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Abstract

Canonical Hedgehog (HH) signaling leads to the regulation of the GLI code: the sum of all positive and negative functions of all GLI proteins. In humans, the three GLI factors encode context-dependent activities with GLI1 being mostly an activator and GLI3 often a repressor. Modulation of GLI occurs at multiple levels, including by co-factors and by direct modification of GLI structure. The GLI code, thus the GLI code, is also regulated by multiple inputs beyond HH signaling. In normal development and homeostasis these include a multitude of signaling pathways that regulate proto-oncogenes, which boost positive GLI function, as well as tumor suppressors, which restrict GLI activity. In cancer, the acquisition of oncogenic mutations and the loss of tumor suppressors – the oncogenic load – regulates the GLI code toward progressively more activating states. The fine and reversible balance of GLI activating GLI\(^A\) and GLI repressing GLI\(^R\) states is lost in cancer. Here, the acquisition of GLI\(^A\) levels above a given threshold is predicted to lead to advanced malignant stages. In this review we highlight the concepts of the GLI code, the oncogenic load, the context-dependence, and different modes of signaling integration such as that of HH and EGF. Targeting GLI directly or indirectly promises therapeutic benefits beyond the direct blockade of individual GLI proteins.

Keywords: GLI transcription factors, Hedgehog-GLI signaling, Cancer, Development, Signaling transduction, Signaling integration, Oncogenes, Stem cells
1. Introduction

The molecular dissection of the Hedgehog (Hh)-Gli signal transduction pathway in insects (e.g. [8–16]), has revealed it to be complex and context-dependent with a surprising number of distinct cellular outputs.

Complexity is found at every level of signaling, from multiple ligands with apparently different and perhaps different properties, multiple membrane components (e.g., PTCH1 vs. PTCH2), intracellular regulators and the existence of three GLI proteins in humans that mediate final responses, to ligand-driven pathway activation. Complexity is also found in the tissue-specific expression of different modulators and in the multiple variations of the canonical pathway found in different species. We are just beginning to understand the meaning of species-specific differences in Hh signaling, but what is clear is that a single-species (e.g., mouse)-centric view is not universally informative. How or why organisms would have evolved multiple Ptc receptors (as in worms), increase the number of Hh ligands or of Gli proteins (as in zebrafish), or constraint HH signaling to primary clia ir and tissues is unclear but likely to have important clues to speciation and the evolution of the morphogenetic plan (reviewed in [15]).

The outputs are numerous since the HH pathway controls aspects of cell proliferation, survival and stemness. How these are orchestrated over time in developing tissues remains unclear. Proteins also regulate and are regulated by tumor suppressors, such as p53 and this reveals another important aspect of HH-GLI signaling: its major role in human cancer (reviewed in [16]).

But perhaps the most intriguing aspect of this and other pathways is their context-dependency. How is it that the same extracellular input can be interpreted differently by responding cells? How is it that reception of a HH ligand can lead to diverse responses in time, space and in different cell types? The complexity of the pathway makes a complete discussion for a review chapter not feasible, on the GLI zinc finger transcription factors, which represent the terminal station of the can signaling path. Whereas other reviews and papers address key aspects of the morphogenetic HH ligands (e.g. [17–20]) we elect to focus this review on 3 key points of the highly context-dependent nature of the HH-GLI pathway, where the history and the molecular make-up of the receiving cell determines the qualitative and quantitative output and biological effect: 1 – The GLI code; of the GLI code by non-HH signals and by the oncogenic load; and 3 – Mechanisms of GLI regulation. In choosing to do so, here we wish to emphasize the fact that the GLI transcription factors act as key determinants in the interpretation of context- and concentration-dependent canonical HH-C development and disease, and that the GLI code is a signaling integration node.

2. The GLI code

The GLI code model [21,22] considers the total GLI function as a balance of positive activative negative repressive (GLI\(^A\)) activities with GLI1 being mostly a positive transcription factor and GLI3 mostly a transcriptional repressor. The GLI\(^A\):GLI\(^R\) ratio is thus critical, being highly regulated and context-specific, and highly dynamic (Fig. 1).

GLI proteins belong to the superfamily of zinc finger transcription factors with five sequen...
of the C2H2 type constituting the sequence specific DNA binding domain. GLI1 (originally identified as an amplified gene in a human glioblastoma cell line [23,24]. Later on and indirectly what turned out to be its fly homolog, Cubitus interruptus (Ci) was identified and placed in the Hh pathway [7,25–27]. GLI1 was not linked to the vertebrate Hh pathway until later [12,28]. The Drosophila genome encodes only one GLI protein, the mouse and human genomes comprise GLI2 and GLI3.

One of the most remarkable features of GLI proteins is that in canonical Hh signaling they can act as both transcriptional activators and repressors [29–33]. The situation is likely to be complex as all GLI proteins can act as activators or repressors in a stage-dependent and target gene-dependent manner [34]. The basic idea of the GLI code is useful as a framework and generally considers GLI1 as an activator and GLI3 mostly as a repressor.

