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Neogenin1 is a Sonic Hedgehog target in medulloblastoma and is necessary for cell cycle progression

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The canonical Sonic Hedgehog (Shh)/Gli pathway plays multiples roles during central nervous system (CNS) development. To elucidate the molecular repertoire of Shh mediators, we have recently described novel transcriptional targets in response to Shh pathway modulation. Among them, we were able to identify Neogenin1 (Neo1), a death dependence receptor, as a new direct Shh downstream regulator in neural precursor proliferation. As appropriate Shh signaling is required for cerebellar growth and alterations cause Shh-driven medulloblastoma (MB), here we have addressed the role of the Shh/Neogenin1 interaction in the context of cerebellar development and cancer. We demonstrate that the Shh pathway regulates Neogenin1 expression in mouse models that recapitulate the Shh MB subtype. We show that the canonical Shh pathway directly regulates the *Neo1* gene acting through an upstream sequence in its promoter both *in vitro* and *in vivo* in granule neuron precursor cells. We also identified and characterized a functional Gli-binding site in the first intron of the human *NEO1* gene. Gene expression profiling of more than 300 MB shows that *NEO1* is indeed upregulated in SHH tumors compared to the other MB subgroups. Finally, we provide evidence that NEO1 is necessary for cell cycle progression in a human MB cell line, because a loss of function of *NEO1* arrests cells in the G2/M phase. Taken together, these results highlight Neogenin1 as a novel downstream effector of the Shh pathway in MB and a possible therapeutic target.

Medulloblastoma (MB), a primitive neuroepithelial tumor, is the most common malignant childhood primary central nervous system (CNS) tumor. Current treatment protocols encompassing surgical resection, chemotherapy and radiotherapy contributed to a better prognosis of MB patients. However, approximately one-third of the MB patients remain incurable and recurrence remains frequent.[1]

Four major MB subgroups including WNT, SHH, Group 3 and Group 4 have been described showing different genetic alterations, pathological features and cerebellar locations. WNT and SHH define the signaling pathways that are deregulated in those subgroups.[2] The molecular pathogenesis of the remaining two groups is less well known, although recent experiments suggest that overexpression of Myc appears to drive "Group 3" MB.[3] Importantly, up to 30% of human MBs provide evidence of abnormal Shh pathway activation.[4]

The Shh pathway has multiple functions throughout development in various tissues. It plays a role in cellular survival, proliferation, tissue morphogenesis and differentiation.[5] Appropriate Shh signaling is also required for normal cerebellar development where SHH in the early postnatal period is produced by the Purkinje cells (PCs) to drive the expansion of granule neuron precursors (GNPs) in the external germinal layer (EGL). The EGL has also been shown to be one of the origins of SHH MB.

Shh is a secreted glycoprotein that activates signaling in target cells by binding to its 12-pass transmembrane receptor Patched 1 (Ptc1/Ptch1), which then derepresses Smoothened (Smo), a seven-pass transmembrane protein and G-coupled coreceptor leading to activation of downstream pathway signaling. This signaling converges in the Gli family of transcription factors (Gli1-3), activating target gene transcription.[6] The Shh pathway was first implicated in the

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What's new?

Abnormal activation of the canonical Sonic Hedgehog (Shh)/Gli pathway has been associated with up to 30% of the human cases of medulloblastoma, which represents the most common malignant primary brain tumor in children. A greater knowledge of the cellular response to Shh pathway activation in the cerebellum is critical for both understanding disease formation and developing new treatments. In this study, the authors identified Neogenin-1 as a novel downstream effector of the Shh pathway that mediates proliferation in both cultured cerebellar progenitors and shh-driven medulloblastoma. The data suggest that targeting Neogenin-1 could offer a promising alternative to current anti-medulloblastoma therapies.

development of MB through the discovery of PTCH1 mutations in a subset of patients[7,8] and mouse models with loss of Ptc1 that developed MB.[9,10] Subsequently, mutations in other members of the Shh pathway such as Suppressor of Fused (SuFu)[11] and Smo[12] have been identified in MB as well. Therefore, understanding the cellular response to Shh pathway activation in the cerebellum is critical to our understanding of MB formation and treatment.

To address the molecular repertoire of Shh target genes, we and other groups have identified a number of transcriptional targets active during embryonic development and in cancer.[13,14] In the cerebellum, these Shh targets include *Ptc1*, *Ptc2*, *Gli1*, *Nmyc*, *CyclinD1*, *Bmi*, *Gpr153*, *Foxo6*, *Yap1* and *Ncor2*.[14–18] In a recent study, we reported neogenin 1 (*neo1*) as a gene controlled by the Shh pathway both *in vitro* and *in vivo* in zebrafish.[13] Neo1, a member of the death dependence family of receptors and part of the immunoglobulin (Ig) superfamily, was originally isolated from embryonic chicken cerebellum as a "deleted in colorectal cancer" homolog. The protein contains four immunoglobulin-like domains followed by six fibronectin domains, a transmembrane domain and an intracellular domain.[19] It binds both Netrins and the Repulsive Guidance Molecule (RGM) protein[20] and has many functions, including axon guidance during vertebrate embryonic development,[21] controlling cell survival and differentiation.[22,23] How Neo 1 regulates these processes is not clear, although it has been demonstrated that under some circumstances the Neo1 intracellular domain is cleaved and transported to the nucleus where it is capable of directly regulating transcription.[24]

Here, we demonstrate that the Shh pathway regulates Neo1 expression in mouse models that recapitulate the Shh MB subgroup. Our data indicate that canonical Shh pathway activation directly regulates the *Neo1* gene in mice and humans, acting through Gli-binding sites (GBSs) present in *Neo1* regulatory regions. Mutation of these sites abolishes responsiveness in murine GNP primary cultures as well as human cancer cell lines. Gene expression analysis performed on a total of 343 patient samples identifies *NEO1* as being upregulated in the Shh MB subgroup. Finally, we present evidence that NEO1 is necessary for cell cycle progression in a human MB cell line, where a loss of function of NEO1 arrests cells in the G2/M phase. Taken together, these results highlight Neo1 as a possible novel therapeutic target in Shh MB.

