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Restore the brake on tumor progression

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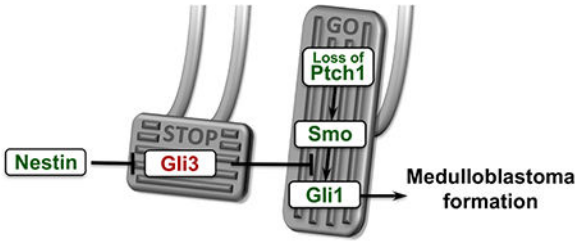
Abstract

Sonic hedgehog (Shh) signaling plays a key role in regulating normal development. The negative feedback mechanism mediated by the transcriptional factor, Gli3, acts to finely tune Shh signaling, providing tight control of normal developmental processes. Hyperactivation of Shh signaling often leads to many human malignancies, including basal cell carcinoma and medulloblastoma (MB). However, how tumor cells sustain the aberrant activation of Shh signaling is still not completely understood. We recently revealed that during MB formation, tumor cells express Nestin, a type VI intermediate filament protein, which maintains uncontrolled Shh signaling by abolishing negative feedback by Gli3. Therefore, Nestin expression is a necessary step for MB formation. These findings highlight the novel function of Nestin in regulating Shh signaling, as well as the important role of a disrupted negative feedback mechanism in MB tumorigenesis. Further, restoration of the intrinsic negative feedback by repressing Nestin expression represents a promising approach to treat MB as well as other Shh signaling associated malignancies.

Graphical abstract. A model for medulloblastoma tumorigenesis

Nestin expression is indispensable for Shh type medulloblastoma tumorigenesis. To achieve this, Nestin augments Shh signaling in medulloblastoma cells by abolishing Gli3-mediated negative feedback mechanism.



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1. Introduction

Proper development of multi-cellular organisms relies on the coordination of a diverse array of signal transduction pathways. Most signaling events are required to stay homeostatically active under precise regulation by activating as well as repressing signals. In many cases, negative feedback mechanisms act to dampen signal transduction, preventing aberrant pathway activation.

Negative feedback regulation is particularly important in the Shh signaling pathway. Shh signaling plays a fundamental role during normal vertebrate development by regulating tissue patterning, cell proliferation and differentiation, as well as fate determination. Shh ligand is a secreted protein, which acts by activating a protein complex, consisting of transmembrane receptors Patched 1 (Ptch1) and Smoothened (Smo). In the absence of Shh ligand, its receptor Ptch1 inhibits pathway effector Smo, preventing the activation of Shh pathway. When Shh ligand is present, its binding to Ptch1 results in the subsequent activation of Smo, which in turn leads to transcription of Gli zinc-finger transcriptional factors and pathway activation [1]. In mammalian cells, there are three Gli protein members - Gli1, Gli2 and Gli3, which are normally tethered to the cytoskeleton. Gli proteins are essential effectors of Shh signaling which determine the pathway outcome. The functions of Gli proteins are regulated by proteolytic processing into balanced full-length transcriptional activators or truncated repressor forms. Gli1 and Gli2 are generally considered to be activators of Shh signaling, while Gli3 predominantlyimparts a repressive function. Full-length Gli3 is mostly processed into its repressor form, which acts as a major negative regulator of Shh signaling transduction [2, 3]. After proteolytic processing, the truncated Gli3 translocates into cell nuclei to repress the activation of Shh pathway (Figure 1). The negative feedback loop mediated by Gli3 plays an important role in ensuring the activation of Shh pathway at physiologic levels.

In the cerebellum, Shh ligand is released from Purkinje neurons and stimulates the proliferation of granule neuron precursors (GNPs) in the external granule layer (EGL). During the first 2-3 postnatal weeks in mice, GNPs exit the cell cycle, differentiate and migrate inward to form the internal granule layer. In parallel, Shh signaling is gradually down-regulated in GNPs, even in the Shh-enriched environment [4, 5]. Aberrant activation of Shh pathway in GNPs caused by loss of Ptch1, drives ectopic proliferation of GNPs. After prolonged proliferation, Shh signaling diminishes in the majority of Ptch1-deficient GNPs. Only a rare population of cells sustains activation of the Shh pathway and ultimately results in the formation of MB [6]. The discrepancy between the normal developmental self-restricted activation of signaling in GNPs and the abnormal proliferation with aberrant pathway activation implies the importance of negative regulation of the Shh pathway. This observation suggests that the disruption of the negative regulation on Shh signaling is critical for MB tumorigenesis.