In the absence of Hh pathway activity positive GLI function is off, GLI1 is not transcribed, GLI code is tipped toward a GLIR output, thus leading to pathway silencing. In this context GLI proteins are proteolytically processed into C-terminally truncated repressors consisting of a repressor domain and the DNA binding zinc fingers, but lacking the C-terminal transactivation domain. There is also evidence for GLI1 isoforms but how these are produced is not clear [35].

GLI processing in the absence of Hh signaling is triggered by sequential phosphorylation of Ci by Protein Kinase A (PKA), Glycogen Synthase Kinase 3-beta (GSK3β) and Casein Kinase 1 (CK1) followed by proteasomal degradation of the C-terminal region [31,33]. Truncated Ci/GLI repressor binds to GLI sites in Hh target promoters, thereby shutting off target gene expression (e.g. [37–39]).

Activation of canonical Hh signaling abrogates GLI processing allowing full-length and active GLI (GLIA) to enter the nucleus and turn on target gene expression. Hh-GLI signaling also has feed-forward and feedback loops. In the latter case, GLI1 directly regulates PATCHED1 (PTCH1), a genetically a SMOOTHENED (SMOH) inhibitor, but it also autoregulates itself. GLI2/3A activity leads to GLI1 transcription, which further positively boosts GLI1 expression. How this apparently close loop is broken is unclear, in order to allow precise and reversible control of the GLI code, which is of utmost proper development and health. It is also unclear how the GLI proteins act since there is evidence that the GLI code will be highly refined and meticulously regulated given that GLI1, GLI2 and GLI3 can act in a combinatorial manner [30,34,41–43].

The importance of the critical and tight regulation of the GLI code is illustrated on the one that varying levels of Hh-GLI will induce different numbers of neural stem cells in normal and homeostasis [35,44–48], and also induce different cell fates in the ventral neural tube in morphogenetic gradient of Hh ligands [8,9,11,49–51]. On the other hand, genetic and/or epigenetic changes leading to irreversible activation of GLI1A, and GLI1 [52], can drive a variety of malignant states ranging from cancers of the brain, skin, breast, prostate and digestive tract to malignancies of the hematopoietic system (e.g. [16,52–60]).

3. Regulation of the GLI code by non-HH signals and by the oncogenic load

The GLI code may be seen as the essential parameter to regulate canonical Hh output. Its...
appeared to be strictly dependent on the presence of specific levels of HH ligands. Indeed, transcription is so far the only general biomarker of a cell's response to HH ligands [12], it diagnostic tool for HH pathway activity [52] and is used to measure the efficiency of SMO clinical samples [61–63].

However, surprising data revealed that the GLI1 code and activity can also be modulated by non-HH signals [64,65]. Such regulation occurs in normal and in disease contexts and here we high examples (Fig. 2).

3.1. Tumor suppressors negatively regulate GLI1 activity in normal development and ho

The first example of tumor suppressors regulating normal GLI activity came from the work p53 negatively regulates GLI1 [35]. Interestingly, GLI1 also regulates p53 [35,66], thus creating a regulatory loop in which the GLI code is subjected to the precise regulation by p53. Modulation of p53 by GLI1 takes place through MDM factors [35,66] and it remains unclear how p53 represses GLI1 although it involves okadaic acid-sensitive protein phosphatases, possibly PP2A [35].

3.2. Loss of tumor suppressors leads to unregulated GLI1 activity.

Loss of p53 is a common occurrence in human tumors and this provokes the unregulated up-regulation of GLI1, thus leading to increased tumor cell proliferation and increased self-renewal of cancer [35]. Similarly, PTEN negatively regulates GLI1 activity in different human tumors that in melanomas [65]. This activity may flow through the action of AKT, which positively regulates GLI1 (see below) and is itself negatively modulated by PTEN [65,67], a repressor of AKT (see below). Many other tumor suppressors have since been found to regulate GLI. For example, loss of the SNF5 or Menin leads to activation of GLI1 [68,69].

3.3. Oncogenes, and the pathways that normally regulate proto-oncogenes, positively n

Not only do common tumor suppressors repress GLI1 but common oncogenes, including FGF, MYC and AKT, positively regulate GLI1 in different tumor types [65,70]. Moreover, regul [71,72] and possibly of AKT [73] by GLI1, may establish positive feed-forward loops. To the idea that it is the stepwise gain of oncogenic events and loss of tumor suppressors – nag levels of GLI1 [64]. These increases then drive GLI1 beyond thresholds that induce chang and behavior, such as the acquisition of metastatic behavior [64,70,71,74].