Results

Neo1 expression is restricted to the proliferative EGL of the developing cerebellum

Neo1 is a cell adhesion molecule and is expressed in a variety of developing tissues, including the CNS. To determine Neo1-positive regions during mouse cerebellar development, we evaluated Neo1 expression levels in the E18.5, P8 and P14 cerebellum (Fig. 1 and Supporting Information Fig. S1). Neo1 expression is found in the developing EGL in all stages analyzed. In the mouse cerebellum, the EGL reaches a maximum thickness during the first postnatal week (P7–P8). During this stage, Neo1 expression on cell somata and processes is mostly restricted to the most outer group of cells in the EGL (o-EGL) where it colocalizes with the proliferative nuclear marker PCNA (Figs. 1*a* and 1*c*, white brackets). The inner EGL (i-EGL), positive for the neuronal marker NeuN (Fig. 1*a*) as well as the internal granular layer (IGL) and positive for NeuN and the granule neuron marker Zic2 (Fig. 1*e*), shows low Neo1 expression. Neo1 expression does not colocalize with Bergman glia within the cerebellum (Fig. 1*g*). Overall, these data show that Neo1 is restricted largely to the o-EGL sublayer and when GNPs postmitotically migrate inward the cerebellum to form granule neurons, these cells no longer express Neo1.

Neo1 is overexpressed in Shh-driven cerebellar tumor mouse models and GNPs

We have previously shown that Neo1 expression can be regulated by the Shh pathway.[13] Yet, the relationship between the expression of Neo1 and the growth of MB has not yet been defined. Given that Shh is the major mitogenic pathway for cerebellar development,[25] we hypothesized that Shh could regulate Neo1 in this context and examined this using two different Shh pathway-driven tumor murine models. The first one corresponds to the conditional hGFAP-Cre-mediated deletion of Ptc1 (hGFAP-Cre/Ptc1$^{lox/lox}$) resulting in activated Shh signaling in neural stem and progenitor cells. In this model, the Ptc1 function is ablated in the ventricular zone between E14.5 and E16.5, resulting in a thickened and disorganized EGL filled with GNP-like cells in later stages.[10] Neo1 expression is evident in the E18.5 EGL in the developing mutant cerebellum in a pattern that resembles the wild-type (wt) expression (Fig. 2*a* and Supporting Information Fig. S1C). At P8 aberrant Shh activation in committed GNPs results in the formation of tumor masses with strong Neo1 labeling that is coexpressed with the proliferation marker

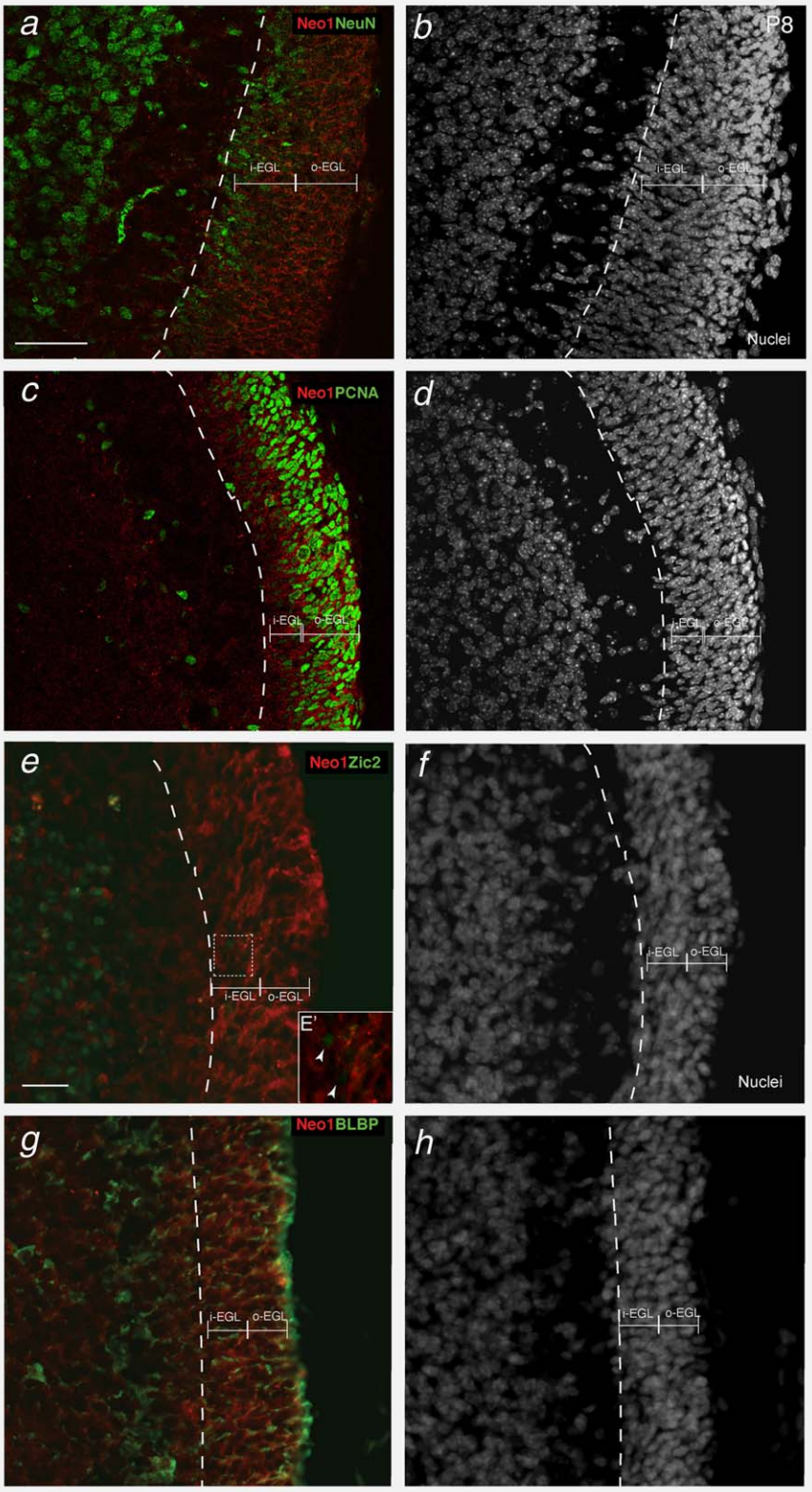

Figure 1. Neo1 is expressed in the EGL of P8 developing cerebellum. High-magnification views of the EGL show Neo1 expression in the o-EGL, with less expression at the i-EGL (*a* and *c*). Postmitotic neuronal markers NeuN (*a*) and Zic2 (*e*) show colocalization with Neo1 in the i-EGL. The proliferative o-EGL shows strong colocalization for Neo1 and the mitotic marker PCNA (*c*). The radial processes of the Bergmann glia are labeled in *g* (BLBP marker). (*b*, *d*, *f* and *h*) Nuclear staining for (*a*, *c*, *e* and *g*), DAPI or TO-PRO3. Bars = 50 μ (*a*–*d*) and 20 μ (*e*–*h*). Brackets delimitates o-EGL and i-EGL boundary. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

