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Another twist to the GLI code

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Abstract

The canonical Hedgehog (Hh) signalling pathway is essential for vertebrate development and its uncontrolled activation is a common occurrence in human cancers. Hh signalling converges in the modification of a family of transcription factors, GLI1, GLI2 and GLI3, to orchestrate a cell type and context-specific transcriptional response. Despite binding to very similar responsive elements, the GLI family members can exert diverse and even opposing functions. A recent article by Tolosa *et al.* (Biochem. J. **477**, 3131-3145, 2020) reveals an unexpected layer of complexity, through physical and functional interaction between GLI1 and GLI2. This commentary discusses the biological significance of the findings and incorporates them into an updated “GLI code”.

The Hedgehog (Hh) signalling pathway has essential roles in patterning of the dorsoventral aspect of the central nervous system, digit specification, vasculogenesis, angiogenesis and lung branching in the vertebrate embryo (1). Postnatally, Hh signalling remains active in many stem and rapid proliferating progenitor cells, with constitutive activation often linked to tumourigenesis (1). The membrane protein PTCH1, which is the receptor for all three Hh ligands (SHH, IHH, and DHH), represses the G protein-coupled receptor Smoothened (SMO) in their absence. Binding of a Hh protein to PTCH1 relieves this inhibition, allowing SMO accumulation in the primary cilium and activation of the GLI family of transcription factors, composed of GLI1, GLI2 and GLI3. GLI2 and GLI3 are constitutively expressed, and the balance of their full length, transcriptional activator forms versus their processed, transcriptional repressor forms is regulated by signals from SMO. In contrast, GLI1 lacks a repressor domain and its expression is only induced by GLI2 and GLI3, upon their activation. GLI1 acts as a transcriptional activator in concert with GLI2 to further amplify the Hh signal (2). Ruiz i Altaba's group coined the term "GLI code" to convey the diverse functions of the three vertebrate GLI isoforms (Fig. 1) (3). In this model, GLI1 is exclusively a transcriptional activator; however, research in the past decade has shown a darker side of GLI1, uncovering its potential to serve as a transcriptional repressor of selected genes, such as *ANO1, CDH1, AQP1* and *SOCS1* (4-6). How can GLI1 mechanistically act as a repressor? Unlike its siblings GLI2 and GLI3, which encode four clusters of phosphorylation sites that are initially modified by PKA, subsequently becoming substrates for GSK3β and CK1, followed by ubiquitylation and partial proteasomal degradation, GLI1 has a reduced number of phosphorylation sites and no degron motifs (7, 8). GLI1 has been only found in full length form, and a repressive function could be explained by acquisition of such through physical interaction with the repressor form of GLI2 or GLI3. The possibility of the formation of heterodimers among the GLI family members has also been suggested by sporadic evidence of synergy between GLI1 and GLI2, despite the ability of all three isoforms to bind to the same responsive elements in the promoters of target genes, containing the canonical GLI binding sequence (GBS) GACCACCCA or slight variations of it (9-11). Indeed, all essential transcriptional functions of GLI2 can be replaced by GLI1 when expressed from the GLI2 locus, and GLI1 is not essential for embryonic development. However, the role of GLI1 in maintaining cancer cells phenotype and/or in maintaining a stem-like state suggests a gain of function during tumour formation and progression that cannot totally be replaced by GLI2. A novel study by Tolosa et al. (6) sheds new light onto these questions and offers mechanistic explanations for the exceptions to the GLI code.

The authors set out to investigate the nature of the cooperativity of GLI1 and GLI2 in the regulation of selected cancer-relevant genes that regulate cell growth (*MYCN*, *CNND1, CNND2, E2F1* and *CDK2*) and survival (*BCL2*, *XIAP*, *BIRC5*) in pancreatic cancer cells and

two non-transformed cell lines. In addition, they compared their transcriptional activity towards two GLI hallmark target genes, *PTCH1* and *PTCH2*. The central question of the study was to distinguish whether dependence of GLI1 and GLI2 for expression of specific target genes is competitive in nature. Could only one of the two GLI isoforms binding to the same genomic elements at any given time, or could they function synergistically, through co-occupancy of the same promoters?