We have recently found that during MB formation, tumor cells gradually express Nestin, a type VI intermediate filament protein. Nestin expression plays a critical role in MB tumorigenesis by sustaining aberrant Shh signaling in MB cells. To achieve this, Nestin impairs the inhibitory regulation of Gli3, driving MB initiation and progression [7]. Our

studies highlight the novel function of Nestin in regulating Shh signaling and the importance of disruption in the negative feedback regulation in the tumorigenesis of MB. In this review, we summarize the expression and functions of Nestin in regulating Shh signal transduction and the negative feedback mechanism in tumorigenesis and the therapeutic implications of the negative feedback.

2. General knowledge of Nestin and its expression in tumors

Nestin, first identified in the neural stem cell (NSC) in 1985, is widely considered a putative marker for stem cells [8, 9]. The human Nestin gene encodes a large protein consisting of 1621 amino acids. Structural organization of the Nestin gene is evolutionarily conserved between human, rat and mouse, indicative of its functional significance [10]. Nestin expression is predominantly regulated by enhancer regions in the first and second introns of the Nestin gene [11, 12]. A 714 bp conserved 3′ portion of the second intron is sufficient to control Nestin expression in progenitors in the central nervous system. An enhancer region of the Nestin gene is found in the second intron and is divided into two separate domains [13]: the 3′ region required to induce Nestin expression in pan-CNS progenitors and the other controlling expression in the midbrain. The putative binding sites located in the second intron of the Nestin gene include Retinoic Acid Receptor (RAR), Retinoid X Receptor (RXR), and Thyroid Hormone Receptor (TR) [14, 15]. In human umbilical vein endothelial cells, Nestin expression was found to be regulated by an element in the first intron [16].

Similar to other intermediate filaments, such as cytokeratin and vimentin, Nestin consists of an α-helical rod domain, flanked by N-terminal ‘head’ and C-terminal ‘tail’ domains. The highly conserved rod domain of the Nestin protein contains several α-helical coils that assemble in antiparallel fashion, resulting in filament formation. The alignment of stable dimers and cohesive forces between adjacent dimers determines the properties of high stability and plasticity in the formed Nestin [17, 18]. Unlike the majority of other intermediate filaments, Nestin was characterized as having an unusually long C-terminal domain and a relatively short N-terminal domain, which define its self-assembly characteristics [17]. The C-terminal domain gives Nestin a remarkable binding capacity to a wide range of proteins and serves as a platform for cell signal integrations. The short N-terminal domain limits the self-polymerization capability in Nestin, leaving its filament formation to be entirely dependent on interactions with other intermediate filament proteins. It is well established that Nestin often forms complexes with vimentin and α-internexin, allowing formation of stable cytoskeletal intermediate filament networks which maintain the structural integrity of the cells [19-21].

Nestin expression is very dynamic and tightly regulated, both spatially and temporally. Although Nestin is commonly utilized as an NSC marker, it is expressed by a variety of progenitor cells, including skin and hair follicle, muscular, renal, hepatic, endothelial, mesenchymal, hematopoetic and neuronal progenitors [22-31]. In general, the expression of Nestin is primarily correlated with the proliferative stage of such progenitors. As cells exit the cell cycle and undergo terminal differentiation, Nestin expression gradually decreases and is replaced by tissue-specific intermediate filaments, such as glial fibrillary acidic protein in astrocytes, α-internexin and neurofilament in neurons, and desmin in myocytes

[32, 33]. Nestin expression is normally down-regulated in mature tissues, but its expression can be driven by conditions resembling developmental processes, such as tissue regeneration, revascularization and wound healing. For example, Nestin expression was observed in regenerating muscle tissue following injury or necrosis, as well as in reactive astrocytes during post-injury glial scar formation [34, 35]. Nestin expression has also been observed in tissues with pathologic conditions, i.e. in the tooth during carious lesion formation or in brain tissue after traumatic injury and ischemia [36].