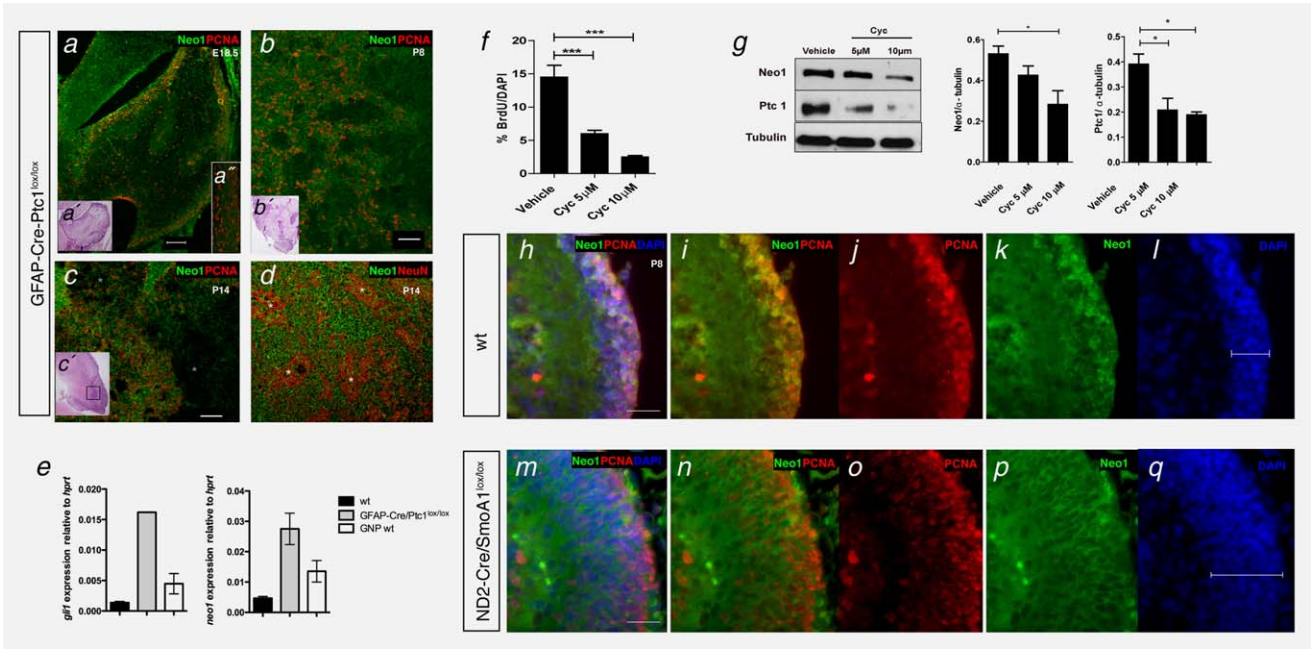

Figure 2. Shh pathway activation upregulates Neo1 in cerebellum. E18.5 GFAP-Cre/Ptc1$^{lox/lox}$ mutant EGL is thicker and disorganized in comparison to its wt counterpart. The EGL expresses PCNA as well as Neo1 (*a*, *a′*, compare with *b*, *b′*). At P8, extensive proliferative PCNA-positive regions inside the developing tumor can be identified (*b* and *b′*). By P14, the tumors cover the entire cerebellum and have extensive proliferation zones, well delimited by Neo1 expression. NeuN is completely excluded from the Neo1 labeling (*d*, white asterisks), confirming that, even in tumors, differentiating cells do not express Neo1. *a′*, *b′* and *c′*: hematoxylin and eosin staining. Gray asterisks: negative staining for Neo1-PCNA. Bar = 100 μ (*a*); 50 μ (*b*–*d*). Isolated wt GNPs from P7 express higher levels of *neo1* compared to total cerebellum; the highest expression, however, is seen in P20 GFAP-Cre/Ptc1$^{lox/lox}$ tumors (*e*). (*f*) Quantification of positive cells for BrdU over total count of DAPI(+) cells. Treatment of P7 GNP cultures with cyclopamine blocks proliferation in a dose-dependent manner. Five different fields were considered in each case in three independent experimental rounds. (*g*) Western blot showing Neo1 and Ptc1 levels in GNPs from control and cyclopamine-treated samples. For (*f* and *g*) statistically significant differences are indicated as $*p < 0.05$; $***p < 0.001$. cyclopamine (cyc). (m-q) P8 EGL from ND2-Cre/SmoA1$^{lox/lox}$ transgenic mice MB (white brackets in *m* and *jq*) shows extended Neo1 and PCNA double-positive territories compared to wt (h-l). Bars = 50 μ. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PCNA. By P14, the tumors cover the entire cerebellum and have extensive proliferation zones, clearly defined by Neo1 expression. This result contrasts with expression of Neo1 in wt at P14, which is restricted to the EGL (Figs. 2*b* and 2*c* and Supporting Information Fig. S1D). Notably, the Neo1-positive cells in tumors are negative for the marker NeuN (Fig. 2*d*).

To evaluate relative differences in transcript levels for *Neo1*, we next performed real-time polymerase chain reaction (PCR), comparing P7 samples, normal cerebella and isolated GNPs from both wt and mutant mice. Of note, *Neo1* RNA levels are substantially higher in P14 tumors from the hGFAP-Cre/Ptc1$^{lox/lox}$ than from wt GNPs (Fig. 2*e*, right panel). Similar relative differences in RNA levels are seen when comparing *Gli1* levels, a well-known canonical Shh pathway readout gene (Fig. 2*e*, left panel). The *Neo1* RNA levels range from 5- to 12-fold higher in the transgenic mouse cerebella compared to wt, whereas *Gli1* ranges from 8- to 13-fold higher. Thus, we conclude that *Neo1* is highly expressed in GNP-like cells and is likely overexpressed in this Shh-driven MB mouse model.

SHH pathway-activated MBs are thought to arise from GNPs in the developing cerebellum that depend on Shh signaling for their expansion during development. As shown in Figure 2*f*, proliferation, measured by BrdU incorporation of GNPs cultures (P7), decreased significantly in the presence of the Smoothened inhibitor cyclopamine in a dose-dependent manner with 10 μM being more effective than 5 μM. Importantly, when we analyzed GNP protein lysates by Western blotting after 48 hr of inhibitor treatment, we detected low levels of Neo1. Neo1 showed a similar dose-dependent reduction in protein levels in response to the drug as seen for Ptc1, a well-known hedgehog target (Fig. 2*g*).

