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Context-dependent signal integration by the GLI code: The oncogen pathways, modifiers and implications for cancer therapy

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Abstract

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

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

1. Introduction

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

Complexity is found at every level of signaling, from multiple ligands with apparently diff 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 fina responses, to ligand-driven pathway activation. Complexity is also found in the tissue – sp of different modulators and in the multiple variations of the canonical pathway found in di

We are just beginning to understand the meaning of species-specific differences in Hh sign is clear is that a single-species (e.g., mouse)-centric view is not universally informative. H organisms would have evolved multiple Ptc receptors (as in worms) for instance, increase 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 t morphogenetic plan (reviewed in [15]).

The outputs are numerous since the HH pathway controls aspects of cell proliferation, surv 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 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-depende that the same extracellular input can be interpreted differently by responding cells? How is of a HH ligand can lead to diverse responses in time, space and in different cell types? Wh 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 morphogeneti HH ligands (e.g. [17–20]) we elect to focus this review on 3 key points of the highly conte nature of the HH-GLI pathway, where the history and the molecular make-up of the receiv 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 GL choosing to do so, here we wish to emphasize the fact that the GLI transcription factors act 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 activ negative repressive (GLI^R) activities with GLI1 being mostly a positive transcription facto mostly a transcriptional repressor. The GLI^A:GLI^R ratio is thus critical, being highly regul 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 (originall identified as an amplified gene in a human glioblastoma cell line [23,24]. Later on and ind what turned out to be its fly homolog, Cubitus interruptus (Ci) was identified and placed in pathway [7,25–27]. GLI1 was not linked to the vertebrate Hh pathway until later [12,28]. V Drosophila genome encodes only one GLI protein, the mouse and human genomes compri GLI2 and GLI3.

One of the most remarkable features of GLI proteins is that in canonical HH signaling they transcriptional activators and repressors [29–33]. The situation is likely to be complex as a can act as activators or repressors in a stage-dependent and target gene-dependent manner the basic idea of the GLI code is useful as a framework and generally considers GLI1 as ar 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 GLI^R output, thus leading to pathway silencing. In this contex 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 transactiva 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 by Protein Kinase A (PKA), Glycogen Synthase Kinase 3-beta (GSK3β) and Casein Kinas followed by proteasomal degradation of the C-terminal region [31,33]. Truncated Ci/GLI r to GLI sites in HH target promoters, thereby shutting off target gene expression (e.g. [37–3 [16,40])).

Activation of canonical HH signaling abrogates GLI processing allowing full-length and a (GLI^A) to enter the nucleus and turn on target gene expression. HH-GLI signaling also has and feedback loops. In the latter case, GLI1 directly regulates *PATCHED1* (*PTCH1*), genet SMOOTHENED (SMOH) inhibitor, but it also autoregulates itself. $GLI2/3^A$ activity leads expression, which further positively boosts *GLI1* transcription. How his apparently close lo unclear, in order to allow precise and reversible control of the GLI code, which is of utmos proper development and health. It is also unclear how the GLI proteins act since there is ev GLI code will be highly refined and meticulously regulated given that GLI1, GLI2 and GL 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 i morphogenetic gradient of HH ligands [8,9,11,49–51]. On the other hand, genetic and/or e changes leading to irreversible activation of GLI^A, and GLI1 [52], can drive a variety of m ranging from cancers of the brain, skin, breast, prostate and digestive tract to malignancies 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 b 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 worl p53 negatively regulates GLI1 [35]. Interestingly, GLI1 also regulates p53 [35,66], thus cre regulatory loop in which the GLI code is subjected to the precise regulation by p53. Modul GLI1 takes place through MDM factors [35,66] and it remains unclear how p53 represses (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 u GLI1, thus leading to increased tumor cell proliferation and increased self-renewal of canc [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 regul below) and is itself negatively modulated by PTEN [65,67], a repressor of AKT (see below tumor suppressors have since been found to regulate GLI. For example, loss of the SNF5 o activation of GLI1 [68,69].

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

Not only do common tumor suppressors repress GLI1 but common oncogenes, including R 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. Tog insures that GLI1 activity will increase as tumor suppressors are lost and oncogenes gainec the idea that it is the stepwise gain of oncogenic events and loss of tumor suppressors – nar oncogenic load – that leads to the acquisition of higher and higher GLI1 levels and thus hig levels of GLI^A [64]. These increases then drive GLI^A 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 fa [75,76], or loss of PTCH1, gain of SMOH activity or increase of SHH levels in sporadic ca [52,53,56,65,70,77–86].

Interestingly, initial evidence for non-HH signaling regulating the GLI code came from stu embryos where GLI2 was found to act in the FGF-Brachyury loop in the early mesoderm [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 betweer

[47], together with the regulation of GLI2 by FGF [87], and the regulation of GLI1 by RA
[65] opened a new chapter on the regulation of the GLI code, in this case by non-HH signa
studies predicted the modulation of the GLI code and of GLI1 by peptide growth factors ac
of RAS, MEK and AKT such as FGF, EGF, and many other ligands that trigger receptor ty
and activate RAS and downstream events. These findings can therefore help to explain wh
and GLI1 in particular, as the final positive feed-forward output, is important in human car
code, and GLI1, act at the tip of a funnel to integrate multiple outputs. Such a funnel idea [
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
pancreatic cancer in mice [88] being required for RAS-mediated tumorigenesis [89], and th
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 c
contexts, such as for instance the EWS/FLI1 fusion oncoprotein [94,95], TGFβ signaling [9
mTOR/S6K1 axis [98], WNT signaling [99] (although *WNT* genes can also be targets and r
GLI function [100,101]) and WIP1 [102].

