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Hedgehog–Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer

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Abstract

The Hedgehog–Gli signaling pathway is involved in the regulation of the proliferation of precursors in different organs of the normal vertebrate embryo. These cells express *Gli1* and may be the target of cancer-causing agents. Many tumor types derived from organs that contain *Gli1*+ precursors appear to consistently express *Gli1*, indicating their origin and/or the presence of an active pathway. Inappropriate pathway activation in a variety of precursor cells in model organisms leads to tumor formation while inhibition of the pathway in human tumor cells leads to a decrease in their proliferation. In the brain we have documented the expression of Gli1 in germinative zones, and a variety of brain tumors express *GLI1*, including medulloblastomas of the cerebellum and a number of gliomas of the cerebral cortex. The requirement for SHH–Gli signaling in the growth of the mouse brain, together with the ability of inappropriate pathway activation in the cerebellum to cause medulloblastomas, and the inhibition of the growth of a number of brain tumors with cyclopamine, a SHH signaling inhibitor, underscores the critical role of the SHH–GLI pathway in brain growth and tumor formation. Moreover, they highlight the components of this pathway as prime targets for drug development, with special emphasis on the GLI proteins. Such reagents would allow a rational therapeutic approach to highly intractable diseases.

Keywords: Hedgehog; Sonic hedgehog; Gli1; Gli2; Gli3; Patched; Smoothened; Tumor; Cancer; Embryo; Progenitor; Stem cell; Brain; Cortex; Cerebellum; Medulloblastoma; Glioma; Mouse; Human; Frog; Model system; Cyclopamine; Small molecule; Inhibitors; Therapeutic agents; Targets; Proliferation

1. Introduction

Brain tumors remain difficult diseases to treat partly because of their heterogeneity. Attempts to describe them have succeeded in providing pathological criteria mostly based on consistent morphological parameters, including cell invasiveness and density. These formal descriptions [1] have helped to provide a framework for the common denomination of tumors in different parts of the world and thus for the possibility to collect data from multiple studies on the same or similar subjects. Such an image-based analysis is not different from those performed for many other types of tumors, including those of the skin [2]. A molecular analysis of brain tumorigenesis, and other types of cancer, is underway [3–7] and this will no doubt provide better and clearer criteria with

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which to classify and understand the pathologies of brain tumors, and cancer in general. So far, these and other studies point to the regulation of the cell cycle and apoptosis as major players in the cancer phenotype as well as to the fact that there is a myriad of genes showing transcriptional alterations in cancer. The identity of some of these genes points to developmental signaling pathways as critical regulators of tumorigenesis.

Cancer growth, however, does not follow normal developmental programs (each using multiple signaling pathways) as no normal tissue or organ is formed. Moreover, if cancer were to follow normal developmental programs one might expect a more restricted genotype–phenotype correlation in tumors. Indeed, from a developmental point of view the fact that apparently similar human brain tumors can harbor mutations in different oncogenes and tumor suppressor genes, and that brain tumors can derive from different causes in mice (reviewed in Refs. [8,9]) is baffling. This is so because normal developmental outputs have a unique or a very limited set of underlying causes and thus mutations in 'developmental' genes cause reproducible and specific embryonic phenotypes. For example, in the mouse, an experimental system that not always recapitulates the human condition, gliomas can be produced by a number of alterations that include the activation of Ras [10], Akt and Ras [11], Ras and loss of Ink4a-Arf [12], EGF signaling [13], EGF and CDK4 signaling [14], EGF signaling plus loss of Ink4-Arf [15], loss of NF1 and p53 [16] or enhanced PDGF signaling with or without loss of Ink4a-Arf [17]. Similarly, medulloblastomas can appear following the combined loss of p53 and Rb [18], loss of p53 and Lig4 [19], loss of PARP1 and p53 [20] or by loss of Patched1 function [21], which is enhanced by loss of p53 but not by that of Ink4a-Arf [22]. The multiplicity of cancer causes (reviewed in Refs. [8,9,23–29]) could be due to the existence of many possible parallel mechanisms that lead to cancer - in opposition to normal development in which redundancy is not the rule; to their possible convergence to critical cellular events, such as the regulation of the cell cycle or apoptosis; and/or to the existence of multiple, yet distinct, target cell populations that yield different, yet morphologically similar tumors, but which require distinct inputs to initiate the tumorigenic program.