Nestin expression has also been reported in various human neoplasms. Nestin is highly expressed in most tumors that originate from undifferentiated precursors and immature progenitors, such as medulloblastoma, neuroblastoma and retinoblastoma [37]. Presence of Nestin has also been confirmed in ductal breast carcinoma and pancreatic ductal adenocarcinoma [38]. In addition to solid tumors, expression of Nestin protein has been found in tumor cells from acute myeloid leukemia and acute lymphoblastic leukemia [39]. In addition, Nestin is widely utilized to label “cancer stem cell” populations in variety of malignancies, including brain tumors, sarcomas, ovarian and prostate carcinoma, lung, breast and pancreatic cancers [38]. Nestin expression is not only present in tumor cells, but also in tumor stroma, particularly vascular endothelial cells. It has been reported that Nestin is involved in the vasculogenesis in tumor tissue [40]. Overall, Nestin expression is associated with malignancy and is indicative of a poor prognosis. In some tumors, including malignant melanoma, schwannomas and gastrointestinal tract tumors, Nestin expression is utilized as a negative predictive marker[41]. Nestin tends to be found in advanced stage tumors. For example, stronger Nestin expression was found in glioblastoma multiforme compared to expression in lower grade gliomas [42]. Although Nestin expression has a strong correlation with malignancy, invasiveness and decreased survival, the functional role of Nestin in the tumorigenesis remains under investigation.

3. Functions of Nestin in Hedgehog signaling and medulloblastoma

MB normally arises on the surface of the cerebellum, but could metastasize to the other parts of the central nervous system. Despite the aggressive tumor treatment including surgical resection, chemotherapy and radiotherapy, 30% of patients with MB still succumb to this disease. Patients who survive MB often suffer with severe long-term effects resulting from treatment, including cognitive deficits and increased incidence of secondary tumors [43, 44]. Therefore, improved approaches to treating MB are urgently needed.

Human MB comprises at least four subgroups based on their distinct genetic/epigenetic profiles: Wnt, Sonic Hedgehog (Shh), Group 3, and Group 4 [45, 46]. The Shh subgroup accounts for about 30% of MB cases, which is caused by aberrant activation of the Shh signaling pathway. We have previously demonstrated that genetic deletion of Ptch1 in cerebellar GNPs leads to MB formation in mice. This indicates that cerebellar GNPs represent the cell of origin for MB. However, the majority of Ptch1-deficient GNPs ultimately differentiate after prolonged proliferation. Only a small proportion of GNPs in the hyperplastic lesions finally develops into MB, suggesting that loss of Ptch1 alone is not sufficient for maintenance of constitutive Shh signaling transduction [6].

Our recent studies revealed that cerebellar GNPs gradually express Nestin after Ptch1 deletion. Loss of Ptch1 causes a transient increase in Shh signaling in GNPs. However, in the absence of Nestin, Shh signaling is eventually dampened by the repressor activity of Gli3 and cells undergo differentiation [6]. In the presence of high levels of Nestin, Gli3 is sequestered and Ptch1-deficient GNPs maintain high levels of Shh signaling and go on to form tumors. We further demonstrated that the C-terminal domain of Nestin can directly bind to Gli3 protein. Physical interaction with Nestin compromises Gli3 phosphorylation and impairs its proteolytic processing. In addition, Nestin tethers Gli3 in the cytoplasm and prohibits its nuclear translocation, thereby blocking the inhibitory effects of Gli3 on Shh signaling [7]. These findings shed light on the molecular mechanisms by which Shh signaling drives MB tumorigenesis. Our data demonstrate for the first time that Nestin functions to maintain and enhance Hh pathway activation leading to malignancy.

4. Negative feedback loops in tumorigenesis and drug resistance

Negative feedback loops are biological mechanisms that normally operate to lessen various types of signaling and thereby precisely tune the signaling outcome. During normal development, various signaling pathways stimulate tissue cell proliferation in order to produce the proper number of cells. The negative feedback loop in each pathway acts to attenuate these proliferative signals. Defects in these critical feedback mechanisms can result in uncontrolled proliferative signaling that eventually lead to the tumor formation.