Finally, interactions between pathways may be balanced by direct transcription factor bind
between GLI repressors and SMAD proteins, the latter being the mediators of normal and c
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 d
interest and its high therapeutic relevance. Here we describe HH-GLI and EGF crosstalk as
of how a cell can integrate apparently parallel signal inputs.

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

Global gene expression studies of human keratinocytes with combined or single activation
EGFR revealed three classes of target gene responses: (i) genes responding to HH-GLI onl
activated or repressed by EGFR only and (iii) genes only or at least preferentially respondi
and simultaneous activation of both pathways [92]. Notably, class III genes, also referred t
target genes or cooperation response genes, contain functional GLI binding sites in their pr
suggesting that signal integration occurs at the level of HH-EGFR target gene promoters [9
important to note that signal cooperation is a selective process as classical HH-GLI target g
PTCH1 or *HHIP* are not affected by parallel EGF signaling in keratinocytes [90,92,93].

In this context, cooperation of EGFR with GLI1 and GLI2 depends on activation of MEK/
while PI3K/AKT function is dispensable downstream of EGFR. MEK/ERK induced phosp
activation of the JUN/AP1 transcription factor is the critical event at the terminal end of th

cascade, inducing binding of activated JUN and GLI to common HH-EGFR target promote cooperatively regulating target gene expression and transformation [92,93]. It is noteworth basically all receptor tyrosine (RTK) pathways (e.g., HGF, VEGF or FGF) activate MEK/E context-dependent as not all of them synergize with HH-GLI in human keratinocytes, poss they fail to activate JUN/AP1 in these cells [90]. So far, only EGFR and PDGFRA [107] si been identified as being able to stimulate both MEK/ERK and JUN/AP1 and synergize wit basal cell carcinoma (BCC) (Fig. 4). Importantly, the beneficial effect of EGFR blockade i 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 colo In this context, enhanced GLI1 represses WNT-TCF targets and repression of WNT-TCF ta dominant-negative dnTCF leads to enhanced HH-GLI targets [71]. This mutually inhibitor distinct from that seen in other contexts between these two pathways (e.g. [100,109]) and i context of the metastatic transition of human colon cancers. Patients with metastases, but n 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 meta 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 advanc thus not recommended.

The interaction between the HH-GLI and WNT-TCF pathways is complex and stage-deper activity, essential for βCATENIN activation of WNT-TCF targets, is required for intestinal for adenomas. (e.g. [111]). However, while it is also required for advanced human colon ca 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]. The to be a functional cross-pathway switch at the metastatic transition. WNT-TCF may keep tu crypt-like state and enhanced HH-GLI together with repressed WNT-TCF may allow tumo and behavior and become metastatic [71].

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

Understanding how WNT-TCF and HH-GLI inputs are integrated is of great importance gi essential functions of both pathways in stem cells, human disease and development. Such i parallel signaling inputs can take place at multiple levels. In the case of WNT-TCF signalir 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 mo 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 GACCAC
The two cytosines flanking the central adenine in the consensus sequence are essential for
the other positions allow a certain degree of variation (Fig. 5A) [117,118]. Sequence-specif
to the *cis*-regulatory region of a GLI target gene mainly involves zinc fingers 4 and 5 whic
extensive base contacts within the 9-mer binding sequence, while fingers 2–3 mainly estab
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). F
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 s
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 therefor
neglected or overseen in many studies. Variations of the consensus sequence while preserv
contribute to subtle differences in DNA–protein binding affinity and hence may have a sig
on the 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
enhanced transcriptional response compared to the consensus motif [117].

Variants of the consensus GLI binding site contribute also to selective target gene activatio
GLI proteins. Although all GLI proteins bind the 9-mer consensus sequence with comparal
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 B
significantly more strongly than GLI1 and systematic analysis of the BCL2 promoter revea
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 interpre
threshold GLI activity levels above which a gene is transcribed or below which the very sa
remains silent. Accordingly, high affinity GLI binding sites in the *cis*-regulatory region of
ensure expression at both high and low levels of GLI activator activity, while targets with l
binding sites will respond to high GLI activity only, as demonstrated for the response of ne
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
differences in the absolute GLI^A protein level or activity determine the context-dependent
responses to HH-GLI [50,51,125] but also differential epigenetic modifications of the *cis*-r
regions of GLI targets affecting GLI-DNA binding affinity. Cell-type specific histone acety
methylations 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 tha
been explored in great detail.

Distinct combinatorial GLI function could also account for the substantial difference and c
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 bin

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 pos approaches will refine our current understanding of context-dependent target gene regulati and high-quality antibodies suitable for the isolation of rare endogenous GLI proteins bour 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 ac repressing co-factors as well as on co-occurrence of other TF that can bind and/or act coop regulate target gene expression (Fig. 5C). It follows that the absence or presence of GLI co cooperative transcription factors within a given cellular context is a major determinant of t transcriptional output.