Paramount in cancer research has been the idea that the mature tumorigenic phenotype results from the progressive acquisition of mutations (reviewed in Ref. [23]). For example, an advanced tumor may have acquired with time mutations that effectively enhance the characteristics of the tumor and thus endow it with a selective advantage, which can be detected in classical transformation assays. The acquisition of these secondary 'hits' would then stand in contrast to those mutations that induce the initial formation of the tumor. In this sense, the two-hit hypothesis (reviewed in Ref. [30]) suggests that cancer cells acquire multiple mutations that may make them eventually independent of the initial events as selection shapes the resulting tumor. This idea has vastly improved our understanding of cancer and has focused attention on factors that when mutated directly misregulate the cell cycle and those that prevent apoptosis, which have been seen as the primary mechanisms to preserve a competent and sustained proliferation status and thus the growth of a tumor.

Recent findings in molecular embryology, however, point to the regulation of the cell cycle as part of patterning programs that instruct cells and their descendants to acquire distinct fates, which include proliferative instructions. For instance, a cell may acquire a given fate through an epigenetic mechanism that includes the necessity to divide in a given lineage a number of times before terminally differentiating, apoptosing or becoming competent to respond to other environmental signals. In this context we have taken the view that cancer is a patterning disease and that tumor cells are playing out abnormal 'developmental' programs. When operational, these programs (which may use multiple signaling pathways and other normal components but with abnormal order, timing, combination or strength) allow the tumor to develop the characteristics proper for its type and location. These are the programs that we call paradevelopmental. Understanding cancer may thus involve, from this viewpoint, the understanding of normal development as well as of paradevelopmental programs, which include cell–cell interactions, motility, adhesion, changes in cell shape, lineage restrictions, response to environmental morphogens, intercellular signaling, etc. We think that in cancer, as in embryogenesis, patterning signals and pathways play critical roles.

Another aspect that may challenge the classical view of cancer is that initiation events are often required for tumor maintenance well after the tumor has formed, progressed and it has been diagnosed. The best examples are those in which a tumor is initiated in transgenic mice through the conditional action of an oncogene. After tumor formation, the oncogene in question is deactivated and, surprisingly, the tumor regresses [31–38]. In these cases, any additional mutations in the tumor did not seem to make it independent from the original initiating event. Nevertheless, one could argue that such a forced expression of a very potent oncogene is enough to promote a rapid and strong tumor response without the slow tumorigenic kinetics that may be more close to the normal case in humans and that would allow for additional mutations to be acquired by the tumor cells. Indeed, the majority of human tumors are thought to be defective in the p53 pathway (e.g. reviewed in Ref. [39]; see also Refs. [40,41]), thus allowing them to escape apoptosis, which would have been normally triggered by the inappropriate proliferative state (reviewed in Ref. [42]). In one study, however, loss of p53 facilitated the development of initiating signaling-pathway independent tumors [38].

Paradevelopmental programs, we propose, are important to drive stem cell lineages, perverted through mutation or epigenetic change, towards a tumorigenic state. It is in these cells, or in any cell with stem cell potentiality—that is self-renewal and generation of more committed progeny—that the initiation events for tumorigenesis may take place as they already have the ability to bypass apoptosis and senescence in response to continued proliferation (discussed in Refs. [29,43,44]). Our focus, therefore, ought to be on the study of patterning pathways that stem cell lineages use and the paradevelopmental programs that cause them to become successfully tumorigenic. In this sense, tumors can be seen as abnormal organ development projects that, nevertheless, display consistent order, morphogenesis and patterning following paradevelopmental programs. It is this consistency in their ontogeny that allows pathologists to classify them morphologically.