Given our observations on GNPs, we hypothesized that a specific increase in Shh signaling in this cell population could account for the expanded Neo1-positive cells *in vivo*. Therefore, we used a second mouse model, the N2-Cre/SmoA1, that forms MB owing to the expression of a constitutively active form of SmoA1 under a 1-kb human NeuroD2 promoter that drives the expression in GNP,[12] resulting in early cerebellar hyperproliferation. It has been reported that MBs form in 94% of homozygous Smo/Smo mice by 2 months of age. Consistent with the thickened EGL that is observed at P8 in N2-Cre/SmoA1 mice (Figs. 2*h* and 2*m*), Neo1 and PCNA expression is also expanded (Figs. 2*h*–2*q*).

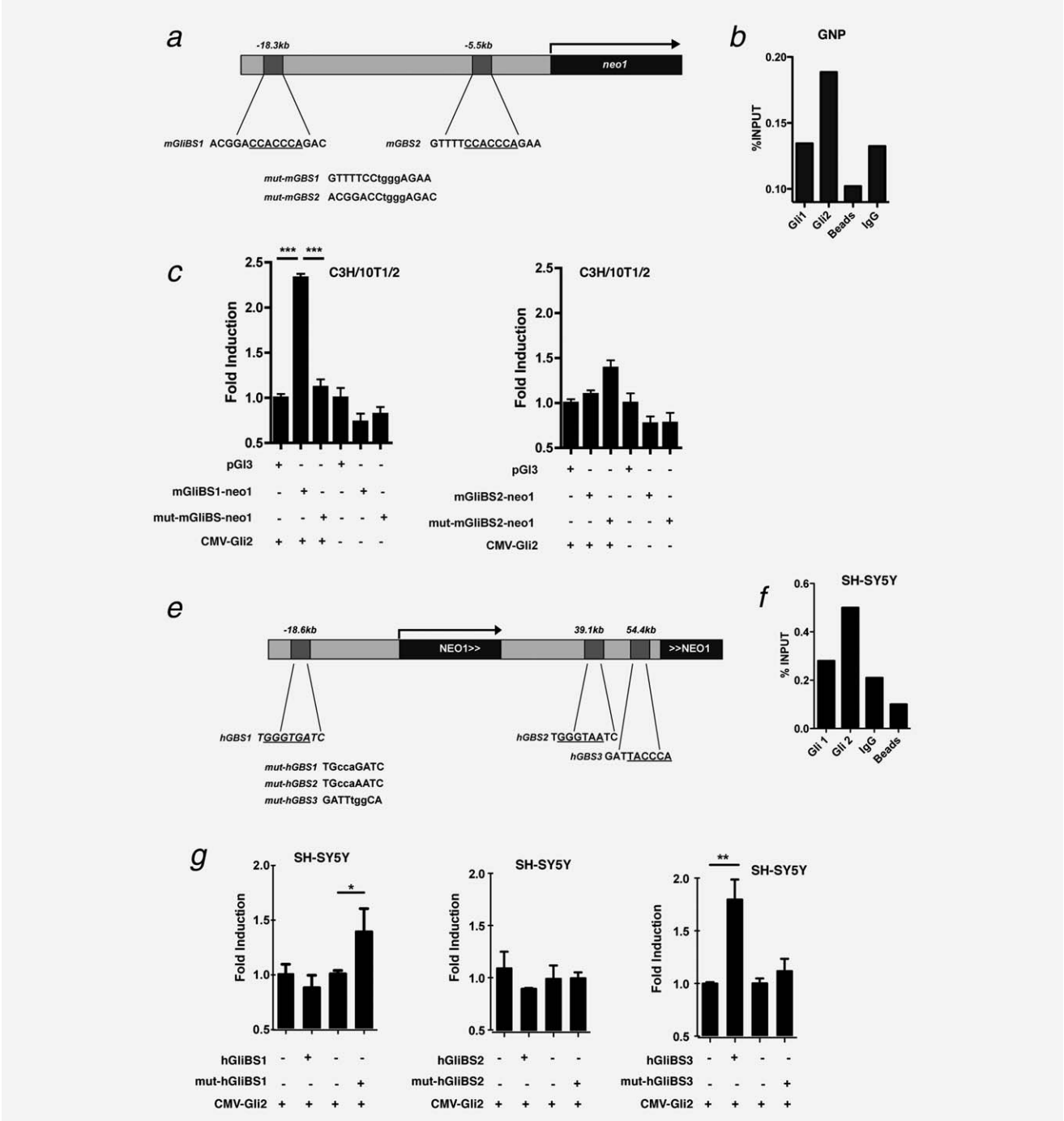

Figure 3. Canonical Shh pathway directly regulates *neo1*. Bioinformatic analysis reveals two putative consensus GBSs in the 5′ proximal regulatory region of *mneo1*; mutant sites (underlined) were designed to test the Gli activation specificity (*a*). ChIP analysis from P8 GNPs demonstrates *in vivo* binding of Gli2 to the 18.3-kb mGliBS1. (*b*) The 18.3-kb mGBS1 from the *neo1* promoter is able to enhance luciferase expression in a promoter context under Gli2 expression, using the Hh-responding cell line CH310T1/2 (*c*). Site-directed mutagenesis in the core GBS abolishes luciferase expression. The −5.5 kb (mGBS2) does not drive luciferase expression (*d*). Bioinformatic analysis reveals three putative nonconsensus Gli-binding sites (hGBS) in the regulatory regions of *hneo1*; mutant sites (underlined) were designed to test the Gli activation specificity (*e*). ChIP analysis from SH-SY5Y cells demonstrates *in vivo* binding of Gli2 to the hGBS3 (*f*). Once stimulated by Gli2 only hGBS3 was able to drive luciferase activity; mutation of the hGBS3 core abolishes this induction (*g*). IgG, Immunoglobulin G, control.

Thus, taken together, our data indicate that the Shh pathway modulates Neo1 expression in the cerebellum, and that a tumorigenic deregulation results in increased Neo1 levels in GNPs.