An example of such an interplay with cofactors that regulates the GLI code is the functiona 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 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 adjac primary neurogenesis, which is triggered by GLI proteins expressed throughout the plate. T leads to the repression of Gli proneurogenic function by Zic2 in restricted domains, thus le definition of domains of neurogenic differentiation [126]. In this context, Zic2 mimics C-te truncated Gli repressors [126]. In a different context Zic2 may mimic positive GLI functio required for ventral forebrain fates: Loss of ZIC2 is associated with human holoprosencepl 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 netwo and NANOG (made from *NANOG1* and *NANOGP8* in human cancer cells) (Fig. 3). As dis p53 negatively regulates GLI1 [35] and GLI1 negatively regulates p53 [35,66]. p53 would active in most cells. However, a further layer of regulation is provided by the homeodomai factor NANOG, which forms a positive feed-forward loop with GLI1 [133]. Interestingly, regulated negatively by p53, establishing a highly dynamic node that will be affected by ar affect GLI1, NANOG and/or p53 [133] (Fig. 3). Regulatory mechanisms involve regulatio proteins by GLI1, protein phosphatase action, direct GLI regulation of *NANOG1* expressio 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 l this is predicted to free the GLI1-NANOG loop from negative regulation, allowing unrestr GLI^A. The essential role of NANOG and HH-GLI is demonstrated by their regulation of cl gliomaspheres and by their absolute requirement for the growth of primary human glioblas orthotopically engrafted in the brains of host mice [133].

Additional mechanisms of GLI code regulation include interactions with CREB-binding pr
Genetic and functional studies first carried out in the fruit fly and later in mammalian cells
CBP as essential co-factor for Ci and GLI3 mediated target gene activation [136]. Haploins
CBP is associated with Rubinstein-Taybi syndrome, a genetic disorder characterized by sev
developmental anomalies with partially striking similarities to defects observed in patients
Greig's cephalopolysyndactyly syndrome, which is caused by mutations in the GLI3 gene [
Given the intrinsic histone acetyl transferase (HAT) of CBP/p300 [140], CBP-GLI interact
to cause epigenetic changes of the *cis*-regulatory region of GLI targets making them more
other transcriptional regulators. In line with this hypothesis, histone acetyl transferase PCA
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
regulate GLI activity under genotoxic stress conditions [142].

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

TBP-associated factor 9 (TAF9) encodes a transcriptional co-activator that directly interact
activator forms GLI1 and GLI2 via their transcriptional activation domain [147]. TAF9 has
enhance the transcriptional activity of GLI, which may play an oncogenic role in lung canc
and chemical inhibition of TAF9-GLI interactions dampen GLI target gene transcription, th
possible therapeutic strategy to target oncogenic HH-GLI signaling downstream of the com
target 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 cofactors
14-3-3 protein. Notably, PKA phosphorylation at amino acid residues distinct from those e
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 a

Studies addressing selected GLI target gene promoters together with global approaches ana
entire landscape of GLI target gene promoters revealed the importance of combinatorial tra
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
inhibition of JUN expression as full transcriptional activation of these targets is likely to re
co-occupancy of their promoter region by GLI and JUN/AP1, similar to the mechanism ac
and EGF signal integration [90,93,150].

Another example of how combinatorial binding of transcription factors controls context-de
output is illustrated by the finding that co-occupancy of selected GLI targets by GLI1 and S
required for full activation of a neural gene expression signature [118].

Motif enrichment analyses identified E-box sequences as frequently co-occurring with GLI GLI target genes expressed in medulloblastoma [120]. It is therefore possible, that E-box b transcription factors cooperate with GLI in the control of tissue specific target gene expres 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-translati modifications

Fine-tuning and reversible activation/termination of HH-GLI signaling is critical to proper and health. As outlined in the introduction of this article, numerous reports have provided showing that precise control of HH-GLI signal strength occurs at nearly every level of the cascade, ranging from the control of ligand production and ligand-receptor interactions do numerous molecular interactions and modifications of GLI proteins eventually determining 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 role in its control by affecting GLI stability, subcellular localization and DNA binding abil (reviewed in [20,40]). To remain focused on the topic of context-dependent GLI activity, w 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 im ability of GLI proteins to regulate target genes by modifying their binding to target promot [156–158].

Atypical Protein Kinase Cι/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 H drug target SMOH by phosphorylating GLI1 at amino acid residues located in the zinc fing binding domain. GLI1 phosphorylated by aPKC displays enhanced DNA binding and max transcriptional activity. Of note, hyperactivation of aPKC in BCC can account for SMOH i resistance, rendering it a promising drug target for the treatment of cancer patients unrespo 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 transcriptional activator of HH acetyltransferase expression, leading to increased HH ligan 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 region of GLI1/2 serve as phosphorylation sites involved in GLI activation. In esophageal 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 S note, the S6K1 phosphorylation site at Ser85 of GLI1 is located in a D-site motif that serve 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 modif 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 re phosphorylating ncPKA sites has not been identified [161]. Whether phosphorylation of nc activates GLI2/3 by disrupting the SUFU-GLI complex or by a different mode also needs t in future studies.