Using a cellular model of pancreatic cancer (PANC1 cells), Tolosa et al. first demonstrated that GLI1 can physically interact with GLI2 and that this interaction does not require DNA binding. Partial deletion analysis revealed that the interaction requires the highly conserved C2H2 zinc finger domain (ZFs 1-5) of both GLI1 and GLI2, and an additional N- or C-terminal stretch of GLI2. A previous study had also shown evidence of GLI2/GLI3 and GLI3/GLI3 physical association, but not of GLI1/GLI3 (12). In that study the first two zinc fingers of GLI3 (ZFs 1-2) where used as bait to pull down C-terminally deleted GLI2 and GLI3, in agreement with the new finding of the requirement of the ZF domain for heterodimerisation of GLI1 and GLI2. The ZFs 3-5 of GLI1 were shown to mediate most of the contact with DNA in a crystal of the GLI1 ZF domain complexed to DNA containing a GBS (13), supporting the findings of that ZFs 1-2 serve as protein-protein interaction domains instead. In addition, older studies revealed that the three GLI family members can also interact through their ZF domain with – and their transcriptional activity enhanced - another family of C2H2-zinc finger transcription factors called ZIC1, ZIC2, and ZIC3 (12, 14). Altogether, these findings show the existence of GLI family heterodimers, GLI-ZIC family heterodimers, and possibly GLI homodimers, adding a new layer of complexity to the GLI code (Fig. 2).

These findings also raise new interesting questions. For example, it seems theoretically possible that GLI1 can also associate with the repressor form of GLI2 (GLI2R), which includes the ZF domain, since the interaction between GLI isoforms does not require the most C-terminal domain lost in GLI2R. If so, would GLI1 act as a transcriptional activator or a repressor? Would they display intermediate activities that could translate the morphogen gradient of Hh ligands *in vivo*? Understanding if the dimers are competent to bind DNA, and they display differential transcriptional activity compared to individual monomers, will be essential to answer these questions.

In agreement with the idea that different homo or heterodimers could preferentially bind to specific promoters, depletion of GLI1 or GLI2 in PANC1 cells or in pancreatic cancer associated fibroblasts resulted in differential downregulation of a number of GLI-target genes. While cell growth and survival genes such as *BCL2*, *MYCN* and *CCND1* were similarly affected by depletion of either GLI1 or GLI2, others showed a skewed preferential reduction in the absence of one of the two GLIs. Of relevance to HH signalling, expression of the receptor and negative regulator PTCH1 seems to be exclusively under the control of GLI1,

while expression of the homolog PTCH2, without capacity to transduce the Hh signal, is under the control of both GLI isoforms. One can speculate that the negative feedback exerted as a consequence of PTCH1 induction would be confined to a high threshold of active GLI1 transcriptional activators, to prevent early signal termination.

In agreement with the relative sensitivity to GLI1 or GLI2 silencing, chromatin immunoprecipitation of GLI1 and GLI2 showed promoter occupancy in line with the observed transcriptional activity. The PTCH1 promoter contained bound GLI1 but much lower levels of GLI2, while similar levels of GLI1 and GLI2 were detected in promoters of genes partially regulated by both isoforms. Nonetheless, when endogenous GLI1 was depleted by siRNA, the occupancy of GLI2 in the *BCL2*, *MYCN* and *CCND1* promoters was strongly reduced, indicating a promoter-specific synergistic function. This intriguing result could not be explained by changes in total GLI2 levels or by significant changes in nuclear trafficking of GLI2, because occupancy of the PTCH1 promoter by GLI2 in the same cells was unaffected. Instead, this suggests that those growth and survival genes (cancer-associated genes) could be preferentially targeted by a GLI1/GLI2 heterodimer. However, binding of a heterodimer to DNA has not been proven yet, and will require future identification of mutants that abolish GLI1-GLI2 contact without disrupting their binding to DNA, or their individual transcriptional activity.