Note that one key contribution to the oncogenic load in a number of cancers, such as basal is the oncogenic mutation of the HH-GLI pathway itself, often through loss of PTCH1 in familial tumors [75,76], or loss of PTCH1, gain of SMOH activity or increase of SHH levels in sporadic cancer [52,53,56,65,70,77–86].

Interestingly, initial evidence for non-HH signaling regulating the GLI code came from studies with frog embryos where GLI2 was found to act in the FGF-Brachyury loop in the early mesoderm [87]. In a separate study, the growth of mouse brain neurospheres was found to be dependent on both HH (SHH) signaling but only after decreasing their levels [46,47]. This synergism between...
together with the regulation of GLI2 by FGF [87], and the regulation of GLI1 by RAS-MEK-AKT opened a new chapter on the regulation of the GLI code, in this case by non-HH signals. These studies predicted the modulation of the GLI code and of GLI1 by peptide growth factors, such as FGF, EGF, and many other ligands that trigger receptor tyrosine kinases and activate RAS and downstream events. These findings can therefore help to explain why GLI1 and GLI2, in particular, as the final positive feed-forward output, is important in human cancer. The GLI code, and GLI1 in particular, act at the tip of a funnel to integrate multiple outputs. Such a funnel idea [53] has strong implications for understanding the logic of signaling but also places the GLI code, a particular, in the line of fire for the development of novel therapies against cancer.

Additional work has shown that oncogenic RAS can regulate GLI1 in the apparent absence of Smo in pancreatic cancer in mice [88] being required for RAS-mediated tumorigenesis [89], and that signaling cannot only synergize with HH-GLI outputs but also modify its outputs [90–93]. Proto-oncogenic and oncogenic inputs have since been shown to regulate the GLI code in contexts, such as for instance the EWS/FLI1 fusion oncoprotein [94,95], TGFβ signaling [96,97], mTOR/S6K1 axis [98], WNT signaling [99] (although WNT genes can also be targets and mediators of GLI function [100,101]) and WIP1 [102].

Finally, interactions between pathways may be balanced by direct transcription factor binding between GLI repressors and SMAD proteins, the latter being the mediators of normal and oncogenic BMP and TGFβ signaling [103,104].

3.4. HH and EGF in human basal cell carcinoma

The integration of HH and EGF signaling [46,47,92] has been intensely studied given its developmental interest and its high therapeutic relevance. Here we describe HH-GLI and EGF crosstalk as an example of how a cell can integrate apparently parallel signal inputs.

HH and EGF signaling synergistically promote oncogenic transformation and integration of signals occur at different levels. SHH can transactivate the EGFR receptor (EGFR) [105]. In addition, EGFR activates the RAS-MEK cascade and this can superactivate GLI1 [65]. Moreover, both pathways can converge on the level of common target gene promoters resulting in selective and synergistic modulation of gene expression (reviewed in [59,106]).

Global gene expression studies of human keratinocytes with combined or single activation of EGFR revealed three classes of target gene responses: (i) genes responding to HH-GLI only, activated by EGFR only and (iii) genes only or at least preferentially respond to simultaneous activation of both pathways [92]. Notably, class III genes, also referred to as target genes or cooperation response genes, contain functional GLI binding sites in their promoters suggesting that signal integration occurs at the level of HH-EGFR target gene promoters [5].

In this context, cooperation of EGFR with GLI1 and GLI2 depends on activation of MEK/PI3K/AKT function is dispensable downstream of EGFR. MEK/ERK induced phosphorylation and activation of the JUN/AP1 transcription factor is the critical event at the terminal end of the
cascade, inducing binding of activated JUN and GLI to common HH-EGFR target promoters, cooperatively regulating target gene expression and transformation [92,93]. It is noteworthy that basically all receptor tyrosine (RTK) pathways (e.g., HGF, VEGF or FGF) activate MEK/ERK context-dependent as not all of them synergize with HH-GLI in human keratinocytes, possibly because they fail to activate JUN/AP1 in these cells [90]. So far, only EGFR and PDGFRα [107] have been identified as being able to stimulate both MEK/ERK and JUN/AP1 and synergize with HH-GLI in basal cell carcinoma (BCC) (Fig. 4). Importantly, the beneficial effect of EGFR blockade in BCC and pancreatic cancer models can be synergistically improved by combined targeting pathways [90,93,108].

3.5. HH-GLI and WNT-TCF in human colon cancer

A second example involves the interaction between HH and WNT signaling in human colon cancer. In this context, enhanced GLI1 represses WNT-TCF targets and repression of WNT-TCF targets by dominant-negative dnTCF leads to enhanced HH-GLI targets [71]. This mutually inhibitory interaction is distinct from that seen in other contexts between these two pathways (e.g., [100,109]) and is context-dependent on the metastatic transition of human colon cancers. Patients with metastases, but not without, harbor local intestinal tumors that display repressed WNT-TCF and enhanced HH as assessed by target gene signatures [71]. This switch, from high WNT-TCF, which drives tumorigenesis (e.g., [110]), to low WNT-TCF and enhanced HH-GLI in advanced and metastatic tumors was totally unexpected and is critical as experimental repression of WNT-TCF or enhancer in xenografts leads to increased metastases in mice [70,71]. Blocking WNT-TCF in advanced cancers is thus not recommended.