Our discussion so far points to several unanswered questions. For example: are stem cell populations the targets of carcinogens? Are there cancer stem cells in all solid tumors as there are in some leukemias [45–47] and possibly in breast tumors [48]? What are the initiation events in human tumors? Are these then required for tumor maintenance and viability? Are patterning pathways involved in all types of cancer? Can paradevelopmental programs be described in detail or do they show enormous redundancy? With these considerations, explanations and speculations in mind we now survey the involvement of the Hedgehog–Gli pathway in brain tumorigenesis.

2. Hedgehog–Gli signaling in tumorigenesis

Since the discovery of the Drosophila Hedgehog (Hh) mutation [49] and gene [50–52], Hh signaling has been found to play multiple roles in development, homeostasis and disease (reviewed in Refs. [53,54]). Three Hh genes are found in vertebrates (Sonic, Desert and Indian Hhs [55–58]) and these act as secreted, intercellular morphogens that affect cell fate, differentiation, survival, and proliferation in the developing embryo and in most if not all organs. In the CNS, Hh signaling is critical for early ventral patterning of the neural tube along the entire neuraxis (reviewed in Ref. [59]) and later on for the development of the dorsal brain, including that of the cerebellum and neocortex (reviewed in Ref. [60]). Hhs act by activating the 7-pass transmembrane protein Smoothened (Smo), which then sends a signal intracellularly (Fig. 1). Activation of Smo appears to occur through the inhibition of the 12-pass transmembrane protein Patched1 (Ptc1), which normally inhibits Smo. Inside the cell, the Smo signal is regulated by a complex of proteins through intricate mechanisms (reviewed in Refs. [61–63], leading to the action of the zinc-finger Gli transcription factors. The three Gli proteins, Gli1-3, act in a combinatorial fashion that is context dependent, with Gli1l/2 being the major positive mediators of Hh signals, and Gli3 having often an antagonistic role (reviewed in Ref. [60]). In mice, Gli2 and Gli3 appear to be the early mediators of Shh signaling [64,65], whereas Gli1 may be an amplifier of the response.

GLI1 was originally identified as an amplified gene in a human glioma line [66] and it can transform cells in vitro in cooperation with E1A [67,68], but its involvement in sporadic tumorigenesis was not substantiated [69,70] until recently ([71–73]; reviewed in

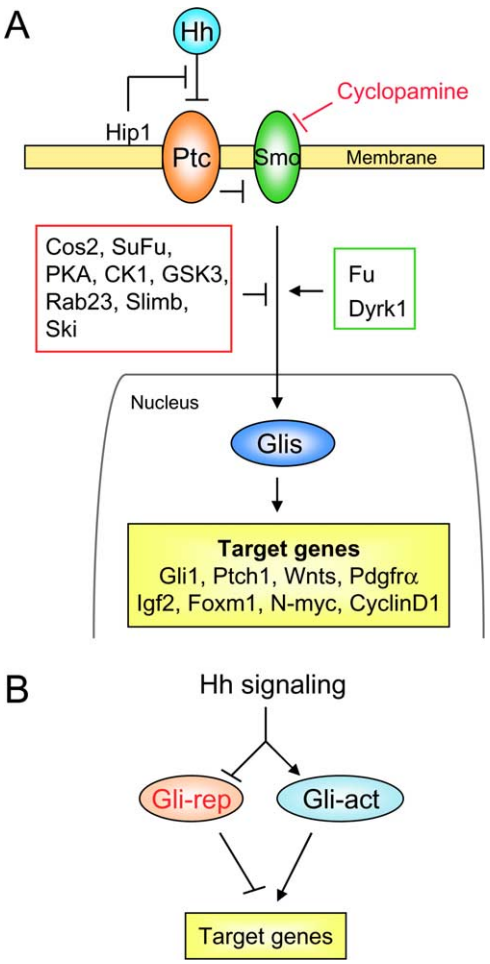

Fig. 1. (A) Diagram of a generalized Hh–Gli signaling pathway derived from knowledge in model systems. (B) Effects of Hh signaling on Gli function. Shh signaling inhibits Gli3 repressor formation and may induce the formation of potent Gli2 activators. Hh binding to Ptch1 inactivates its repression of Smo, which then sends an intracellular signal modulated by multiple components. This leads to the regulation of the zinc finger Gli proteins. See reviews by Ingham and McMahon [53], Ho and Scott [62], Ruiz i Altaba et al. [29] and Mullor et al. [54] for details. T bars show inhibitory interactions. Arrows show positive interactions. Cyclopamine inhibits signaling by acting on Smo. See text.