What does this novel information reveal about a potential biological role of the GLI1/GLI2 heterodimer? If heterodimers are competent to bind to DNA, as suggested by the lack of dissociation of the GLI1/GLI2 heterodimer by GANT61 and the functional and positional studies, they might fine-tune GLI transcriptional outputs. Another scenario is that GLI1/GLI2 heterodimers differentially accumulate in the cell nucleus, either by increased nuclear shuttling or reduced exit, or have a different affinity to the Sufu chaperone (15). Furthermore, the heterodimers could be more resistant to phosphorylation by PKA, GSK3β and CK1, or less efficient ubiquitylation substrates.

All three GLI isoforms are substrates of extensive post-translational modifications (PTMs) that regulate their activity, stability, and subcellular localisation. In addition to the inhibitory phosphorylation by PKA, GSK3β and CK1, stimulation of their transcriptional activity by MEK, JNK, aPKCι/λ and other unknown kinases has been suggested (7, 16-18). Furthermore, acetylation, ubiquitylation and SUMOylation are known to regulate GLI activity and stability (19, 20). Does the dimerization of GLI transcription factors affect the repertoire of PTMs achievable? Is ciliary trafficking of GLI2 and GLI3 affected by potential dimerisation with each other? Finally, is dimerisation of GLIs a requirement for binding to DNA akin to bHLH type transcription factors clamping of DNA?

An oncogenic role for GLI1 and GLI2 has been reported by many independent studies in different cancer types. Only a selected group of malignancies – SHH-type medulloblastoma,

basal cell carcinoma, and rhabdomyosarcoma - arise by constitutive activation of the canonical HH pathway through *PTCH1*, *SMO*, or *SUFU* mutations. However, GLI1 is upregulated in many other cancer types and subtypes by SHH or IHH upregulation or by crosstalk with other cancer-associated signalling pathways, such as EGFR, IL6/STAT3, and TGFβ/SMAD signalling (3; 21-23). Silencing of GLI1 in cancer cells reduces cell proliferation and tumour growth, inhibits expression of stemness markers, and increases apoptosis (24-27). In light of the new findings of Tolosa et al., an attractive hypothesis is that expression of some key oncogenic GLI1 target genes might not be achievable at a similar level by a redundant function of GLI2.

In summary, the new evidence indicates that the GLI code is much more complex than previously thought, through the existence of heterodimers of GLI in addition to GLI/ZIC dimers, whose characteristics are just beginning to be unveiled. Of particular interest is their role in tumorigenesis and the potential therapeutic effect of targeting the dimers interface.

Abbreviations

Hh	Hedgehog
SMO	Smoothened
GLI	Glioma-associated oncogene homolog
PKA	cAMP-dependent protein kinase
GSK3β	Glycogen synthase kinase 3β
CK1	Casein kinase 1
GBS	GLI Binding Sequence
ZIC	Zinc finger protein of the cerebellum
PTMs	Post-translational modifications