The interaction between the HH-GLI and WNT-TCF pathways is complex and stage-dependent: TCF activity, essential for βCATENIN activation of WNT-TCF targets, is required for intestinal tumorigenesis (e.g., [111]). However, while it is also required for advanced human colon cancer in vitro [110] it is not required in vivo [71]. Moreover, HH-GLI is dominant: enhanced GLI1 suppression of PTCH1, rescue the deleterious effects of TCF blockade by dnTCF [71]. This interaction is a functional cross-pathway switch at the metastatic transition. WNT-TCF may keep the cancer in a crypt-like state and enhanced HH-GLI together with repressed WNT-TCF may allow tumors to change fate and become metastatic [71].

Modeling such interactions in mice has revealed that Hh-Gli signaling is a parallel requirement for intestinal tumor initiation can be initiated by loss of Apc but it is fully rescued by concomitant in the intestine [112,113].

Understanding how WNT-TCF and HH-GLI inputs are integrated is of great importance for the essential functions of both pathways in stem cells, human disease and development. Such parallel signaling inputs can take place at multiple levels. In the case of WNT-TCF signaling evidence for binding of βCATENIN, the final output of canonical WNT pathway and both C′-terminally deleted repressors and GLI1 [71,114]. Whether this interaction is the key mode of integration remains to be determined.

4. Mechanisms of GLI regulation
4.1. Context-dependent regulation of GLI activity by modulation of DNA binding

GLI proteins regulate target gene promoters by binding the consensus sequence GACCACCA. The two cytosines flanking the central adenine in the consensus sequence are essential for GLI binding, while the other positions allow a certain degree of variation (Fig. 5A) [117,118]. Sequence-specific DNA binding to the cis-regulatory region of a GLI target gene mainly involves zinc fingers 4 and 5 which establish extensive base contacts within the 9-mer binding sequence, while fingers 2–3 mainly make contacts with the phosphate backbone. Extensive protein–protein contacts between fingers apparently contribute to the overall stability of the DNA binding domain [119] (Fig. 5B). Fingers 1 and 2 also provide protein–protein interaction sites to form GLI2, GLI3 and ZIC2 complexes [34].

Although global chromatin immunoprecipitation analyses and in vitro GLI-DNA binding studies confirmed the consensus sequence as dominant binding site for GLIs [38,39,115,116,120], of GLI binding sequences with 1–2 base pair substitutions is underappreciated and therefore neglected or overseen in many studies. Variations of the consensus sequence while preserving protein–protein interaction affinity and hence may have a significant impact on transcriptional output in response to defined GLI activator levels [117,118,121]. For substitution of the consensus cytosine at position 7 for adenine results in a GLI binding site with even enhanced transcriptional response compared to the consensus motif [117].

Variants of the consensus GLI binding site contribute also to selective target gene activation. Although all GLI proteins bind the 9-mer consensus sequence with comparable affinity, repressor and activator forms bind the same sites [37], and different GLI proteins affect the genes differently [34,41]. For example, GLI2 induces expression of the direct GLI target BCL2 significantly more strongly than GLI1 and systematic analysis of the BCL2 promoter reveals that one of the three validated GLI binding sites accounts for the preferential response to GLI2 [122].

In line with the documented morphogenetic activity of HH-GLI signaling e.g., in the neura in [123,124] variations in binding site affinity are likely to play a major role in the interpretation of threshold GLI activity levels above which a gene is transcribed or below which the very same gene remains silent. Accordingly, high affinity GLI binding sites in the cis-regulatory region of GLI targets ensure expression at both high and low levels of GLI activator activity, while targets with low affinity binding sites will respond to high GLI activity only, as demonstrated for the response of ne ene patterning genes controlled by GLI [118]. High affinity binding may be generated by GLI sequence variants and/or multiple repeats of the binding motif. This also suggests that not absolute GLI protein level or activity determine the context-dependent responses to HH-GLI [50,51,125] but also differential epigenetic modifications of the cis-regulatory regions of GLI targets affecting GLI-DNA binding affinity. Cell-type specific histone acetylations and/or CpG methylation patterns of GLI target gene promoters are thus likely both the qualitative and quantitative response to GLI [118], an area in the HH-GLI field that has not yet been explored in great detail.

Distinct combinatorial GLI function could also account for the substantial difference and cell dependency of GLI1 regulated gene networks in the early embryo [30,34,41], as well as in developing cerebellum and in medulloblastoma [120]. A genome-wide survey of GLI1 binding...
revealed numerous GLI1 binding sites in both the normal and malignant tissues, though the expression pattern diverged significantly between normal and malignant cells [120].