Ref. [29]). As for HH signaling in general, it was first implicated in tumor formation when *PTCH1* was identified as the gene mutated in the familial Gorlin's or Basal Cell Nevus syndrome [74,75].

Perhaps the most significant findings on the Hh–Gli pathway relating to brain tumors are (i) that it is required for the maintenance and viability of a variety of human tumors, including some gliomas and medulloblastomas [72,76], and that in model systems, (ii) it is involved in tumor initiation [21,72,76], and (iii) it normally acts on neural precursors (Fig. 2; [21,72,78–80]) and on cells with neural stem cell properties [81,82].

These findings in brain tumors (reviewed in Refs. [27,29]), appear to parallel those of this pathway in other tumors, such as basal cell carcinomas of the skin in humans and animal models ([71,73–75,83–91]; reviewed in Refs. [29,92,93]), and possibly small cell lung cancers [94]. These and other studies (e.g. [95,96]) also highlight the expression of *Gli1* as a good universal marker of a cell's response to Hh signaling [95,96]: *GLI1* is consistently expressed in BCCs and other SHH–GLI pathway-related tumors [71,72,94,97–100].

In the CNS, *Gli1* is expressed in germinal populations (Fig. 2), such as the ventricular/subventricular zones in the developing neocortex, the external germinal layer of the perinatal cerebellum and in the svz of the lateral ventricle and dentate gyrus of the hippocampus in adults (reviewed in Ref. [29]). And it is here where Hh signaling, mostly through Sonic hedgehog (Shh), is active [72,78–82]. Indeed, Shh signaling affects *Gli1*$^+$ neural precursors in

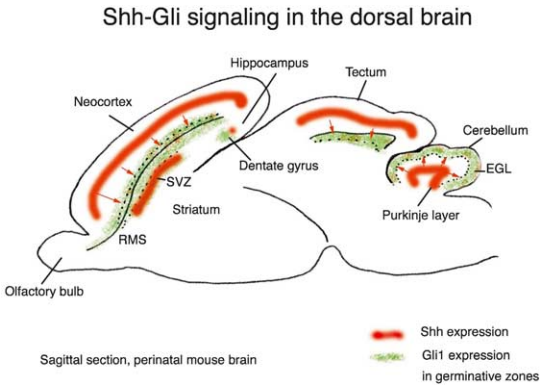

Fig. 2. Schematic representation of the dorsal sites of *Shh* (red) and *Gli1* (green) expression in a sagittal section of a perinatal mouse brain. Red arrows show proposed induction of *Gli1* expression in germinative zones by Shh. Note that these may express *Gli1* as seen in neurospheres and in the SVZ of the lateral ventricle [82], and in the early EGL [78]. Note that *Gli1* and *Shh* are coexpressed (green with red splotches) in the SVZ and in the cortex [82], and that early EGL cells transiently express *Shh* [78]. This diagram is modified from Ref. [29]. EGL, external germinal layer; RMS, rostral migratory stream; SVZ, subventricular zone of the lateral ventricle.

germinative zones that include stem cells [72,82], and most germinative zones in the perinatal and adult brain express *Gli1* (e.g. [72]). This includes the subventricular zone of the lateral ventricle in adults, which has been proposed to be a source of adult brain tumors [101].