Shh/Gli directly regulates Neo1

To investigate whether the apparent transcriptional regulation of Neo1 by Shh was direct or indirect, we applied a web-based

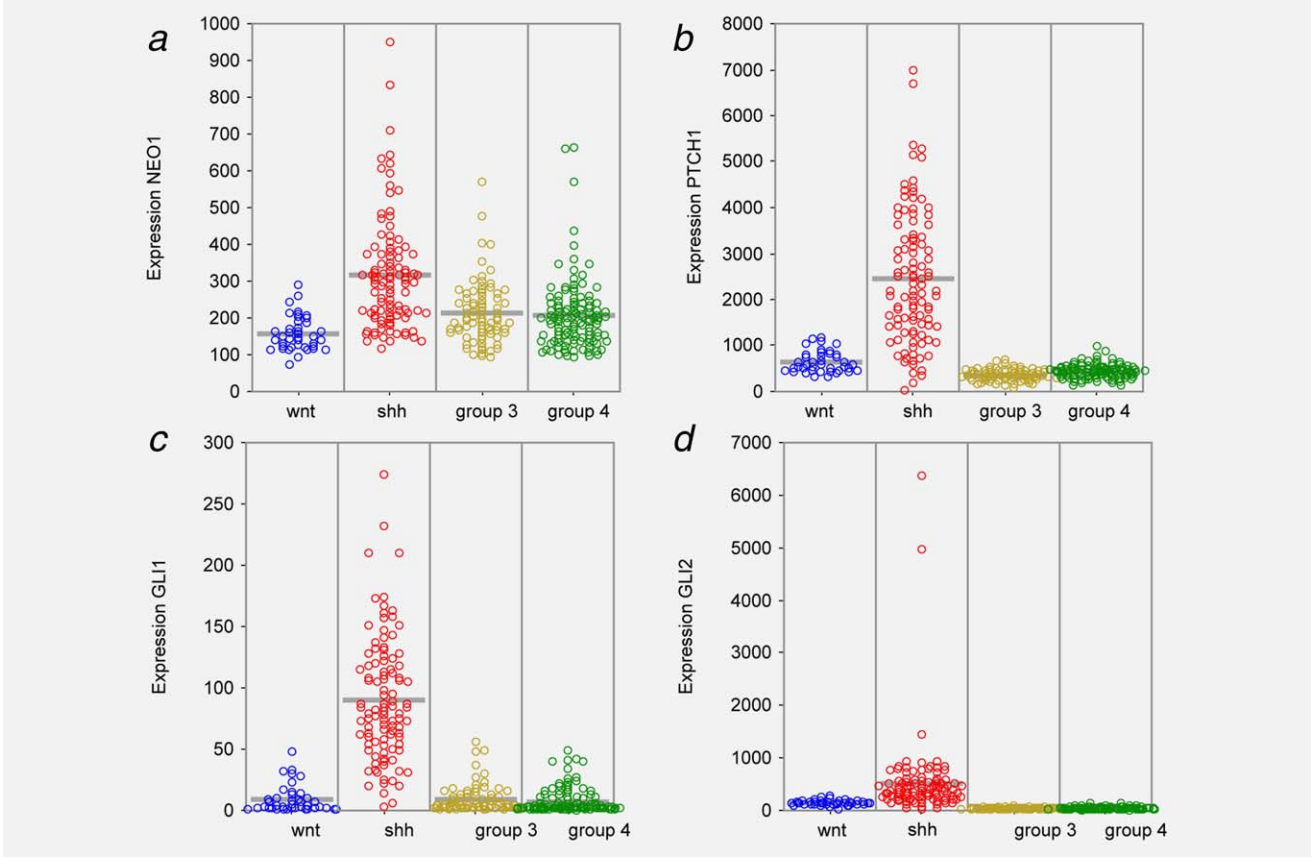

Figure 4. Upregulation of *NEO1* expression in human MB. Box plot showing *NEO1* mRNA expression obtained from an array profiling of 343 human medulloblastomas in comparison to fetal and adult normal cerebella. Note the high levels of expression in the SHH-associated MB. Expression of other hedgehog target genes (*Gli1*, *Gli2* and *PTCH1*) confirms that the expression pattern distribution of NEO1 is consistent with being a defining feature of the SHH tumor group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bioinformatic approach to identify putative GBSs in the *Neo1* promoter. We detected two possible sites that possess the consensus GBS sequences at 18.3 kb (mGBS1) and 5.5 kb (mGBS2) upstream of the *Neo1* gene (Fig. 3*a*). Given these observations, we next addressed whether the putative GBSs were likely to be functional in GNPs. Accordingly, we performed chromatin immunoprecipitation (ChIP) in P7 GNPs using antibodies to both murine Gli1 and Gli2. Gli2 but not Gli1 bound to the mGBS1, whereas the mGBS2 did not bind Gli2 in this context (Fig. 3*b* and data not shown). To evaluate their functionality as enhancers, we isolated and cloned these fragments upstream of a minimal promoter driving the luciferase reporter gene with mutated versions in the consensus core as controls. To evaluate the Gli dependence, a full-length form of Gli2 was used that activates a GBS tandem repeat, as already described.[6] Using the Shh-responding cell line CH310T1/2 it was verified that Gli2 expression was able to generate luciferase activation using the 18.3-kb sequence. As predicted from the ChIP data, the putative 5.5-kb GBS did not activate the reporter in response to Gli2 expression (Supporting Information Fig. S3 and Fig. 3*c*). Overall, these results support the previous ChIP findings and corroborate that Gli2 factor is able to bind to the *Neo1* promoter in P7 GNPs. Further, these data suggest that the Shh pathway regulates *Neo1* expression in the cerebellum during development.

To further extend our findings to the human Neogenin1 gene, we performed an *in silico* analysis revealing three putative GBS in the *NEO1* sequence: hGBS 1, located 18.6 kb upstream from the translation start, and hGBS 2 and hGBS 3, which are located within the first intron, 39.1 and 54.4 kb from the translation start, respectively (Fig. 3*e*). The GLI transcriptional factor recognition and binding to these putative GBS was analyzed through ChIP using the human neuroblastoma cell line SH-SY5Y. Although both Gli1 and Gli2 bind to a GBS present in the promoter of PTCH1 (Supporting Information Fig. S2), we found that Gli2 recognized only hGBS 3 (Fig. 3*f*). Finally, to evaluate the *NEO1* GBS functionality, each site was placed upstream of the luciferase reporter gene. Concordant with the ChIP result, only the hGBS3 induced luciferase expression upon Gli2 stimulation, whereas in mutated versions this effect was abolished (Fig. 3*g*).

These results support the ChIP findings and corroborate the data from our analysis of murine Neo1 that the Gli2 transcription factor is able to bind to the *Neo1* promoter in P7 GNPs. Further, they suggest that Shh control of *Neo1* expression is likely conserved between murine and human species.

Neo1 is overexpressed in human SHH-MB

Next, we carried out gene expression analysis in a MB cohort of 343 samples consisting of 40 WNT, 103 SHH, 79 Group 3 and 121 Group 4 tumors. NEO1 is overexpressed in SHH-driven MBs when compared to other MB subgroups (Fig. 4), suggesting NEO1 as a target of Shh signaling and further underlining the data from our murine MB studies. For comparison we also present the expression profile in those same tumors for the established SHH target genes Gli1, Gli2 and PTCH1, indicating that the expression pattern distribution of NEO1 is consistent with being a defining feature of the SHH tumor group. We considered the possibility that within the SHH MB group there existed a subgroup where NEO1 expression might define clinicopathological features. We applied a number of standard statistical approaches to this question and no significant associations were found (data not shown).