Although global ChIP approaches successfully and reliably identified classical HH-targets novel targets, it should be noted that these studies were performed with epitope tagged and GLI [38,39,118,120], which may fully mimic endogenous GLI function. It is therefore possible that future approaches will refine our current understanding of context-dependent target gene regulation, once reliable and high-quality antibodies suitable for the isolation of rare endogenous GLI proteins become available.

4.2. Context-specificity of the GLI code by interactions with co-factors

Specificity and activity of transcription factors (TF) heavily depend on interactions with activating or repressing co-factors as well as on co-occurrence of other TF that can bind and/or act cooperatively to regulate target gene expression (Fig. 5C). It follows that the absence or presence of GLI co-operative transcription factors within a given cellular context is a major determinant of transcriptional output.

An example of such an interplay with cofactors that regulates the GLI code is the functional interaction between Zic and Gli proteins [126] (Fig. 3). The Zic factors are nuclear proteins with a GL finger domain [127] that can recognize GLI binding sites albeit with different affinities [12] and can modify GLI outputs [126,128] and can interact through the first two zinc fingers [34,129]. Neural plate of frog embryos, Zic2 is expressed in specific longitudinal bands that are adjacent to zones of primary neurogenesis, which is triggered by GLI proteins expressed throughout the plate. It leads to the repression of Gli proneurogenic function by Zic2 in restricted domains, thus defining domains of neurogenic differentiation [126]. In this context, Zic2 mimics C-terminally truncated Gli repressors [126]. In a different context Zic2 may mimic positive GLI function required for ventral forebrain fates: Loss of ZIC2 is associated with human holoprosencephaly paralleling the association of this malformation with loss of SHH [131] or GLI2 [132].

A second case that exemplifies a different form of interaction is the cross-functional network and NANOG (made from NANOG1 and NANOGP8 in human cancer cells) (Fig. 3). As discussed above p53 negatively regulates GLI1 [35] and GLI1 negatively regulates p53 [35,66]. p53 would be active in most cells. However, a further layer of regulation is provided by the homeodomain factor NANOG, which forms a positive feed-forward loop with GLI1 [133]. Interestingly, p53 is regulated negatively by p53, establishing a highly dynamic node that will be affected by an affect GLI1, NANOG and/or p53 [133] (Fig. 3). Regulatory mechanisms involve regulation of GLI1, protein phosphatase action, direct GLI regulation of NANOG1 expression of microRNAs [35,66,134,135]. Thus, in adult cells expressing NANOG, likely stem cells cells, the GLI code will be modulated by additional positive mechanisms. As p53 is often lost in cancer, this is predicted to free the GLI1-NANOG loop from negative regulation, allowing unrestricted activity of GLI1. The essential role of NANOG and HH-GLI is demonstrated by their regulation of gliomaspheres and by their absolute requirement for the growth of primary human glioblastomas orthotopically engrafted in the brains of host mice [133].
Additional mechanisms of GLI code regulation include interactions with CREB-binding protein (CBP). Genetic and functional studies first carried out in the fruit fly and later in mammalian cells identified CBP as an essential co-factor for Ci and GLI3-mediated target gene activation [136]. Haploinsufficiency of CBP is associated with Rubinstein-Taybi syndrome, a genetic disorder characterized by severe developmental anomalies with partially striking similarities to defects observed in patients with Greig's cephalopolysyndactyly syndrome, which is caused by mutations in the GLI3 gene [136–139].

Given the intrinsic histone acetyl transferase (HAT) of CBP/p300 [140], CBP-GLI interactions are likely to cause epigenetic changes of the cis-regulatory region of GLI targets making them more accessible to other transcriptional regulators. In line with this hypothesis, histone acetyl transferase PCAF interacts with GLI1 and enhances HH-GLI target gene expression in medulloblastoma cells by promoting H3K lysine modifications [141]. However, when functioning as ubiquitin ligase, PCAF can negatively regulate GLI activity under genotoxic stress conditions [142].

Further evidence for epigenetic modifications in context-dependent GLI activity comes from studies of SAP18, a component of the histone deacetylase complex. Recruitment of SAP18 to GLI via negative GLI regulator Suppressor of Fused (SUFU) [143] is crucial for efficient repression of target genes [144,145]. Like SAP18, Atrophin (Atro) has been identified in fish and flies as a GLI/Ci cofactor required for target gene repression via recruitment of histone deacetylases [146].

TBP-associated factor 9 (TAF9) encodes a transcriptional co-activator that directly interact with GLI activator forms GLI1 and GLI2 via their transcriptional activation domain [147]. TAF9 has been shown to enhance the transcriptional activity of GLI, which may play an oncogenic role in lung cancer and chemical inhibition of TAF9-GLI interactions dampen GLI target gene transcription, a possible therapeutic strategy to target oncogenic HH-GLI signaling downstream of the common HH drug SMOH [147].