A number of distinct phosphorylation events in the N-terminal region of GLI control full C suggests that the N-terminus of GLI proteins serves as integration domain for multiple sign 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 [162]. It follows that this integration domain plays a major role in the fine-tuning of GLI ac 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 transcriptional activity. Acetylation of GLI2 at Lys757 by the histone acetyl transferase p30 negative regulatory modification in HH signaling [157]. Interestingly, acetylated GLI2 disp significantly reduced recruitment to chromatin and consequently only weak activator poten the acetylation site is C-terminal of the DNA binding domain it is unlikely that acetylation DNA binding affinity. Rather, deacetylation may favor the interaction of GLI with chromat proteins and therefore enhance its recruitment to target gene enhancers/promoters. Indeed, promotes deacetylation of GLI1/2 via inducing class I histone deacetylases (HDAC), whicl identified as important step in the activation of GLI target gene expression [157,158].

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

5. Outlook

Whereas great progress has been made to understand how the GLI proteins act (e.g., reviev [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 the in cells receiving simultaneous inputs, how their activity can be affected by and affect epig how they are protected from cleavage or modification, or even how the pathway is effectiv when required.

Documenting the full range of inputs and factors that can modulate their activities in multi developmental, homeostatic and disease contexts will require much effort but will certainly Such knowledge may allow us to begin to understand the logic of signaling in developmen hopefully also in evolution. Thus, we promote the idea that these analyses must be carried possible species and cell types in order to compare and contrast mechanisms and outcomes 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 dise specifically in cancer, through pathway corruption and the oncogenic load. Such knowledg lead us to design novel and more efficient therapies against multiple forms of deadly cance those 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 th molecular approaches to block positive GLI function, reverting the GLI code, could be hig

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 [64,65,90,9 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 i 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 beer effective, but clearly the number of recent examples such as those mentioned above along identification of small molecule GLI antagonists [166,167] provide ample proof-of-concep 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, particu amenable to small molecule targeting, as well as studies addressing their context-dependen an area of intense future research with significant impact on several medical areas such as regeneration and wound healing.