After Hh pathway activation, Gli3 primarily functions to suppress further signal transduction. MB cells express Nestin, allowing them to evade the negative regulation of Gli3 on Shh signaling. This results in uncontrolled activation of the Shh pathway and sustained MB progression. The process of tumor formation is often likened to a car speeding out of control. If the gas pedal is stuck, a functioning brake can still bring the car to a full stop. If the brake is disconnected at the same time the gas pedal is stuck, the car will continue uncontrolled. In a sense, the same thing happens during MB formation. Analogous to pushing the gas pedal, activation of the Hh pathway by Ptch1 deletion drives the proliferation of cerebellar GNPs. However, the brake of Shh signaling, Gli3 acts to repress further pathway activation, so that the majority of GNPs eventually stops dividing and differentiates. Only a rare population of Ptch1 deficient GNPs expresses Nestin, abolishing the functions of Gli3 and thereby the negative feedback mechanism on Shh signaling. As a result, tumor is finally developed. Both Nestin expression (disconnected brake) and loss of Ptch1 (stuck gas pedal) are indispensable for MB tumorigenesis (lost control of car) (Graphical abstract). Our findings highlight the importance of disruption of these negative feedback mechanisms in the process of tumorigenesis.

Disruption of negative feedback loops has been also observed in other signaling pathway associated tumorigenesis. For example, Ras GTPase serves as intrinsic negative feedback to ensure transient activation of Ras signaling [47]. The oncogenic effects of Ras do not result from a hyperactivation of its signaling powers, instead mutations in the Ras gene compromise the inhibitory capability of Ras GTPase [48]. In addition, PTEN phosphatase counteracts PI3K by degrading its product, phosphatidylinositol (3,4,5) triphosphate (PIP3). Loss-of-function mutations of PTEN induce tumorigenesis of many human malignancies

through activation of PI3K pathway. Such compromised negative feedback loops in proliferative signaling pathways are widely present among human cancer cells and serve as an important means by which these cells achieve proliferative advantage [48].

Negative feedback is also involved in drug resistance during tumor treatment. In the RAF/MEK/ERK signaling cascade, the negative feedback from ERK to RAF effectively attenuates the pathway activity. MEK inhibitors weaken the feedback signal and upregulate RAF compensating for the initial loss of ERK activity [49]. Thus, tumor cells often exhibit resistance to sole treatment with MEK inhibitors. Thus, a combination of MEK and RAF inhibitors is currently considered a standard strategy for melanoma treatment. Negative feedback has also been found to underlie resistance against drugs targeting the PI3K/Akt/mTOR pathway, which is hyperactivated in many types of tumors. In normal cells, stimulation of IGF activates insulin receptor substrates (IRS1/2) that trigger the downstream PI3K/Akt/mTOR cascade. mTOR represses the interaction between IRS 1/2 and IGF receptor, generating a negative feedback loop from mTOR to IRS 1/2. mTOR inhibitors often lead to relief of the feedback, which causes increased IRS signaling and induction of the kinases upstream of mTOR, PI3K and Akt [50]. This is commonly believed to be the mechanism of tumor cell resistance to mTOR inhibitors.

5. Tumor treatment by restoring the negative feedback

Mutations and dysregulation of the Shh pathway often lead to tumorigenesis and accelerated tumor growth in many tissue types. Basal cell carcinoma and MB are two well-recognized tumors with mutations in components of the Shh pathway. Inappropriate activation of the Shh pathway has been implicated in the development of several other types of cancer including lung, prostate, breast and pancreas [51, 52]. In addition, Shh signaling can also drive tumor initiation and progression through stimulation of cancer stem cells or alteration of the surrounding stroma [52]. The Shh signaling pathway has been recognized to be one of the most important therapeutic targets in tumor treatment.

As a critical effector of Shh signaling, Smo has been the primary target for development of Hh pathway inhibitors. Smo inhibition prevents the downstream activation of Gli transcription factors, leading to suppression of those genes associated with cancer growth and progression [53]. Vismodegib and Sonidegib are approved by the FDA to treat advanced or metastatic basal cell carcinoma. Several other Smo antagonists, including XL139, Glasdegib, Taladegib and Saridegib are currently utilized as a monotherapy or in combination in clinical trials to treat a wide array of cancers, including MB, small cell lung cancer, metastatic pancreatic cancer, metastatic prostate cancer, recurrent glioblastoma and hematological malignancies [54]. Although initial findings suggest that while available Smo antagonists exhibit initial therapeutic efficacy, there is a rapid emergence of drug resistance [55, 56]. In many cases, resistance arises as a consequence of mutations in Smo that prevent binding of the antagonists or genetic events that activate downstream components of the Shh pathway [57, 58]. Additionally, Smo antagonists globally repress Shh signaling, even in normal cells. In particular, MB is a brain tumor that mainly affects children and Shh signaling is necessary for normal development of many tissues in patients. Thus, Smo antagonists can cause severe developmental side effects, such as bone defects and endocrine

disorders [59]. Smo antagonists are contraindicated during pregnancy as they are teratogenic, embryotoxic and fetotoxic. Other adverse reactions include alopecia, muscle spasms, weight loss, fatigue, GI disturbance and arthralgias. Therefore, novel inhibitors with improved efficacy and fewer toxicities are still required to treat Shh pathway associated malignancies.