Furthermore, direct interaction of GLI3 with MED12, a subunit of the RNA Polymerase II Mediator, enhances the transcriptional response to GLI activator by reversing the Mediator repression of HH target genes [148].

Transcriptional activity of GLI proteins can be negatively regulated by binding to cofactor 14-3-3 protein. Notably, PKA phosphorylation at amino acid residues distinct from those responsible for repressor formation promote association of 14-3-3 with GLI2 and GLI3, thereby repressing transcriptional activity independent of the intrinsic N-terminal repressor domain of GLI2 and GLI3. Studies addressing selected GLI target gene promoters together with global approaches analyzing the entire landscape of GLI target gene promoters revealed the importance of combinatorial transcription factor binding in context-dependent HH-GLI target gene regulation (Fig. 5C). For instance, activation of a subgroup of direct GLI target genes such as IL1R2, JUN/AP1, or ARC was observed even when JUN expression was full transcriptional activation of these targets is likely to require co-occupancy of their promoter region by GLI and JUN/AP1, similar to the mechanism accounting for HH and EGF signal integration [90,93,150].

Another example of how combinatorial binding of transcription factors controls context-dependent output is illustrated by the finding that co-occupancy of selected GLI targets by GLI1 and 3 is required for full activation of a neural gene expression signature [118].
Motif enrichment analyses identified E-box sequences as frequently co-occurring with GLI binding sites in medulloblastoma [120]. It is therefore possible, that E-box binding bHLH transcription factors cooperate with GLI in the control of tissue specific target gene expression and cancer development, a model that still needs to be confirmed in future studies.

4.3. Modulation of GLI DNA binding affinity and transcriptional activity by post-translational modifications

Fine-tuning and reversible activation/termination of HH-GLI signaling is critical to proper development and health. As outlined in the introduction of this article, numerous reports have provided evidence showing that precise control of HH-GLI signal strength occurs at nearly every level of the canonical HH signaling cascade, ranging from the control of ligand production and ligand-receptor interactions down to numerous molecular interactions and modifications of GLI proteins eventually determining their molecular phenotype by controlling gene expression in response to pathway activity [20,22,40,54,151].

At the level of GLI code, a number of post-translational modifications of GLI proteins play a fundamental role in its control by affecting GLI stability, subcellular localization and DNA binding ability (reviewed in [20,40]). To remain focused on the topic of context-dependent GLI activity, we concentrate here on GLI modifications that directly affect the intrinsic GLI transcriptional activity.

Post-translational modifications of GLI proteins result in drastic modifications of activity. Phosphorylation and acetylation of GLI1/2 at specific amino acid residues have a major impact on the ability of GLI proteins to regulate target genes by modifying their binding to target promoters [156–158].

Atypical Protein Kinase C/λ (aPKC) has been identified as both a HH-GLI target gene and regulator of GLI activity in basal cell carcinoma. aPKC acts downstream of the essential HH drug target SMOH by phosphorylating GLI1 at amino acid residues located in the zinc finger binding domain. GLI1 phosphorylated by aPKC displays enhanced DNA binding and maximal transcriptional activity. Of note, hyperactivation of aPKC in BCC can account for SMOH resistance, rendering it a promising drug target for the treatment of cancer patients unresponsive to classical HH pathway inhibitors targeting SMOH [156]. aPKC (also referred to as PRKCI) HH-GLI signaling also by phosphorylating the transcription factor SOX2. Phospho-SOX2 acts a potent transcriptional activator of HH acetyltransferase expression, leading to increased HH ligand and cell-autonomous HH-GLI activation in lung squamous cell carcinoma [159].

In addition to aPKC phosphorylation of GLI1, several serine and threonine residues in the N-terminal region of GLI1/2 serve as phosphorylation sites involved in GLI activation. In esophageal cancer cells, activation of mTOR/S6K1 signaling leads to S6K1-mediated phosphorylation of Ser85 in GLI1 transcriptional activity by disrupting its interaction with the negative GLI regulator SUFU. Note, the S6K1 phosphorylation site at Ser85 of GLI1 is located in a D-site motif that serves as a kinase binding site required for phosphorylation and activation of GLI1 by JNK and ERK. S6K1 phosphorylation may therefore not only interfere with SUFU binding but also modify GLI phosphorylation by MAP kinases.

A cluster of non-consensus PKA phosphorylation sites (ncPKA) in close proximity to the
site has also been shown to regulate GLI2/3 activation, though the GLI activating kinase responsible for phosphorylating ncPKA sites has not been identified [161]. Whether phosphorylation of ncPKA activates GLI2/3 by disrupting the SUFU-GLI complex or by a different mode also needs to be addressed in future studies.