Taken together, these data comprise the first observations of *NEO1* upregulation in human MB.

NEO1 loss of function induces cell cycle arrest at G2/M

To elucidate the possible role of Neo1 in MB, we performed *NEO1* knockdown experiments using lentiviral shRNA and the human MB cell line DAOY, which is predicted to be of the SHH subtype. Silencing NEO1 expression (Figs. 5*a* and 5*b*) resulted in an increased number of cells in G2/M cell cycle stage compared to the control scrambled counterpart and fluorescent shNEO1-transduced cells demonstrated a reduced rate of BrdU incorporation (Figs. 5*c* and 5*d*). To examine whether *NEO1* knockdown induces cell cycle arrest in late G2 or M phase,[26] we quantified the number of histone H3 phosphorylated (H3P)-positive cells. Interestingly, shNEO1 cells accumulated H3P label relative to the scrambled control (Supporting Information Figs. S3A–S3AC, left panel), even when there are fewer cells expressing the shNEO1 lentivirus after 48 hr of treatment (Supporting Information Fig. S3C, right panel), indicating that cells are likely in a prolonged state G2/M arrest. Despite the observed G2/M arrest, we still found mitotic figures and condensed chromosomes (Supporting Information Figs. S3D–S3F). Finally, we verified that *NEO1* knockdown does not induce premature cell differentiation (data not shown).

Discussion

In the last few years, detailed genomic information along with the engineering of different murine models have helped to uncover important mechanisms in MB etiology, which may be exploited for therapeutic purposes. Here, we show that Neo1 is a novel direct Shh downstream mediator in cerebellar growth and in Shh-driven MB.

Neo1 is a Shh-regulated target expressed in the o-EGL

During cerebellar development, Shh produced by the PCs acts as a potent mitogen, signaling to the EGL and increasing the number of the proliferating GNPs, thus promoting the growth and foliation of the complete cerebellum. Expression of Neo1 is spatially restricted to the proliferative o-EGL during postnatal cerebellum development. This, in principle, was unexpected owing to the previously defined roles of Neo1 in axon guidance. However, there is evidence that Neo1 is expressed in proliferating CNS zones such as neurogenic progenitors[27] and in cells displaying stem cell characteristics within the adult human SVZ.[28] Within the EGL, a number of hedgehog targets, previously demonstrated to promote cellular growth, are expressed including Gli1,[25] Nmyc, CyclinD1,[15] C-Myc[29] and Bmi1.[17] Neo1 localization studies presented here suggest that its expression is downregulated in the mitotically quiescent i-EGL population when the inward migration of maturing granule neurons begins. Therefore, the spatial expression pattern of Neo1 is consistent with that of a putative Shh target gene. A functional mGBS for *Neo1* was located at −18.3 kb of *Neo1* origin, whereas for human NEO1 we defined a similar element in the first intron. Long distance enhancers have been described for the members of the Shh pathway,[30,31] and many GBSs located near to the gene do not appear to contribute to transcriptional events,[14] as was the case of the −5.5 kb consensus mGBS identified here for *Neo1*. It has been reported that weak GBS can be acting along with other nonconsensus GBS to drive strong transcriptional activation.[32] This possibility or the utilization of the −5.5 kb consensus mGBS in a different cellular context cannot be ruled out. In the o-EGL and i-EGl interface, for instance, a Gli-mediated differential regulation might contribute to fine-tuning the Neo1 spatial restriction. Other direct Shh/Gli targets could be following a similar regulatory pattern. Interestingly, we identified that Neo1 was likely directly regulated by Gli2 and not by Gli1, which, if confirmed in other systems, would make Neo1 the first Gli2-specific target identified to date.

Possible roles of Neo1 in normal cerebellar development and MB tumorigenesis

Apart from its initial role as a Netrin receptor participating in pathfinding axon guidance, there appear to be multiple roles for Neo1 in different aspects of embryonic development. For instance, as a signal for neural tube closure and dorsal brain formation[33] or as a regulator of gene transcription.[24] From the discovery that the RGM ligand interacts with Neo1,[20] it has been suggested that RGM/Neo1 interactions function as a dependence ligand/receptor couple, regulating cell survival through a DAP kinase-dependent mechanism.[22,34] Importantly, RGM A is expressed in the ventricular zone throughout the embryonic brain. Thus, RGM A-Neo1 interactions may regulate progenitor survival or proliferation within the proliferative zones of the developing CNS (28).

In studies of breast cancer, there has been a suggestion that NEO1 expression is inversely correlated with mammary carcinogenicity,[35] and there is also evidence that shows a growth inhibition in NEO1 loss of function in human ovarian epithelial cells.[36] Although there are reports indicating that NEO1 is expressed in different human MB cell lines,[37] to date no detailed analysis has been performed.