In our studies, Nestin expression represents a necessary step for MB tumorigenesis and it promotes MB growth by augmenting Shh signaling by abolishing Gli3 negative feedback. Therefore, targeting Nestin in MB cells could restore the inhibitory functions of Gli3 and repress hyperactivation of Shh pathway. We have shown that inhibition of Nestin expression effectively blocks MB formation in mice, indicating that Nestin expression represents a promising therapeutic target to treating MB. Further, disruption of the negative feedback of Shh signaling is only present in MB cells and the Gli3 inhibitory mechanism is intact in normal cells. Indeed, Nestin expression is present only in MB cells, but not normal GNPs. Thus, recovery of negative feedback by repressing Nestin expression could cause much less on-target toxicity, compared to Smo antagonists. Since Nestin regulates Gli3 activity downstream of Smo, inhibition of Nestin expression may be effective for treatment of drug-resistant MB as well as other Shh pathway associated malignancies.

The functions of Nestin in tumorigenesis were not recognized until recently. As an intracellular cytoskeletal protein, Nestin itself may not represent an ideal pharmaceutical target. Therefore, no inhibitors antagonizing the functions of Nestin are currently available in preclinical studies and/or in clinical trial. On the other hand, Nestin expression is not limited to tumor cells, but also found in normal stem cells, especially NSCs. It was previously reported that Nestin was required for the self-renewal of NSCs [33]. Direct inhibition of Nestin functions could cause toxicities in NSCs, precluding clinical utilization of Nestin itself as a therapeutic target in MB treatment. However, mechanisms underlying Nestin induction in tumor cells may represent an attractive target for MB treatment. Our previously studies suggest that Nestin expression in MB cells relies on the unique tumor microenvironment [7]. Ongoing studies in our laboratory are to address the cellular and molecular basis for Nestin expression in tumor cells during MB development. Elucidation of mechanisms for Nestin expression in tumor cells and normal NSCs could help to develop a promising approach to treat MB by targeting Nestin induction.

6. Conclusion

Though Nestin is widely used as a marker for NSCs, recent studies have revealed that Nestin participates in the regulation of many signaling pathways [60] [61]. The wide and dynamic expression pattern of Nestin encourages further investigation of the diverse functions of Nestin during normal development and pathological processes. It is becoming clearer that Nestin and other cytoskeletal proteins play important roles in a number of signaling events by direct contribution in signaling transduction or provision of ample surface for protein-protein interaction. Therefore, cytoskeletal proteins could potentially be utilized as targets to manipulate cell signaling activity to resolve a variety of diseases.

Our previous studies revealed that Nestin promoted the tumorigenesis of Shh pathway-associated MB by inhibiting Gli3. However, Nestin expression was also detected in non-Shh type of human MB including Wnt type, group 3 and group 4, where Shh pathway is not involved in the tumor initiation and progression [7]. Nestin may modulates other signaling pathways that are essential for tumorigenesis of non-Shh type MB, which are warranted for future studies.

Negative feedback mechanisms are crucial in dynamic regulation of developmental signaling. Recent studies highlight the important role of disrupted negative feedback in tumorigenesis. These findings significantly further our understanding of the molecular basis underlying tumor initiation and progression and provide novel targets to explore therapeutically for tumor treatment. Because negative feedback dramatically alters drug sensitivity and dose-responsiveness, for tumor treatment to be successful, it is critical to surpass drug resistance by careful understanding of feedback loops of signaling pathways.