A number of distinct phosphorylation events in the N-terminal region of GLI control full GLI activation. This suggests that the N-terminus of GLI proteins serves as integration domain for multiple signals from distinct pathways such as PI3K/AKT, mTOR/S6K or FGF/MEK/ERK signaling [65,98,162]. An integration function of the N-terminal region, deletion of the GLI1 N-terminus abolishes activation of FoxA2 (HNF3β) in the neural tube [30] and its hyperactivation in response to FGF treatment [162]. It follows that this integration domain plays a major role in the fine-tuning of GLI activity in normal tissues and importantly, also in the irreversible activation of GLI in cancer cells.

Besides phosphorylation, acetylation of GLI is another parameter in the complex regulation of GLI transcriptional activity. Acetylation of GLI2 at Lys757 by the histone acetyl transferase p300 is a critical negative regulatory modification in HH signaling [157]. Interestingly, acetylated GLI2 displays significantly reduced recruitment to chromatin and consequently only weak activator potential. The acetylation site is C-terminal of the DNA binding domain, it is unlikely that acetylation directly affects DNA binding affinity. Rather, deacetylation may favor the interaction of GLI with chromatin proteins and therefore enhance its recruitment to target gene enhancers/promoters. Indeed, promotes deacetylation of GLI1/2 via inducing class I histone deacetylases (HDAC), which is identified as an important step in the activation of GLI target gene expression [157,158].

In summary, the remarkable progress in our understanding of GLI modifications highlights context-dependent reversible post-translational modifications as critical determinants of GLI activity. Here, selected kinases (MAPK, S6K1 and aPKC) and deacetylases (HDAC) act as positive regulators, while acetylases (p300), PKA [161] and as yet unidentified phosphatases control the termination of HH signaling via GLI inactivation (Fig. 6). In cancer, a number of these components are deregulated, thus the oncogenic load that regulates the GLI code.

5. Outlook

Whereas great progress has been made to understand how the GLI proteins act (e.g., reviewed [7,21,58,59,64,163]), much remains to be understood. For example, it is not clear what are endogenous concentrations of GLI proteins, how they interact with co-factors, how can they be modified in cells receiving simultaneous inputs, how their activity can be affected by and affect epigenetic changes, how they are protected from cleavage or modification, or even how the pathway is effectively turned off when required.

Documenting the full range of inputs and factors that can modulate their activities in multiple developmental, homeostatic and disease contexts will require much effort but will certainly be worth it. Such knowledge may allow us to begin to understand the logic of signaling in development and hopefully also in evolution. Thus, we promote the idea that these analyses must be carried out on all possible species and cell types in order to compare and contrast mechanisms and outcomes in the quest to extract essential signaling principles as well as specific solutions for each system.
A more anthropocentric goal is to understand how the GLI code is perverted in human disease, specifically in cancer, through pathway corruption and the oncogenic load. Such knowledge lead us to design novel and more efficient therapies against multiple forms of deadly cancer of the brain, intestine, lung, skin, pancreas and other organs. Indeed, the involvement signaling in normal stem cell lineages and in cancer stem cells [54] raises the possibility for molecular approaches to block positive GLI function, reverting the GLI code, could be highly beneficial. For example, the discovery of aPKC, PI3K/AKT, mTOR/S6K or EGF signaling (see above oncogenic load and, importantly, as druggable GLI modulators has already pointed out pos how to design novel combination treatments with improved therapeutic benefit [64,65,90,95,164,165]. However, despite the increasing number of studies of GLI regulation in health and disease, beginning to realize the remarkable complexity of context-dependent regulatory processes GLI code. The identification and in depth analysis of modifiers of the GLI code will guide development of better rational combination treatments by synergistically targeting the core pathway itself, and its modifiers. This will also open therapeutic opportunities to tackle the relapse and drug resistance, as exemplified by the successful targeting of aPKC in SMOH: resistant basal cell carcinomas [156].

We are now entering an era where the GLI transcription factors and their modulators are be center stage as drug targets. Targeting transcription factors for cancer therapy has long been effective, but clearly the number of recent examples such as those mentioned above along the identification of small molecule GLI antagonists [166,167] provide ample proof-of-concept therapeutic relevance of such an approach. Given the essential function of GLIs in normal stem cells, the systematic identification and functional analysis of GLI modulators, particularly those amenable to small molecule targeting, as well as studies addressing their context-dependence, are an area of intense future research with significant impact on several medical areas such as tissue regeneration and wound healing.

