Distinct and regulated activities of human Gli proteins in *Drosophila*

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In both vertebrates and *Drosophila*, limb development is organized by a posteriorly located source of the signalling protein Hedgehog (Hh) [1–4]. In *Drosophila*, the expression of Hh target genes is controlled by two opposing activities of the transcriptional regulator Cubitus interruptus (Ci), which activates target genes in response to Hh signalling but is converted into a repressor form in the absence of Hh [5–10]. Three homologs of Ci (Gli1, Gli2, and Gli3) have been implicated in mediating responses to Sonic hedgehog (Shh) in vertebrates [11,12]. Much attention has been devoted to the expression pattern of *GLI* genes; *GLI1* is induced by Shh, whereas *GLI3* transcription appears to be repressed by Shh signalling [13–15]. The regulation of *GLI* gene expression is therefore one important mechanism by which *GLI* genes organize pattern. It is not well understood, however, whether Shh signalling also controls the activities of Gli proteins post-translationally and whether these activities have activating or repressing effects on target genes *in vivo*. Here, we have subjected the human proteins Gli1 and Gli3 to the precise and well-defined Hh signalling assay of *Drosophila* wing development and established that Gli1 functions as an activator and Gli3 as a repressor of Hh target genes; that the activating transcriptional activity of Gli1 and the repressing activity of Gli3 are both subject to Hh regulation *in vivo*; and that the combined activities of Gli1 and Gli3 can substitute for Ci in controlling Hh target gene expression during embryonic and larval development.

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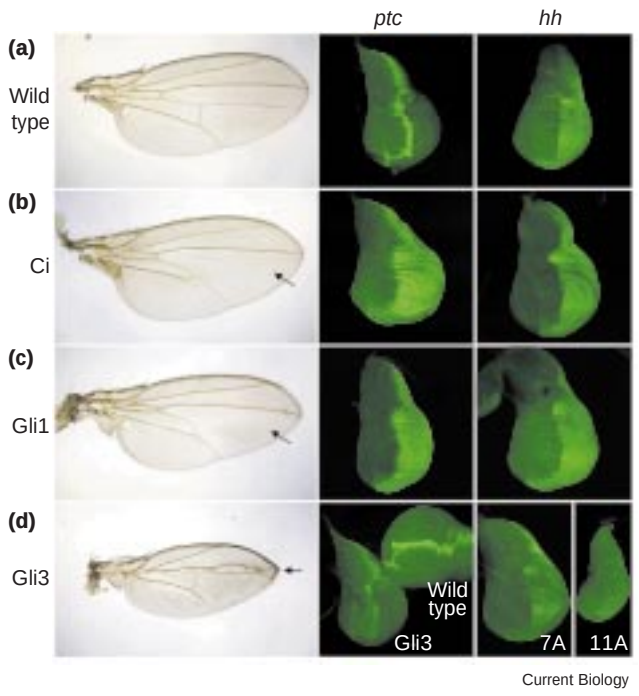
Results and discussion

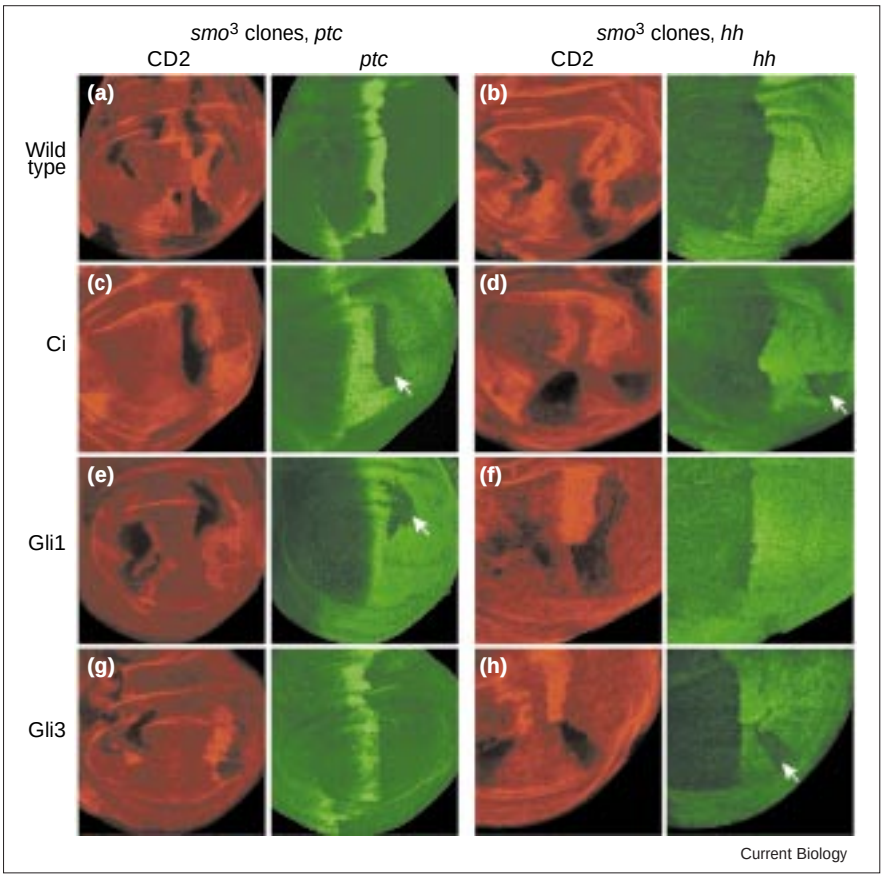
The coding regions of human *GLI1* and *GLI3* and *Drosophila ci* were each modified to encode identical amino-terminal and carboxy-terminal epitope tags and placed under the control of the weak, ubiquitously active tubulin α1 promoter, and of a Gal4-dependent promoter, which produces higher expression levels in combination with the imaginal-disc-specific Gal4 driver gene *C765*. For each construct, transgenic lines were obtained. Protein expression was monitored in the wing imaginal disc using