Experimental manipulation of Gli signaling shows that overexpression of Gli1 in the CNS of tadpoles leads to tumor formation [72], using the word tumor here to describe at least abnormal hyperplasia but without necessarily invoking neoplasia or a transformed state. It is not yet known if Gli1 is sufficient to induce brain tumors in the mouse. However, Gli1 induces epidermal tumors in the tadpole skin that have a molecular similarity to basal cell carcinomas [71] and misexpression of Gli1 or Gli2 in mouse skin leads to basal cell carcinomas development [73,89]. Interestingly, the formation of tumors in the tadpole brain through the injection of human *GLI1* RNA (and thus protein) is dependent on the activation of the endogenous pathway as an anti-sense oligonucleotide specific for the endogenous frog *Gli1* RNA co-injected with the human *GLI1* RNA completely suppresses tumor formation [72]. This result suggests that a positive feed-back loop is created and required for tumor progression, and suggests that a somatic epigenetic event results in a expression change that can drive tumorigenesis. It also leads to the idea that the HH–GLI signaling pathway may not only be active in brain (and other) tumors, as *GLI1* is specifically and consistently expressed in these tumors (Fig. 3; [72]), but also be involved in their viability. To test this possibility with human brain tumor cell lines and primary cultures we used cyclopamine, a plant-derived drug that selectively inhibits the Hh–Gli pathway by suppressing the activity of Smo (Fig. 1; [102–104]). We found that brain tumor growth is inhibited by cyclopamine providing the basis for a rational treatment of many brain tumors [72]. Subsequently, Berman et al. [76] elegantly showed that cyclopamine inhibits the growth of medulloblastoma mouse allografts. Together, these studies provide a sound basis for a therapeutic approach and open a new way of viewing brain tumorigenesis. Indeed, we proposed that blockade of HH–GLI signaling may inhibit the growth of many different types of brain tumors as these may derive from *GLI1*+ germinative zones

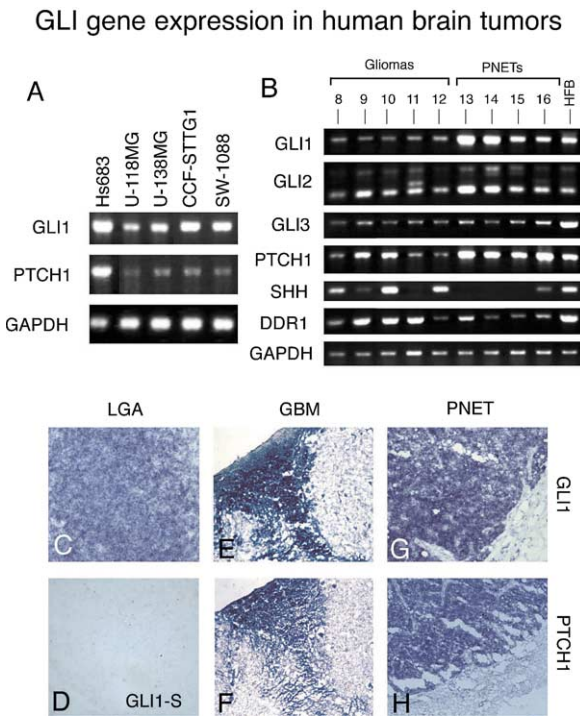

Fig. 3. Expression of *GLI* genes in brain tumor cell lines (A) and primary brain tumors (B) by RT-PCR and by in situ hybridization of *GLI1* in a low grade astrocytoma (LGA, C), also showing a sense control (D), a glioblastoma multiforme (GBM, E) and a primitive neuroectodermal tumor (PNET, G). *PTCH1* expression is also detected overlapping that of *GLI1* in a GBM (F) and a PNET (H). The PNET is a medulloblastoma from the cerebellum. *DDR1*: *Discoidin Domain Receptor 1*; Expression of the housekeeping gene *GAPDH* serve as internal control. See Ref. [72] for further details. HBF, human fetal brain RNA.

where SHH signaling is important to maintain a proliferative state [72].