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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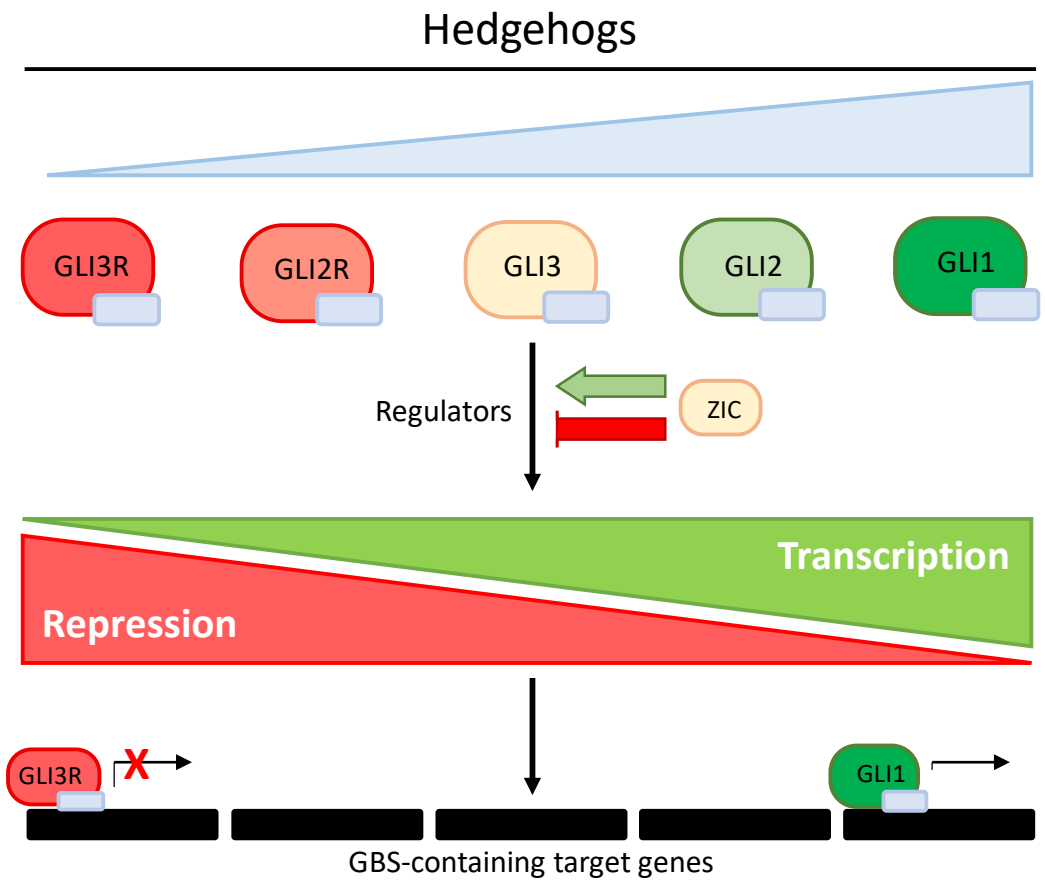
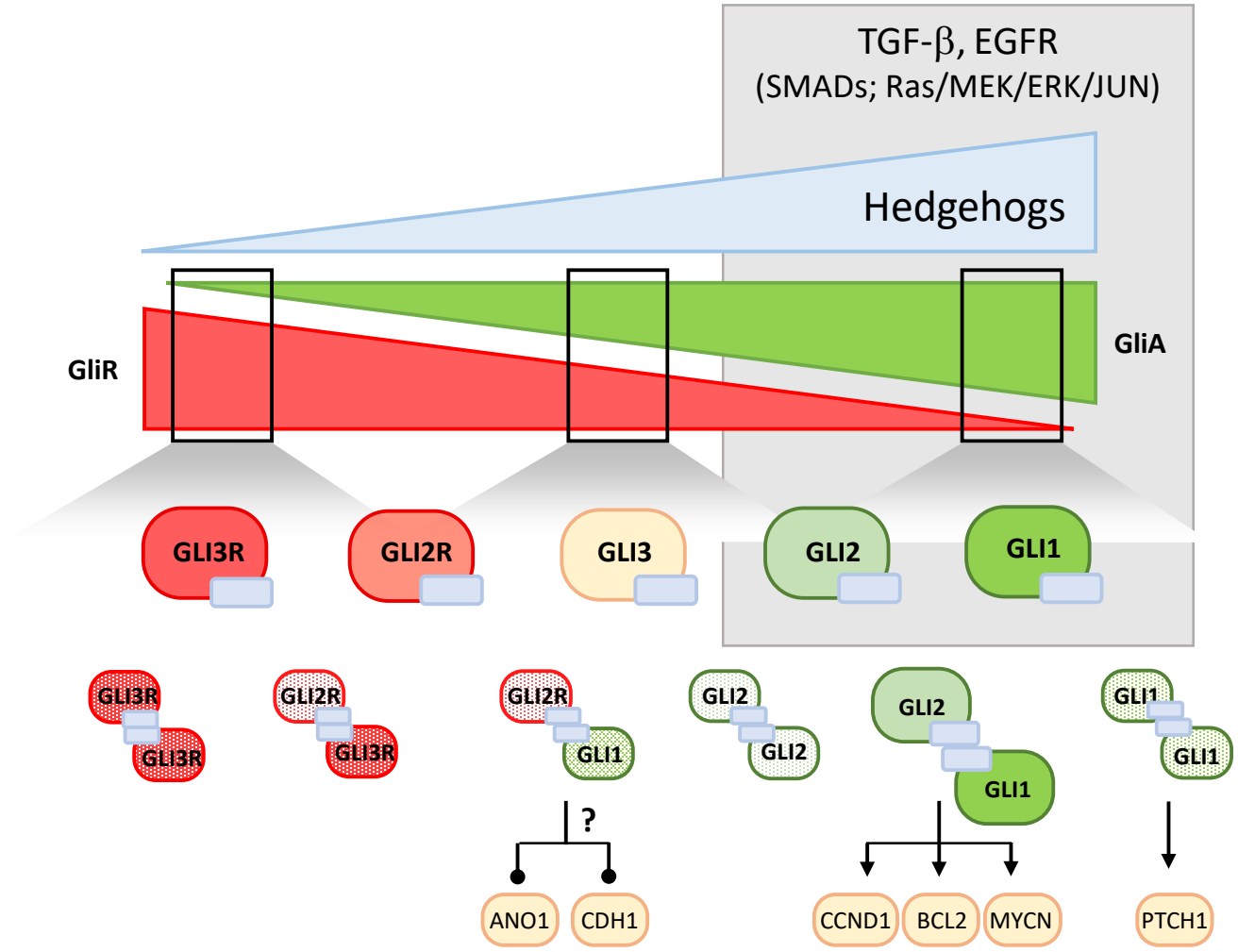
Figure legends