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Fig. 1

THE GLI CODE

Model for the GLI code and its morphogenetic activity leading to the creation of context-dependent diversity of HH ligands is interpreted, canonically, by a combinatorial and context-specific distribution of repressor and activator activities of the three GLI proteins, the GLI code. Note that GLI1 and GLI2 have strong activating action and GLI3 is a strong repressor in many contexts. Combinatorial GLI activities are then modified by positive or negative modifiers leading to differential regulation of target genes, which may either respond to create graded levels of expression of specific genes or induce specific genes in given thresholds. The output of these genetic changes is then the creation of spatially and/or temporally distinct outputs and behaviors.
Control of the GLI code by the oncogenic load. (A) Under normal homeostatic conditions a fine-tuned signaling as well as of parallel proto-oncogenic (e.g., EGF, FGF, PDGF, etc.) and tumor-suppressive path precisely controlled levels of GLI^A/GLI^R. The balance can be tipped one way or another, thus allowing controlled ON-OFF switch. For simplicity, feed-forward and feedback regulatory loops are not included the loss of tumor suppressors and the presence of mutant oncogenes lead to the massive deregulation of to a constitutively active ON state (GLI^A). Note that given the stable genetic changes resulting from gene GLI code is no longer under homeostatic control.
Fig. 3
A working framework for the GLI code as a node for signal integration. Multiple signaling inputs from including but not restricted to HH, EGF, FGF, TGFβ, can converge on GLI regulation, changing the GL can also take place above, through crosstalk (gray arrows). The position of the different components is other but shown as examples of the types of components involved in the signaling cascades. The GLI transcriptional regulatory node, is then modulated by additional context-dependent inputs (arrow and T proteins) that include a negative feedback loop with p53 [35] and a positive feed-forward regulatory loop [133]. The outcome, through differential regulation of target genes, is context-dependent and includes cell survival, proliferation migration and metabolic regulation. This framework can help not only to concept behavior resulting from multiple signaling events but also design multi-target therapies to increase effic resistance. Note that each input also has divergent pathways not shown in the scheme.
Modes of HH-EGF signaling integration. (A) Canonical HH-GLI signaling activated by binding of SHH-
PTCH results in ciliary localization of SMOH and subsequent GLI activation (GLI\(^{A}\)). HH-GLI signaling activates classical GLI targets including HHIP and GLI1 but fails to induce HH-EGFR cooperation target Concomitant activation of HH-GLI and EGF/PDGF signaling (EGFR or PDGFR\(A\)) can lead to synergism\([46,47]\). Such interactions can result in (i) cross talk between SHH and EGFR in neural stem cells\([105]\) of GLI1 activity by RAS/MEK signaling in melanomas and other tumor cells\([65]\), and/or (iii) synergistic basal cell carcinoma and pancreatic cancer by selective activation of HH-EGFR target genes such as C\(\beta\) SOX9 and TGFA\([90,92,93]\). In the latter case, integration of HH-EGFR signaling occurs at the level of gene promoters. Activation of EGF signaling induces the RAS/RAF/MEK/ERK cascade eventually leading to GLI1 or/and of the JUN/AP1 transcription factor. JUN synergizes with GLI activator forms by co-occupying target gene promoters leading to synergistic transcriptional activation of HH-EGFR targets and enhance (e.g., BCC and pancreatic cancer).
GLI DNA binding and context-dependent target gene regulation. (A) Consensus 9-mer GLI DNA binding motif calculated from experimentally validated GLI binding sites. The motif was generated with a set of 22 experimentally validated GLI binding sites using WebLogo3 [168]. Positions 4C and 6C are essential for DNA binding.
other positions allow a certain degree of sequence variation resulting in distinct target gene activation efficiencies. (B) 3D model of the GLI DNA binding domain composed of five zinc fingers and its interaction with the consensus sequence. Note that fingers 4 and 5 form extensive base contacts thereby determining binding specificity (Protein Databank ID 2GLI; [119]). (C) Non-exhaustive models of context-dependent target gene activation. Here (GLI\(^A\)) and GLI repressor forms (GLI\(^R\)) binding the same target sequences refer to the GLI code. (i) Classical target gene activation model with GLI\(^A\) binding to the promoters of canonical targets such as \textit{PTCH1} or \textit{HHIP}. (ii) Context-dependent interactions of GLI\(^A\) with co-activators (CoA) or (iii) of GLI\(^R\) with co-repressors (CoR) modifies the GLI code and expression of HH-GLI targets. (iv) Context-dependent combinatorial binding of GLI\(^A\) and cooperating transcription factors (TF) (e.g., JUN, SOX2) to common target promoters can also result in synergistic modulation of
Post-translational modifications regulate GLI transcriptional activity. Fine-tuning of GLI activity by phosphorylation/dephosphorylation and acetylation/deacetylation. Left: fully activated GLI transcription factor with multiple phosphorylated serine/threonine residues in the N-terminal region and the DNA binding domain. De-acetylation promotes DNA binding affinity and transcriptional activity, respectively. Several kinases (MAPK, S6K, aPKC) and deacetylases catalyze the activation of GLI, while phosphatases, PKA and acetyltransferases negatively regulate GLI activity. Note that PKA phosphorylation of the two amino acid residues C-terminal of the DNA binding domain negatively regulates the transcriptional activity of GLI without affecting processing or stability.

Non-consensus PKA phosphorylation sites involved in GLI activation.