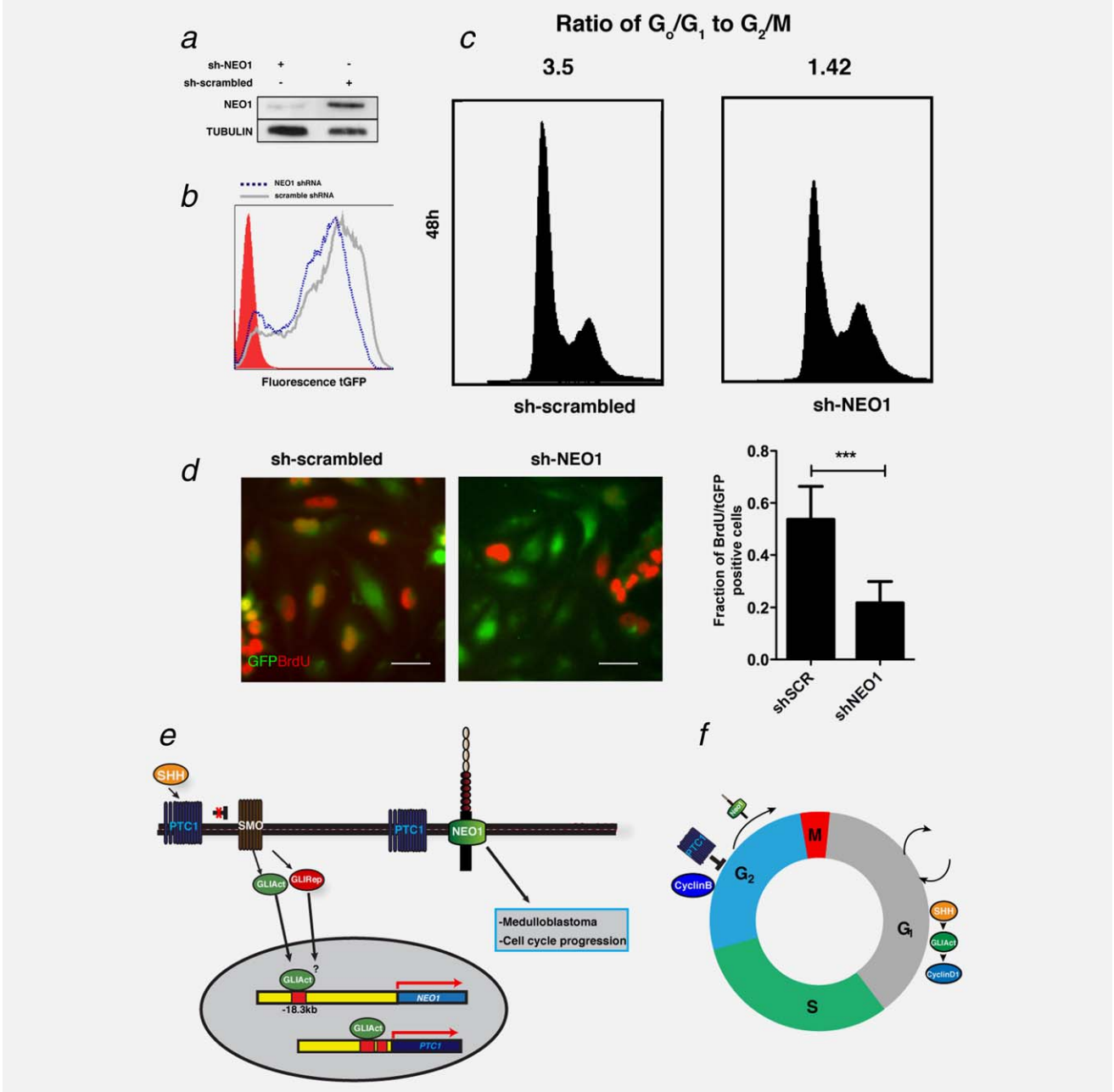

Figure 5. NEO1 loss of function arrests cells in G2/M. The fluorescent (*b*) lentiviral NEO1shRNA downregulates NEO1 expression in Western blot analysis (*a*). FACS analysis shows an increase in G2/M cells under NEO1 shRNA lentiviral infection. For the control cells the percentage values were G1: 53.3, S: 24.98 and G2: 15.27. For the sh-NEO1-transduced cells data were G1: 40.26, S: 26.13 and G2: 28.3. (*c*) BrdU incorporation decreases under NEO1 shRNA treatment. Bar = 20 μ (*d*, right panel). $p < 0.0001$. (*e*) During cerebellar development, activated SHH pathway upregulates Neo1 through the binding of GLI2 to a specific regulatory site. We cannot rule out the possibility of other GLI binding in the same or new sites in other cellular contexts. This NEO1 upregulation is enhanced in the case of MB or other cancer types. (*f*) The SHH pathway has been connected with different cell cycle pathway components as CyclinD1 and CyclinB in G1 and G2 phases, respectively. We propose that Neo1 is necessary for cell cycle progression at G2-M level, and that this relationship might be regulated by the Shh pathway. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Importantly, we found that Neo1 is expressed in a Shh-dependant murine MB model, and that Neo1 is necessary to permit the cell cycle progression (Figs. 5*e* and 5*f*). Indicating conserved roles between development and MB, we also observed that Shh-induced human MB possessed high NEO1 levels.

Crosstalk of different, previously unrelated pathways may drive tumorigenesis, probably recapitulating physiological developmental processes. Notably, the ability of Neo1 to trigger apoptosis in the absence of its ligands has been considered as a "safeguard" mechanism preventing primary tumor

proliferation within a tissue depleted of its ligands (Netrin, RGM).[38] Tumor cells constitutively overexpressing the ligand could therefore escape this proapoptotic regulation. It would be interesting to ascertain if the Shh and the Netrin-RGM-Neo1 pathway are related in different contexts. Further research is required to elucidate potentially additional roles of NEO1 in cancer progression, such as regulation of invasiveness, angiogenesis or cooperation with the tumor formation.

Neo1 as an interactor with the Shh pathway

Shh signaling controls the brain size partly by controlling the proliferation of neural stem/progenitor cells. Here, we demonstrate that in MB, Shh acts through Gli2 to transcriptionally regulate *Neo1* expression within the nucleus. Neogenin1 may be acting by sustaining the cell cycle completion during abnormal cell growth in an opposite manner as the cell cycle interaction reported for Ptc1 and cyclin B (Fig. 5*b*).[39] In other contexts, as, for example, in the neural tube, Neo1/RGM acts as a dependence receptor/ligand system.[22] Thus, the roles of Neo1 appear to be dependent on the complex biologic or cellular context.

A hallmark of Shh signaling is the upregulation of *Ptc1*, which functions as a negative feedback that restricts the activity of Shh. We and other authors have shown that Neo1 is expressed in proliferative zones of dorsal brain where the Shh pathway is active. It has been recently reported that Neo1 acts as a negative regulator for the Shh pathway during limb development.[40] Neo1 could therefore also be part of a negative feedback in the Shh signal transduction cascade. Neo1 induced by Shh could act in parallel with Ptc1 to attenuate Shh signaling in the CNS. The upregulation of Neo1 we observed in the hGFAP-Cre/Ptc1$^{lox/lox}$ and the N2-Cre/SmoA1 cerebellum could be the result of a deregulated Shh-dependent activation of the Neo1 pathway leaving this inhibitory loop to no longer be functional in tumors. It will be of interest to address the mechanism and regulation of this process and how generally this interaction could be operating to regulate cell growth and differentiation.

Neo1 pathway as a new therapeutic target for MB

In several recent studies, others and we have shown that MB is not a single disease, but in fact comprises clinically and molecularly diverse tumor subgroups (51).[2]

Currently, the most attractive target for rational therapy of SHH-MB is the SHH pathway itself. Indeed, multiple pharmaceutical companies have developed small-molecule inhibitors of the SHH pathway coreceptor SMO. Nevertheless, administration of Smo inhibitors provides only temporary antitumor activity.[2] Targeting of additional pathway components in combination to Smo inhibition seems to be pivotal to avoid drug resistance. Here, we have demonstrated that Shh is a direct regulator for the multifunctional receptor Neo1, present in tumor cells, and is necessary for cell cycle progression in a MB cell line. Our data strongly suggest that *in vivo* loss of function will result in mitotic arrest of MB cells, and that this approach would be applicable to the treatment of SHH subtypes, if not more broadly. This may be a particular opportunity because the majority of tumors occur after the cerebellum has matured and NEO1-expressing GNPs would not be targeted by an anti-NEO1-based therapeutic approach. However, as been discussed, the mechanism of action of Neogenin1 is unclear and nuclear transcriptional targets have not been defined so with current knowledge envisaging a specific NEO1-based treatment will require a much more detailed understanding of the function of NEO1 and its ligands.