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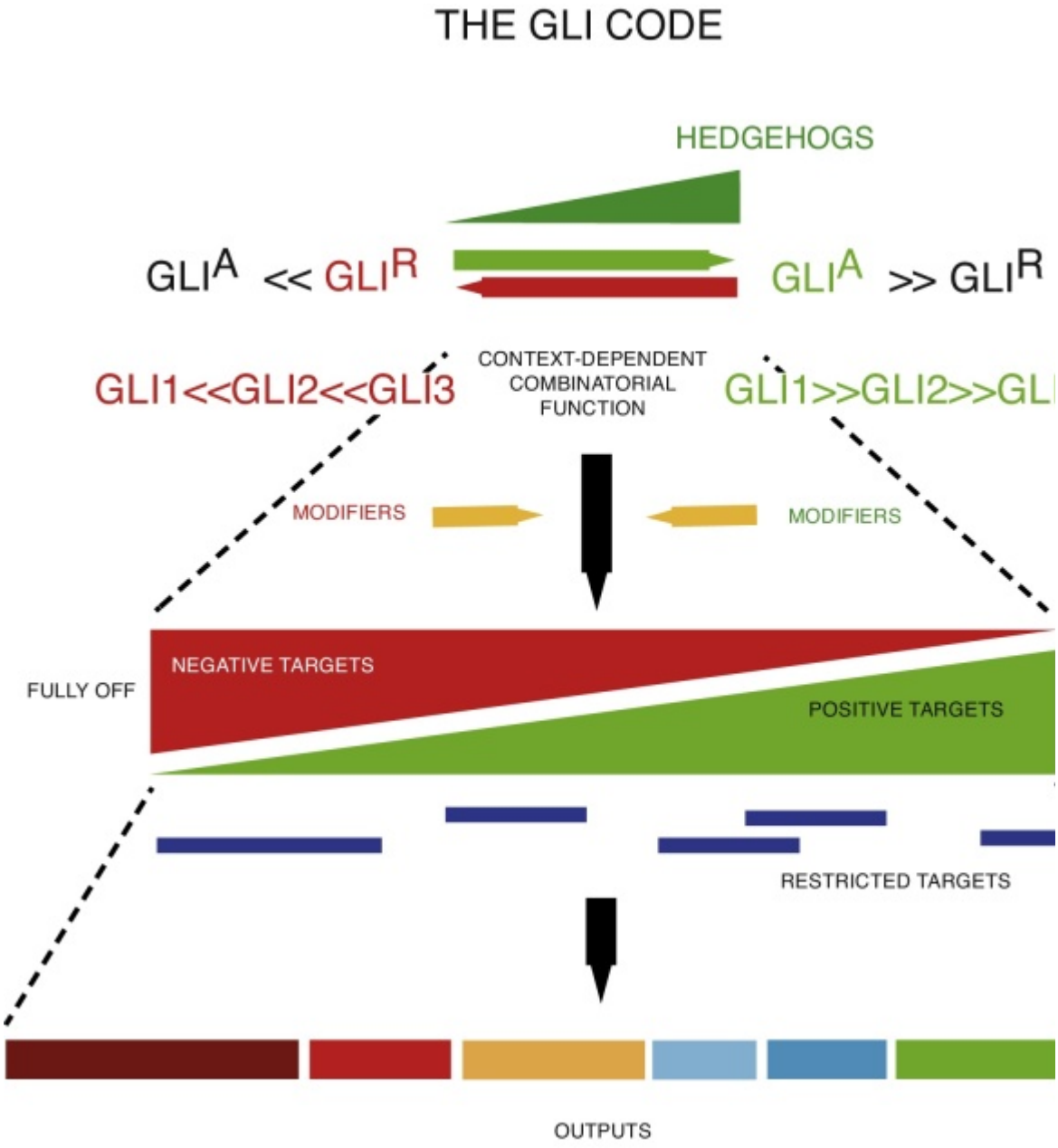
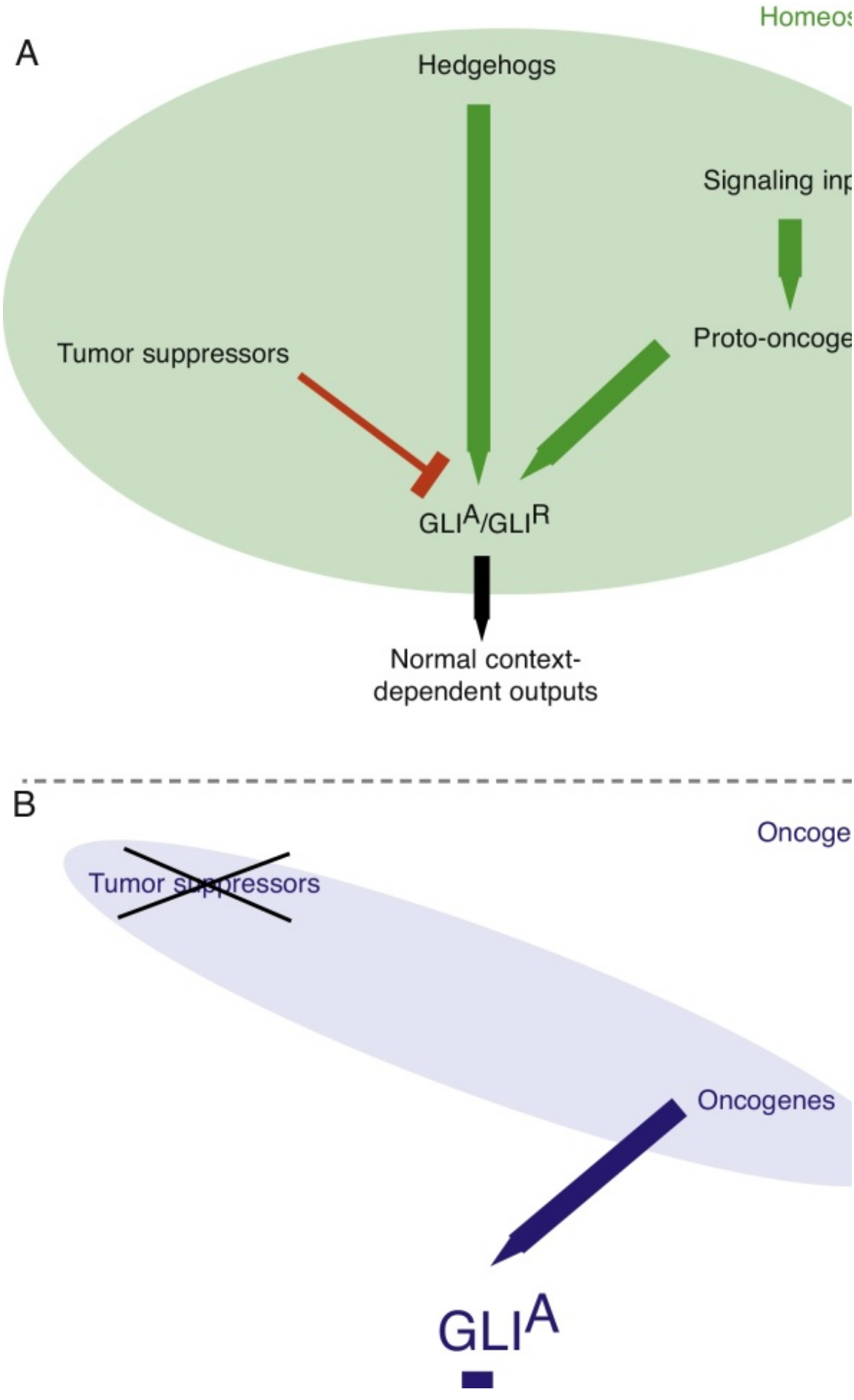
Figures and Tables

