1999; 286(5440):785–790. [PubMed: 10531066]
38. Markant SL, Wechsler-Reya RJ. Personalized Mice: Modeling the Molecular Heterogeneity of Medulloblastoma. *Neuropathology and applied neurobiology*. 2011
39. Matrone A, Grossi V, Chiacchiera F, Fina E, Cappellari M, Caringella AM, Di Naro E, Loverro G, Simone C. p38alpha is required for ovarian cancer cell metabolism and survival. *Int J Gynecol Cancer*. 2010; 20(2):203–211. [PubMed: 20169663]
40. Michael LE, Westerman BA, Ermilov AN, Wang A, Ferris J, Liu J, Blom M, Ellison DW, van Lohuizen M, Dlugosz AA. Bmi1 is required for Hedgehog pathway-driven medulloblastoma expansion. *Neoplasia*. 2008; 10(12):1343–1349. 1345p following 1349. [PubMed: 19048113]
41. Miralbell R, Tolnay M, Bieri S, Probst A, Sappino AP, Berchtold W, Pepper MS, Pizzolato G. Pediatric medulloblastoma: prognostic value of p53, bcl-2, Mib-1, and microvessel density. *J Neurooncol*. 1999; 45(2):103–110. [PubMed: 10778725]
42. Northcott PA, Fernandez LA, Hagan JP, Ellison DW, Grajkowska W, Gillespie Y, Grundy R, Van Meter T, Rutka JT, Croce CM, Kenney AM, Taylor MD. The miR-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by N-myc in sonic hedgehog-treated cerebellar neural precursors. *Cancer Res*. 2009; 69(8):3249–3255. [PubMed: 19351822]
43. Oliver TG, Gräsfeder LL, Carroll AL, Kaiser C, Gillingham CL, Lin SM, Wickramasinghe R, Scott MP, Wechsler-Reya RJ. Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. *Proc Natl Acad Sci U S A*. 2003; 100(12):7331–7336. [PubMed: 12777630]
44. Oliver TG, Read TA, Kessler JD, Mehmeti A, Wells JF, Huynh TT, Lin SM, Wechsler-Reya RJ. Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. *Development*. 2005; 132(10):2425–2439. [PubMed: 15843415]
45. Parathath SR, Mainwaring LA, Fernandez LA, Campbell DO, Kenney AM. Insulin receptor substrate 1 is an effector of sonic hedgehog mitogenic signaling in cerebellar neural precursors. *Development*. 2008; 135(19):3291–3300. [PubMed: 18755774]
46. Parathath SR, Mainwaring LA, Fernandez LA, Guldal CG, Nahle Z, Kenney AM. beta-Arrestin-1 links mitogenic sonic hedgehog signaling to the cell cycle exit machinery in neural precursors. *Cell Cycle*. 2010; 9(19):4013–4024. [PubMed: 20935513]
47. Pizem J, Cor A, Zdravcevic Z, Popovic M. Prognostic significance of apoptosis in medulloblastoma. *Neurosci Lett*. 2005; 381(1-2):69–73. [PubMed: 15882792]
48. Raffel C. Medulloblastoma: molecular genetics and animal models. *Neoplasia*. 2004; 6(4):310–322. [PubMed: 15256053]
49. Rausch O, Marshall CJ. Cooperation of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways during granulocyte colony-stimulating factor-induced hemopoietic cell proliferation. *J Biol Chem*. 1999; 274(7):4096–4105. [PubMed: 9933603]
50. Remke M, Hielscher T, Korshunov A, Northcott PA, Bender S, Kool M, Westermann F, Benner A, Cin H, Ryzhova M, Sturm D, Witt H, Haag D, Toedt G, Wittmann A, Schottler A, von Bueren AO, von Deimling A, Rutkowski S, Scheurlen W, Kulozik AE, Taylor MD, Lichter P, Pfister SM. FSTL5 Is a Marker of Poor Prognosis in Non-WNT/Non-SHH Medulloblastoma. *J Clin Oncol*. 2011; 29(29):3852–3861. [PubMed: 21911727]
51. Roussel MF, Hatten ME. Cerebellum development and medulloblastoma. *Curr Top Dev Biol*. 2011; 94:235–282. [PubMed: 21295689]
52. Schiffer D, Cavalla P, Chio A, Giordana MT, Marino S, Mauro A, Migheli A. Tumor cell proliferation and apoptosis in medulloblastoma. *Acta Neuropathol*. 1994; 87(4):362–370. [PubMed: 7912469]
53. Schuller U, Heine VM, Mao J, Kho AT, Dillon AK, Han YG, Huillard E, Sun T, Ligon AH, Qian Y, Ma Q, Alvarez-Buylla A, McMahon AP, Rowitch DH, Ligon KL. Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer cell*. 2008; 14(2):123–134. [PubMed: 18691547]
54. Sotelo C. Cellular and genetic regulation of the development of the cerebellar system. *Prog Neurobiol*. 2004; 72(5):295–339. [PubMed: 15157725]