Figure 1. The canonical Hedgehog signalling response is mediated through the differential repressor and activator functions of the three GLI transcription factors. A concentration gradient of Hh ligands elicits step-wise changes in the modifications and processing of each isoform. Light blue rectangles represent the Zinc Finger (ZF) domain of GLIs, which mediate DNA binding to GLI-binding sequences (GBS) in the promoters of Hh-regulated genes.

Figure 2. Updated model for the GLI code. The transcriptional output depends on the balance of the 1) expression level, 2) processing into repressor forms, 3) positive and negative posttranslational modifications, and 4) the presence of additional protein interactors. The gradient of Hh ligands regulates GLI1 expression and GLI2 and GLI3 PTMs and processing, while other signalling pathways activated by TGF-β and EGFR alter the balance through upregulation of GLI2 and phosphorylation changes of GLI1 and GLI2. Based on the new findings of Tolosa et al. (6) and earlier reports, dimerization of GLI isoforms through their Zinc Finger (ZF) domain adds another level of transcriptional activity regulation. The proposed specific function of various GLI homo- and heterodimers is indicated by their effects on target genes. GLI1/GLI2 dimers have been identified in (6). We propose the existence of other homo- and heterodimers (shaded shapes), based on reported biochemical interaction of the individual ZF domains, or unexpected functional outputs. Note: GLI1/GLI3 heterodimers are not included in the model because their ZF domains do not show interaction in the same assays. Light blue rectangles represent the Zinc Finger (ZF) domain of GLIs, which mediate binding to GBS elements and protein-protein interaction.


Hedgehogs
GLI3R
GLI2R
GLI3
GLI2
GLI1
Regulators
ZIC
Transcription
Repression
GLI3R
X
GLI1
GBS-containing target genes


TGF-β, EGFR
(SMADs; Ras/MEK/ERK/JUN)
Hedgehogs
GliR
GliA
GLI3R
GLI2R
GLI3
GLI2
GLI1
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ANO1
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BCL2
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