Fig. 1



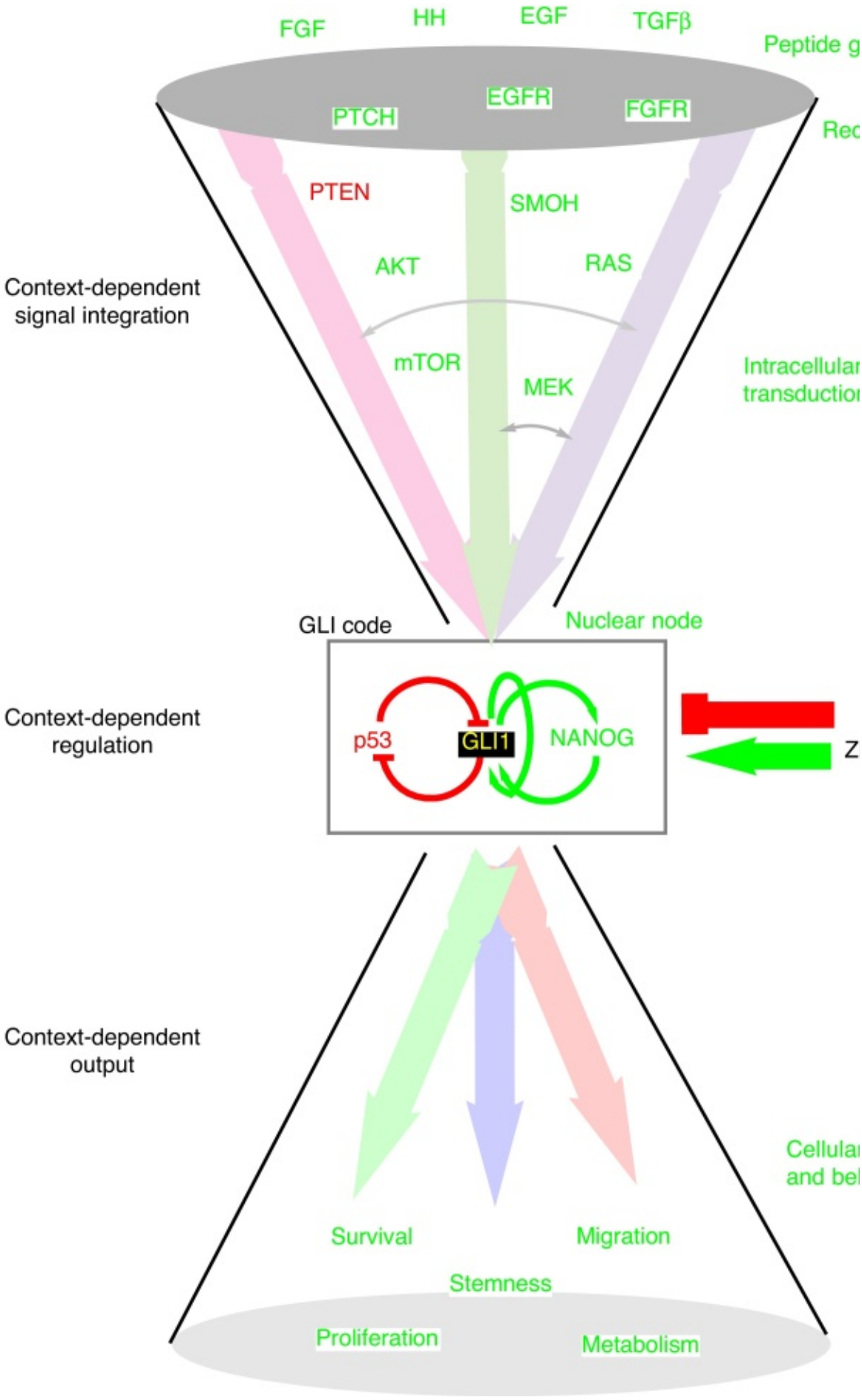
Model for the GLI code and its morphogenetic activity leading to the creation of context-dependent div
of HH ligands is interpreted, canonically, by a combinatorial and context-specific distribution of repress
activities of the three GLI proteins, the GLI code. Note that GLI1 and GLI2 have strong activating actio
strong repressor in many contexts. Combinatorial GLI activities are then modified by positive or negati
leading to differential regulation of target genes, which may either respond to create graded levels of ex
specific genes or induce specific genes in given thresholds. The output of these genetic changes is then
spatially and/or temporally distinct outputs and behaviors.

Fig. 2


Homeos
A
Hedgehogs
Signaling inp
Proto-oncoge
Tumor suppressors
GLI^A/GLI^R
Normal context-
dependent outputs
B
Oncoge
Tumor suppressors
Oncogenes
GLI^A