55. Spassky N, Han YG, Aguilar A, Strehl L, Besse L, Laclef C, Ros MR, Garcia-Verdugo JM, Alvarez-Buylla A. Primary cilia are required for cerebellar development and Shh-dependent expansion of progenitor pool. *Dev Biol.* 2008; 317(1):246–259. [PubMed: 18353302]
56. Suzuki DE, Ariza CB, Porcionatto MA, Okamoto OK. Upregulation of E2F1 in cerebellar neuroprogenitor cells and cell cycle arrest during postnatal brain development. *Vitro Cell Dev Biol Anim.* 2011; 47(7):492–499.
57. Takekawa M, Kubota Y, Nakamura T, Ichikawa K. Regulation of stress-activated MAP kinase pathways during cell fate decisions. *Nagoya J Med Sci.* 2011; 73(1-2):1–14. [PubMed: 21614932]
58. Thornton TM, Rincon M. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *Int J Biol Sci.* 2009; 5(1):44–51. [PubMed: 19159010]
59. Vaillant C, Monard D. SHH pathway and cerebellar development. *Cerebellum.* 2009; 8(3):291–301. [PubMed: 19224309]
60. White DE, Burchill SA. Fenretinide-dependent upregulation of death receptors through ASK1 and p38alpha enhances death receptor ligand-induced cell death in Ewing's sarcoma family of tumours. *Br J Cancer.* 2010; 103(9):1380–1390. [PubMed: 20877355]
61. Wright JW, Alt JA, Turner GD, Krueger JM. Differences in spatial learning comparing transgenic p75 knockout, New Zealand Black, C57BL/6, and Swiss Webster mice. *Behav Brain Res.* 2004; 153(2):453–458. [PubMed: 15265642]
62. Yang ZJ, Ellis T, Markant SL, Read TA, Kessler JD, Bourbonlous M, Schuller U, Machold R, Fishell G, Rowitch DH, Wainwright BJ, Wechsler-Reya RJ. Medulloblastoma can be initiated by deletion of Patched in lineage-restricted progenitors or stem cells. *Cancer cell.* 2008; 14(2):135–145. [PubMed: 18691548]
63. Yauch RL, Dijkgraaf GJ, Alicke B, Januario T, Ahn CP, Holcomb T, Pujara K, Stinson J, Callahan CA, Tang T, Bazan JF, Kan Z, Seshagiri S, Hann CL, Gould SE, Low JA, Rudin CM, de Sauvage FJ. Smoothed mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. *Science.* 2009; 326(5952):572–574. [PubMed: 19726788]
64. Yong HY, Koh MS, Moon A. The p38 MAPK inhibitors for the treatment of inflammatory diseases and cancer. *Expert Opin Investig Drugs.* 2009; 18(12):1893–1905.
65. Zhao M, Liu Y, Bao M, Kato Y, Han J, Eaton JW. Vascular smooth muscle cell proliferation requires both p38 and BMK1 MAP kinases. *Arch Biochem Biophys.* 2002; 400(2):199–207. [PubMed: 12054430]
66. Zhao Q, Kho A, Kenney AM, Yuk Di DI, Kohane I, Rowitch DH. Identification of genes expressed with temporal-spatial restriction to developing cerebellar neuron precursors by a functional genomic approach. *Proc Natl Acad Sci U S A.* 2002; 99(8):5704–5709. [PubMed: 11960025]
67. Zu X, Zhang Q, Cao R, Liu J, Zhong J, Wen G, Cao D. Transforming growth factor-beta signaling in tumor initiation, progression and therapy in breast cancer: an update. *Cell Tissue Res.* 2011