Experimental Procedures

Mouse models

All work involving mice was performed with approval and according to guidelines of the University of Chile and University of Queensland Ethics Committee. Mouse models used were C57BL/6, Ptc1 conditional mice[41] crossed with the GFAP-Cre line[42] and SmoA1 conditional mice crossed with the N2-Cre line.[12]

RNA extraction and real-time PCR assays

Total RNA was isolated from cerebella at postnatal day 7 (P7) using RNeasy kit (Qiagen, Hilden, Germany) and stored at −80°C until further processing. Total RNA (2 μg) was reverse transcribed using Superscript III system (Invitrogen, Carlsbad, CA). The quantitative PCR reaction was carried out using custom Taqman probes using *hprt* as internal control (Life Technologies, Grand Island, NY) for the evaluated genes and quantified by the comparative C(T) method[43] using a AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's suggestions.

Immunohistochemistry and immunofluorescence

Immunohistochemistry analysis was carried out on 6-μm-thick paraffin sections of cerebellum at E18.5 or P14. Brain samples were fixed in 4% paraformaldehyde overnight. Antigen retrieval of deparaffinized wax tissue sections or defrosted cryosections was performed by boiling in antigen unmasking solution (Vector Laboratories, Burlingame, CA). Sections were blocked in 4% horse serum, 1% BSA and 0.2% Triton-X in phosphate-buffered saline (PBS) before primary antibody incubation overnight at 4°C. Slides were incubated with secondary antibodies for 1 hr at room temperature. For immunofluorescence, DAPI counterstain (Sigma Aldrich, St Louis, MO) was performed for 5 min before mounting with Fluorescence Mounting Media (Dako, Carpentaria, CA). For histological analysis, deparaffinized and rehydrated sections were stained in Hematoxylin (Vector Laboratories) and Eosin Y (Sigma Aldrich) and mounted.

Antibodies

Antibodies used were anti-BrdU (Dako), anti-BLBP (Abcam, Cambridge, MA), anti-Neo1 (H-175 and C20, Santa Cruz Biotechnology), anti-betaIII tubulin (Promega Corporation, Madison, WI), anti-Phospho-Histone H3 (Cell Signaling, Danvers, MA), anti-zic 2 (kindly provided by Dr. R. Segal,

Harvard Medical School), anti-Ptc11 (G-19, Santa Cruz Biotechnology, Dallas, TX) and anti-PCNA (Invitrogen). Fluorescent secondary antibodies used were anti-rabbit Alexa488 (Invitrogen) and anti-mouse Alexa555 (Invitrogen).

Chromatin immunoprecipitations

This assay was performed as described, with several modifications.[44] Briefly, the tissue was crosslinked in 1% formaldehyde, homogenized and sonicated on ice. The cell extracts were harvested by centrifugation and immunoprecipitated with anti-Gli1, anti-Gli2 or anti-IgG, and Protein-A-Agarose (Santa Cruz Biotechnology). The precipitated DNA fragments were purified by phenol/chloroform extraction and used for PCR using the following primers: mGliBS1 (forward 5′-GCT TTCCCAGAACTTGCTATG-3′; reverse 5′-ACAGACAGACC CACCAGGAC-3′); mGliBS2 (forward 5′-AACCAGTTTTCC ACCCAGAA-3′; reverse 5′-TCTGGGCTACAAACCACCTC-3′); hGliBS1 (forward 5′-GGTCTCCACCTGCTTACCTG-3′; reverse 5′-CCAACTCCATACCCCAAAGA-3′); hGliBS2 (forward 5′-GC CAGGATTTGTGATTACCG-3′; reverse 5′-GGTGACTAATCC AGGGAACAGA-3′); hGliBS3 (forward 5′-AAGGTGATCTCG AAGATTGATGA-3′; reverse 5′-GGACATCTCCTTTGCAAAA CTT-3′). An independent ChIP-positive control was performed with the human PTCH1 promoter, using the following primers: (forward 5′-GAAGCCGAGGATGCACAC-3′; reverse 5′-CTGT CAGATGGCTTCGGTTT-3′).

Reporter constructs

Fragments from the mouse and human neo1 enhancers were PCR-cloned driving a minimal promoter and luciferase reporter gene in the reporter vector pGL3-Promoter (Promega). The mutated versions m5.5GliBS and m18.3GliBS were created using Quikchange II (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions.

Lentivirus preparation

Lentivirus were prepared, amplified and purified using the procedures described in Ref. [45]. Briefly, HEK 293T cells were triple transfected with pCMV-VSV-G, p8.91 and pGIPZ-shRNA (Openbiosystems, Huntsville, AL). Viral supernatant was harvested 48 hr after transfection, filtered through a 0.45-mm cellulose acetate filter and ultracentrifuged at 25,000*g* for 2 hr at 4°C in a Beckman refrigerated centrifuge. The viral pellet was resuspended in 0.5 ml of PBS and stored at −80°C. Western blot experiments were performed using anti-Neo1-C20 (Santa Cruz Biotechnology) and anti-alpha tubulin (Sigma).

Luciferase reporter assays

Firefly luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and included a Renilla (Ren) luciferase construct (pRL-SV40; Promega) as an internal control.

GNP cell isolation

GNP primary cultures were prepared from pooled P7/P8 cerebella of mice according to the procedures described in Ref. [46] and used without further passages.

Microscopy

Confocal images were taken on a Zeiss LSM 510 META. Fluorescence microscopy was performed using an Olympus BX-51 microscope.

FACS analysis

DAOY cells were treated with lentiviral NEO1 shRNA or control sh-scramble for 24 and 48 hr. The treated cells were fixed and stained with propidium iodide. At least 20,000 stained cells were analyzed using FACS. The percentages were calculated after eliminating the cell debris, using the FlowJo software, under Dean-Jett-Fox (DJF) fitting model, obtaining G0/G1 and G2/M peaks.

Human tumor collection and expression analysis

We used publicly available[47–49] and newly generated gene expression profiles of in total 343 cases (Kool and Pfister, unpublished data) to analyze the expression of NEO1. All expression profiles were generated using total RNA isolated from fresh frozen tumor material hybridized to Affymetrix U133 plus2.0 arrays according to the manufacturer's instructions. Gene expression profiling and data analysis for *NEO1* with tumor subgrouping were performed using the R2 software (http://r2.amc.nl).

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