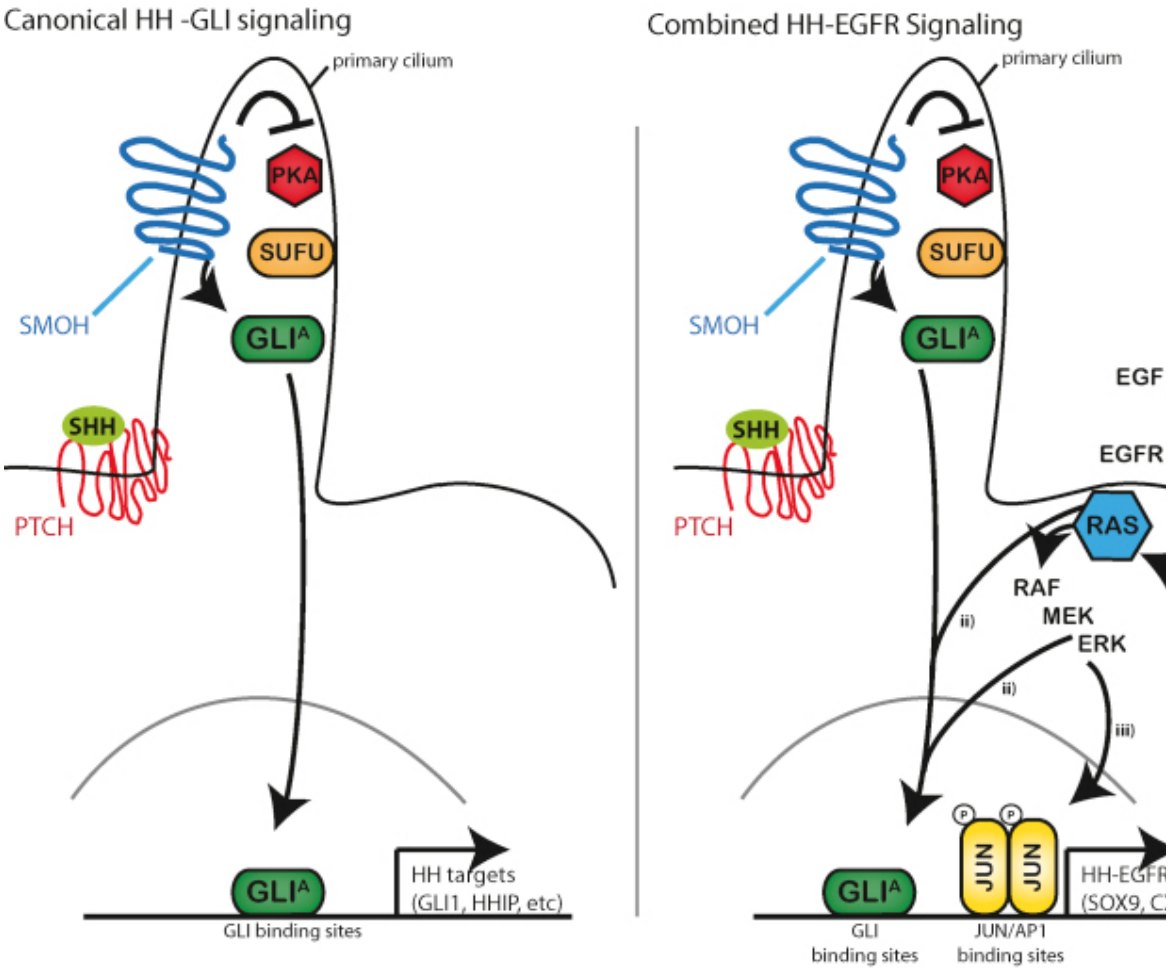
Control of the GLI code by the oncogenic load. (A) Under normal homeostatic conditions a fine-tuned b signaling as well as of parallel proto-oncogenic (e.g., EGF, FGF, PDGF, etc.) and tumor-suppressive pat precisely controlled levels of GLI^A/GLI^R. The balance can be tipped one way or another, thus 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 ger GLI code is no longer under homeostatic control.

Fig. 3


FGF
HH
EGF
TGFβ
Peptide g
EGFR
PTCH
FGFR
Rec
PTEN
SMOH
AKT
RAS
Context-dependent
signal integration
mTOR
MEK
Intracellular
transductio
GLI code
Nuclear node
Context-dependent
regulation
p53
GLI1
NANOG
Z
Context-dependent
output
Cellular
and bel
Survival
Migration
Stemness
Proliferation
Metabolism

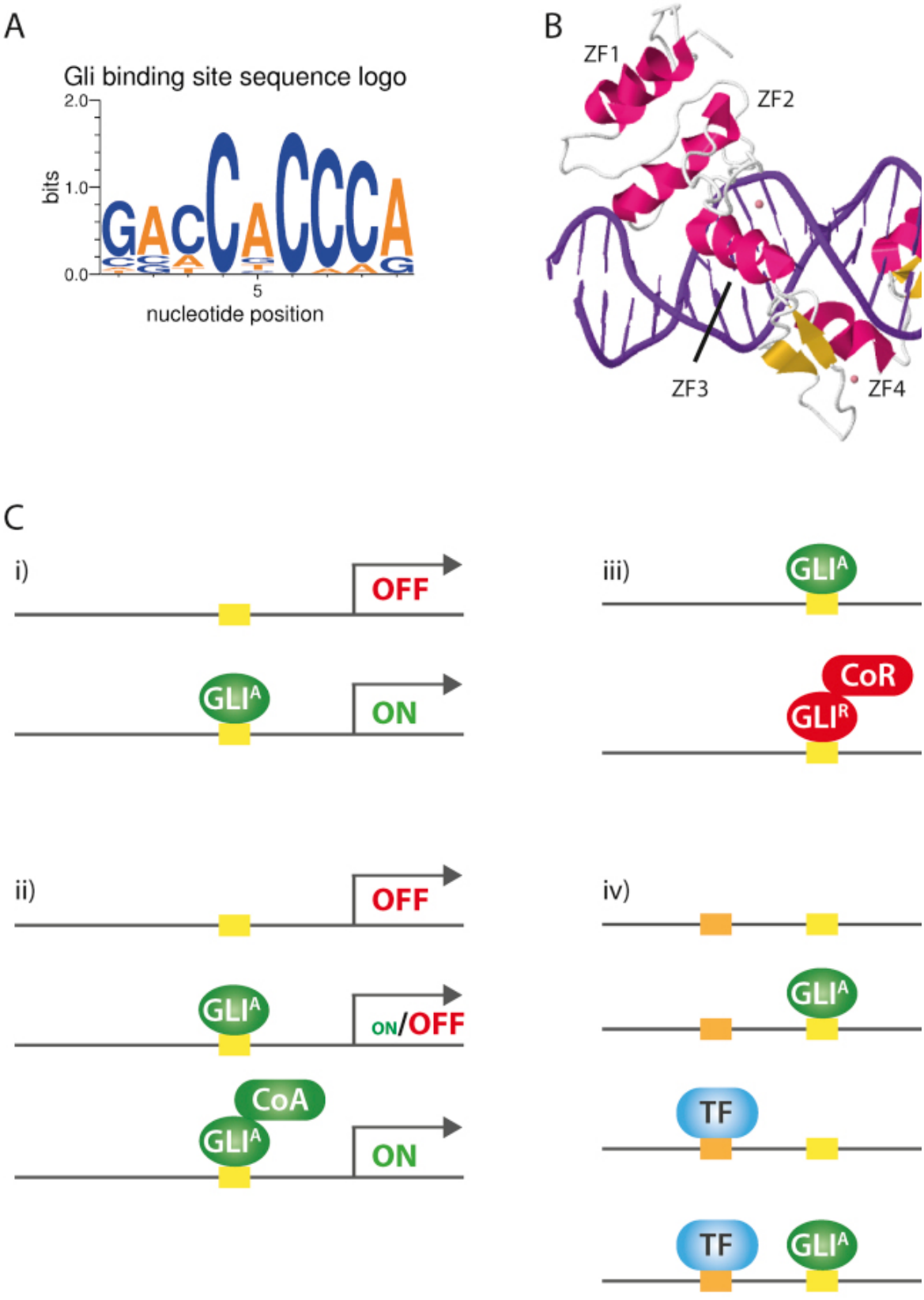
A working framework for the GLI code as a node for signal integration. Multiple signaling inputs from
including but not restricted to HH, EGF, FGF, TGFβ, can converge on GLI regulation, changing the GL
can also take place above, through crosstalk (gray arrows). The position of the different components is r
other but shown as examples of the types of components involved in the signaling cascades. The GLI co
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 loo
[133]. The outcome, through differential regulation of target genes, is context-dependent and includes cl
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.