Fig. 1.

p38 pathway is active in CGNPs and this activity is dependent on Shh-dependent proliferation. **a** Protein lysates from PN 5 CGNPs cultured with (+) or without (-) Shh for 48 hours were probed with antibodies against p38α, phospho-p38α (p-p38α, Thr180/Tyr182), upstream kinases MKK3 and ASK1 as well as cyclin D2 to assess proliferation and Shh pathway activity by western blot analysis. Lysates were normalized for total protein and β-tubulin was used as a loading control. **b** Protein lysates from PN 5 CGNPs cultured with (+) or without (-) Shh for 48 hours were probed with antibodies against phospho-JNK (p-

JNK, p46 and p54 SAPK/JNK, Thr183/Tyr185) and phospho-c-Jun (p-c-Jun, Ser63) by western blot analysis. β -tubulin was used as a loading control. **c** CGNPs from PN 5 mice were cultured for 2 days with (Shh) or without (V) Shh. The cultures were pulsed with BrdU as described in Materials and Methods and immunofluorescence staining against BrdU (red), p38 α (green), and phospho-p38 α (p-p38 α , green) was performed. DAPI staining (blue) was used to label all nuclei. BrdU-positive CGNP clumps characteristic of proliferating CGNPs in response to Shh were observed in the cultures with Shh. White scale bars indicate 100 μ m. **d** Quantification of proliferation by BrdU and levels of p38 α and phospho-p38 α (p-p38 α) staining in **c** normalized to DAPI. (**) indicates a p-value < 0.001 with two-tailed t-test between V and Shh sample. **e** Western blot analysis of protein lysates from PN 5 CGNPs cultured with Shh for 24 or 48 hours (24h, 48h) or without (–) Shh for 48 hours and SANT-2 for the duration of the experiment (48h) or the last 24 hours (24h). Cyclin D2 levels are indicative of Shh pathway activity and CGNP proliferation. β -tubulin was used as a loading control. **f** CGNPs were cultured with (+) or without (–) Shh either without (–) or with bFGF for 10, 24, and 48 hours and protein lysates were analyzed for cyclin D2 and p38 levels in western blots. β -tubulin was used as a loading control.

Fig. 2.

p38 is active in the developing cerebellum and proliferating CGNPs. Representative medial cerebellar sections from PN 3, 5, and 7 mice are shown with immunohistochemical DAB staining against **a** p38α and **b** phospho-p38α. Strong staining for both p38α and p-p38α is indicated with brown color intensity in the EGL region of the cerebellar folia. Scale bars indicate 100 μm.

Fig. 3.

Shh-driven mouse medulloblastoma has high p38 activity. **a** Protein lysates from the brains of adult Smo A1 mice with medulloblastomas were analyzed for cyclin D2, phospho-p38 α , and cleaved PARP levels by western blot analysis. First two lanes are from the same mouse brain, where adjacent non-tumor tissue (A.n-T1) was separated from the medulloblastoma (MB1) tissue before protein extraction. Similarly, lanes 3 and 4 are from the same brain. β -tubulin was used as an internal control for each sample. **b** Tissue section from an adult SmoA1 mouse with a medulloblastoma containing tumor and adjacent non-tumor tissue was