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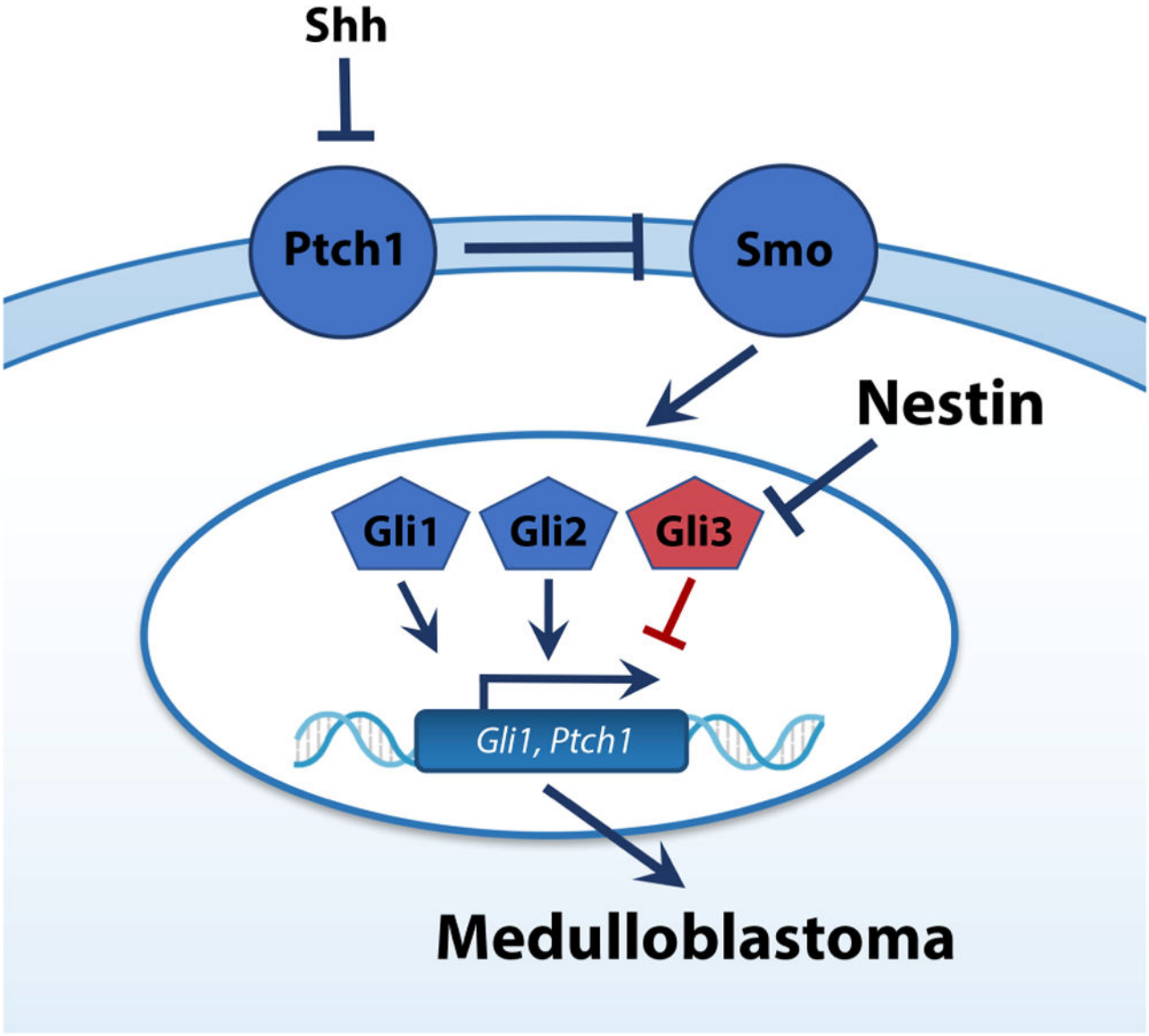

Figure 1. Nestin augments Shh signal transduction by inhibition its repressor, Gli3

Shh ligand interacts with its antagonizing receptor Ptch1, which activates the effector protein, Smo, leading to nuclear translocation of the Gli family of transcriptional factors: Gli1, Gli2 and Gli3. While Gli1 and Gli2 act primarily as transcriptional activators, Gli3 blocks subsequent transcription of Shh pathway target genes (*Gli1, Ptch1*, etc.), acting as an essential negative regulator of Shh signaling transduction. Recent work from our laboratory has demonstrated that Nestin mediates Shh pathway associated tumorigenesis by abolishing the inhibitory functions of Gli3 on Shh signaling [7].