These results fit with the previous demonstration that germline loss of Ptc1 function—a negative regulator of Hh signaling—lead to medulloblastoma development in the cerebellum of mutant mice [21,27]. Loss of Ptc1 leads to the activation of the pathway and thus to Gli function and *Gli1* expression. Why such mice do not develop other HH–GLI-related tumors remains unknown. For example, they also do not develop BCCs (unless irradiated, [89]; a treatment that also increases the incidence of medulloblastomas [105]), suggesting that modifiers in different tissues may affect the phenotype. Similarly, patients with Gorlin's syndrome in which one copy of *PTCH1* is mutated, show a higher than normal incidence of BCCs

and medulloblastomas and other tumors [106,107], but apparently not gliomas, even though the growth of at least some human gliomas is inhibited by cyclopamine treatment [72]. Thus, augmented or sustained activation of the HH–GLI pathway may thus be the critical initiation event in many human brain tumors (and in tumors from other organs with *GLI1* $^{+}$ germinative zones).

The cause of pathway activation may often be mutation of PTCH1 [106–111] (as in BCCs (reviewed in Refs. [27,29])), SMOH ([109]; see also Ref. [87] for BCCs) or even other components of the pathway, such as Suppressor of Fused [112]. In the case of PTCH1, however, it appears that loss of heterozygosity [113] is not required for pathway activation [114]. Although the role of PTCH2 is unclear, it may also be mutated in different tumors [115,116]. Indeed, it is predicted that many other mutations, or epigenetic changes (discussed in Ref. [29]), could directly or indirectly affect GLI function, and thus apparent pathway activation. This is why looking at *GLI1* expression bypasses the need to sequence all the different pathway components, many of which are still unknown or not identified in humans.

GLI1 expression is a reliable marker of cells responding to HH signaling and increased levels of *GLI1* may be correlated with tumor grade [98]. This does not appear to be the case with the levels of *SMOH* [117] or *PTCH1*, which is also upregulated by Shh signaling [118] and in tumors [119]. However, Gli1 is not required for mouse development, being redundant with Gli2/3 [120], or for medulloblastoma formation in mice [77], but it remains uncertain if this is so in humans. Mutations in GLI1, GLI2 or GLI3 have not been found associated with any human tumors so far (e.g. [121]), but there are other cases of transcription factors associated with tumorigenesis for which mutations have also not been found (reviewed in Ref. [122]).

It is also not clear if pathway activation per se is sufficient for tumor initiation. The described experiments in model systems argue the case, but the incidence of tumors in *Ptch* $+/-$ mice dramatically increases to 100% when p53 is mutated [22], and at least for human BCCs, mutations in both genes have been detected [123–125]. Is it that the same cell population can initiate tumorigenesis better in *Ptc1* $+/-$; *p53* $-/-$ mice as compared to *Ptc1* $+/-$ mice? Or is it that additional cells are now recruited to the pool of tumor-forming cells in the doubly mutant mice? This is unclear, but the most likely candidate to give rise to a medulloblastoma in these mice are cells that normally respond to Shh signaling, that is, the granule neuron precursors in the EGL. These cells express *Gli1* and their response to Shh is tightly regulated as they follow a precise program of proliferation in the cerebellar cortex, followed by differentiation and inward migration. Tumor initiation in this case may thus be seen as a result of inappropriate maintenance of the response to SHH signals. This, together with the possibility that *Gli1* $^{+}$ neural precursors have stem cell properties leads us to the question of stem cells and cancer.

It is interesting to note that *PTEN* [126–129] and *N-MYC* (e.g. [7,130–132]), genes associated with brain tumors, also normally function in the regulation of neural progenitor proliferation [133–136], suggesting the possibility that the different pathways involved in brain tumorigenesis, for example, may converge in a yet unidentified manner on the regulation of neural stem cell properties. In fact, *N-Myc* is a target of Shh signaling [7,136] and *c-Myc* is highly expressed in medulloblastomas [137,138]. Similarly, medulloblastoma formation by loss of p53 and PARP1 leads to the downregulation of *Ptc1* and the upregulation of *Gli1* [20], and BCC formation in *Notch* $^{-/-}$ mice leads to the upregulation of *Gli2* [139].