Fig. 4



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 signalin activates classical GLI targets including *HHIP* and *GLI1* but fails to induce HH-EGFR cooperation targ Concomitant activation of HH-GLI and EGF/PDGF signaling (EGFR or PDGFRA) can lead to synergis [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) synergist basal cell carcinoma and pancreatic cancer by selective activation of HH-EGFR target genes such as CX 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 leac of GLI1 or/and of the JUN/AP1 transcription factor. JUN synergizes with GLI activator forms by co-oc target gene promoters leading to synergistic transcriptional activation of HH-EGFR targets and enhance (e.g., BCC and pancreatic cancer).

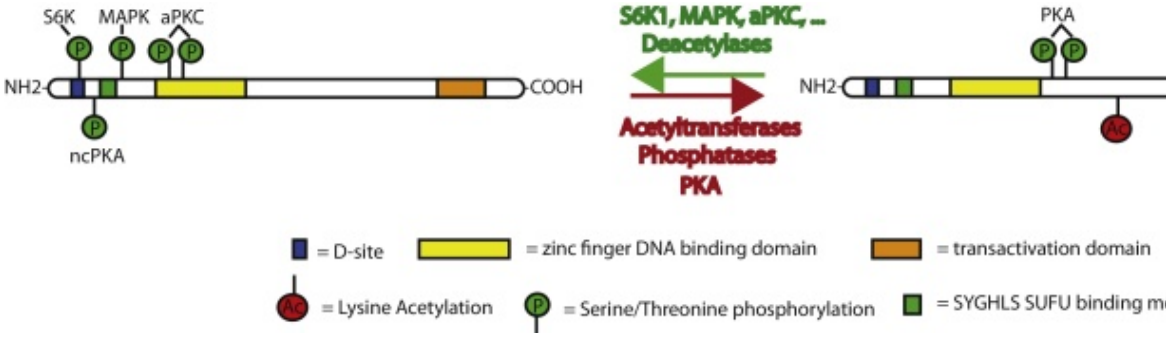
Fig. 5



GLI DNA binding and context-dependent target gene regulation. (A) Consensus 9-mer GLI DNA bindi calculated from experimentally validated GLI binding sites. The motif was generated with a set of 22 ex 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 e
model of the GLI DNA binding domain composed of five zinc fingers and its interaction with the conse
sequence. Note that fingers 4 and 5 form extensive base contacts thereby determining binding specificit
Databank ID 2GLI; [119]). (C) Non-exhaustive models of context-dependent target gene activation. He
(GLI^A) and GLI repressor forms (GLI^R) binding the same target sequences refer to the GLI code. (i) Cl
activation model with GLI^A binding to the promoters of canonical targets such as *PTCH1* or *HHIP*. (ii)
dependent interactions of GLI^A with co-activators (CoA) or (iii) of GLI^R with co-repressors (CoR) mo
and expression of HH-GLI targets. (iv) Context-dependent combinatorial binding of GLI^A and cooperat
factors (TF) (e.g., JUN, SOX2) to common target promoters can also result in synergistic modulation of

Fig. 6



Post-translational modifications regulate GLI transcriptional activity. Fine-tuning of GLI activity by phosphorylation/dephosphorylation and acetylation/deacetylation. Left: fully activated GLI transcriptio multiple phosphorylated serine/threonine residues in the N-terminal region and the DNA binding domai de-acetylation promotes DNA binding affinity and transcriptional activity, respectively. Several kinases aPKC) and deacetylases catalyze the activation of GLI, while phosphatases, PKA and acetyltransferase regulate GLI activity. Note that PKA phosphorylation of the two amino acid residues C-terminal of the domain negatively regulates the transcriptional activity of GLI without affecting processing or stability non-consensus PKA phosphorylation sites involved in GLI activation.