immunostained for Ki67 (red) and phospho-p38 α (green). DAPI staining (blue) was used to label all nuclei. The internal granule layer (IGL) and the molecular layer (MOL) in adjacent non-tumor region (top panels) were devoid of Ki67 and phospho-p38 staining. White dashed lines delineate the IGL. The tumor (lower panels) had the characteristic Ki67-positive cells, most of which also have high levels of p-p38 α unlike the cells of the normal adult cerebellum. Scale bars indicate 100 μ m. **c** Representative immunohistochemistry staining of phospho-p38 α of human SHH subtype medulloblastoma biopsies on tissue microarray. First column shows one of two p-p38 α -low medulloblastomas, the following 4 columns are representative p-p38 α -high tumors, as judged by intense brown staining. Bottom panels (40x magnification) are close up of top panels (10x magnification), where individual cells can be detected. Scale bars indicate 50 μ m.

Fig. 4.

p38 activity is required for CGNP proliferation. **a** Western blot analysis of protein lysates from CGNPs cultures exposed to lentiviruses expressing shRNAs targeting GFP or p38α/ MAPK14 (p38-1 and p38-2). CGNPs were incubated with viruses at time of plating for 4-12 hours and protein lysates were collected at 48 hours. Both p38-1 and p38-2 shRNAs were predicted to knock down p38; however, only the former yielded a successful knockdown, allowing us to use p38-2 as an additional control. β-tubulin was used as a loading control. **b** Quantification of proliferation of cultures infected with lentiviruses for p38 knockdown as

measured by Ki67 staining normalized to DAPI staining. CGNPs were cultured with or without Shh and either with lentiviruses expressing the control shRNA, targeting GFP, or p38-1 shRNA, targeting p38/MAPK14. Cells were immunostained for Ki67 and GFP. DAPI staining was used to label all nuclei. Ki67 positive cells were normalized to all cells using DAPI staining. (Also see Supp. Fig. ESM 2 for representative fields used for quantification.) This quantification is an underestimate of the reduction in proliferation caused by p38 knock down, as the proliferation in all 5 selected fields in each culture was compared, rather than only those cells that are successfully infected (GFP-positive). We rarely observed any cells that double-positive for Ki67 and GFP. (**) indicates a p value < 0.0001 in two-tailed t-test between Vehicle and Shh cultures, (*) indicates a p value < 0.001 in two-tailed t-test between Shh cultures with GFP shRNA and p38-1 shRNA. Though there is a slight effect of simply infecting CGNPs with an unspecific shRNA (GFP shRNA), this effect is not statistically significant. **c** CGNPs were cultured with (Shh) or without (V) Shh for 48 hours and treated with 0 μ M (lane 2), 5 μ M (lane 3), or 10 μ M (lane 4) of SB 203580 (SB) for the last 24 hours of culture. Protein lysates were probed for p38 α , phospho-p38 α , JNK, phospho-JNK, cyclin D2, and cleaved PARP in western blot analysis. β -tubulin was used as a loading control. Cyclin D2 indicates CGNP proliferation levels and cleaved PARP is an indicator of apoptosis. **d** Quantification of proliferation by immunofluorescence for Ki67 in wild-type CGNPs cultured with or without (Vehicle) Shh for 48 hours. Three Shh cultures were also treated with 5 μ M, 10 μ M, or 20 μ M SB203580 for the last 24 hours of culture. DAPI staining was used to label all the nuclei and Ki67 levels were normalized to DAPI staining to determine the percentage of cells positive for Ki67 using Image J as described in Materials and Methods. The difference of proliferation between Vehicle and Shh culture (**) has a p value of $p < 0.002$ with a two-tailed t-test, and between Shh and Shh + 20 μ M SB 203580 culture (*), $p < 0.01$. **e** Quantitative PCR was performed on cDNA from total RNA from CGNPs cultured without Shh (V), with Shh (Shh), with Shh and 10 μ M SB203580 (Shh+SB). CGNPs were in culture for 48 hours while SB203580 treatment was for the last 24 hours of culture. *Gli1* and *N-myc* levels were determined using Taqman probes as described in Materials and Methods. *HPRT1* was used as the internal control for normalization. The amount of *Gli1* decrease observed with SB203580 is similar to the decrease observed with Shh inhibitors cyclopamine and SANT-2 [45].