Additional targets of SHH–GLI function in tumorigenesis include *Cyclins D* and *E* [140], *FOXM1* [141], *Wnts* [99,142] and *Igf2* [143]. Of these, IGF2 function is indispensable for tumor formation in *Ptch1* heterozygous mice [143], but all may be required to set in motion the paradevelopmental program that leads to the formation of a recognizable tumor. For example, Wnts may orchestrate tumor morphogenesis, yielding the recognizable forms of different tumors, and cyclins may drive enhanced cell cycle progression and thus contribute to the hyperproliferative state characteristic of tumors. Erratic tumor types may be the result of misregulation of such programs, or even the occurrence of multiple programs (each involving multiple signaling pathways) at once.

The finding that a number of human brain tumors require an active HH signaling pathway for growth, as

indicated by the cyclopamine experiments [72,76], suggests that it may be possible to attack different kinds of brain tumors, deriving from different cell populations at different developmental stages, with the same agent. Perhaps then many paradevelopmental programs used by brain tumors are build on the bases of HH–GLI signaling, inducing tumors in stem cells or in cells with stem cell properties. If this is so, our goal should be to find out how many and what kinds of tumors are sensitive to HH pathway blockade, whether inhibition of GLI function (as the last elements of the pathway) is most efficient, which GLI proteins are required, and what may the side effects be. The consequences derived from inhibiting Hh signaling, such as possible problems with hair follicle growth or the replenishment of the gastric mucosa, which require active Hh signaling (e.g. [144,145]), may be tolerable side effects in the face of the usual brain tumor prognosis. Moreover, pregnant females of various species treated with cyclopamine and related active compounds, and which gave birth to cyclopic or cebocephalic animals—thus demonstrating that they had a systemic Hh pathway blockade, did not appear to suffer severe symptoms themselves (e.g. [146,147]).

But, after all, why would the HH–GLI pathway be so critical for brain tumors and tumors from other systems (e.g. [145,148])? Since brain tumors come in so many different types, could there be a common Achilles' heel in their provenance from *Gli1*$^{+}$ germinative zones? Why would developing animals depend so heavily in a little diversified pathway? Compared to the multiple FGFs, TGFbetas or Wnts and their receptors, for example, the Hh pathway, with three *Hhs* expressed in mostly non-overlapping manners, two *Ptc* genes and a single *Smo*, seems non-diversified (and thus prone to catastrophic events). Perhaps then we could ask what is the selective advantage on non-diversification? One possible answer is that this pathway is a general and critical sensor of the cell's well-being. By that we mean that it senses the cell's neighbors (through intercellular Hh signals), its community (through the levels of Hhs acting as morphogens (reviewed in Ref. [59])), possibly the cell's metabolic comfort (through the levels of cholesterol required for Hh processing and for signal reception (reviewed in Ref. [53])), possibly the state of the cell's cytoskeleton and thus shape or form possibly through the microtubule-associated Costal2 protein [149], the integration of other signaling inputs, through the participation of PKA, CK1 and GSK3, in Gli regulation (reviewed in Ref. [150]), and the regulation of Glis by other inputs (e.g. [151]). The Hh–Gli pathway may thus be a unique and pivotal sensor of a cell's wellness, operating for instance in cases of proliferation as well as in cases of survival. Perhaps it is this major function that has to be perverted and maintained to allow tumors to initiate and grow, through the misperception of a cell's own well-being and environment, that is, through erroneous patterning, leading to growth of recognizable tumors following paradevelopmental programs.

Finally, as the last elements and mediators of Hh signals, the zinc-finger Gli transcription factors are prime targets for disease control. Rational anti-cancer therapies should target Gli activity (discussed in Ref. [29]), other transcription factors acting downstream of signaling pathways involved in tumorigenesis (reviewed in Ref. [122]), and not only upstream membrane elements such as Smo [91,152,153]. The ability of the PKA, GSK3 and CK1 kinases to phosphorylate Glis, thereby inducing their proteolysis and silencing the pathway [154,155] suggests possible initial targets. Whether inhibitors can be found that are safe and able to kill tumor cells in patients through the inactivation of Gli function, and have only temporary side effects, remains an open challenge, but one that we hope has a better fate than that which befell Troy.

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