Figure 1



Distinct activities of human Gli proteins. Phenotypes resulting from the uniform expression of Gli proteins in adult wings (left column) and in the wing imaginal discs (right columns) are shown. The expression of Ci target genes *ptc* and *hh* was visualized using *lacZ* reporter genes [20,21] inserted into the respective loci. Expression of β-galactosidase was then detected using an anti-β-galactosidase antibody and a fluorescein-isothiocyanate-conjugated secondary antibody (green). (a) In the wild type, *ptc* transcription is upregulated in response to Hh signalling in a thin stripe of anterior cells at the anterior–posterior boundary. Expression of *hh* is seen only in posterior-compartment cells. (b,c) The phenotypes caused by misexpressing (b) Ci and (c) Gli1 are very similar. The cubital vein L4 is interrupted (arrow) and *ptc* is upregulated ectopically in the posterior compartment. Both effects are consequences of ectopic Hh signal transduction in posterior-compartment cells. (d) When Gli3 is misexpressed, wing size is reduced and veins L3 and L4 are partially fused (arrow). Expression of *ptc* is reduced (compared with an adjacent wild-type control disc). Expression of *hh* is also reduced or almost abolished, depending on the strength of expression; the two discs shown are from two lines (7A and 11A) that differ in their levels of transgene expression. Transgenes were driven either by the tubulin α1 promoter (adult wings in (b,c)) or using the Gal4 driver line *C765* (all other panels). Discs are shown with anterior to the left and dorsal to the top.

Figure 2



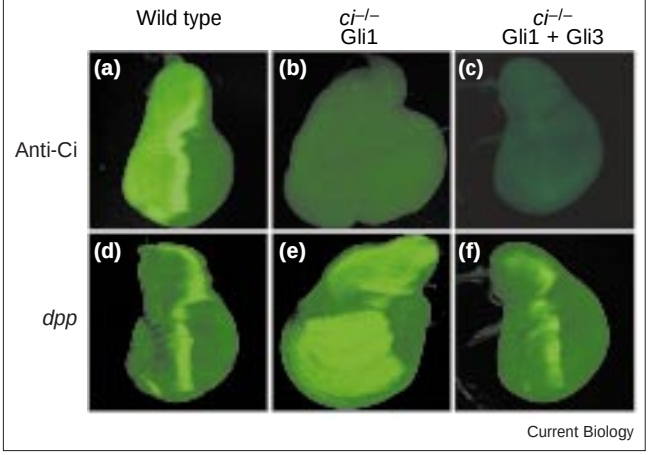
Regulation of Gli protein activities by Hh signalling *in vivo*. Regulation of Gli activity was assayed by comparing expression of Ci target genes in Hh-transducing posterior-compartment cells and in *smo* mutant posterior-compartment cells, in which Hh signalling is abolished. The *smo*^{3} mutant cell clones are marked by the loss of expression of the CD2 marker gene [22]. Our analysis focuses on the posterior compartment (the right half of the disc), because cells in it are exposed to Hh ligand and do not express Ci. Each pair of panels shows an experimental disc in which the expression of CD2 (left panel, detected using an anti-CD2 antibody and a Texas-red-conjugated secondary antibody, red) and *ptc* or *hh* (right panel, detected as in Figure 1) was monitored by double staining. (a,b) Wild-type negative control. Expression of *ptc* is off in the posterior compartment (no activator present), and *hh* expression is on (no repressor present). Neither (a) *ptc* nor (b) *hh* expression is affected in *smo* mutant clones. (c,d) Positive control overexpressing Ci. Ci has both activator and repressor activities, and both activities are controlled by Hh signalling. (c) Ectopic expression of Ci in posterior-compartment cells upregulates *ptc* expression in *smo*$^{+/-}$ cells but not in *smo*$^{-/-}$ cells (arrow). Thus, Ci activator activity is dependent on Hh transduction [9,21]. (d) Conversely, *hh* expression is repressed in *smo*$^{-/-}$ cells (arrow) but not in neighboring *smo*$^{+/-}$ cells. Thus, Ci has repressor activity in the absence of Hh transduction [7,9]. (e,f) Overexpression of Gli1. (e) Like Ci, Gli1 activates *ptc* expression only in *smo*$^{+/-}$ but not *smo*$^{-/-}$ cells (arrow). Its activator activity is thus strictly regulated by Hh signalling. (f) No repressor activity of Gli1 is detectable, irrespective of the state of Hh signalling. (g,h) Overexpression of Gli3. (g) Gli3 has no activator activity, but (h) represses *hh* expression strongly in *smo*$^{-/-}$ cells (arrow) and weakly in *smo*$^{+/-}$ cells. Gli3 therefore possesses weak repressing activity in Hh-transducing cells and strong repressing activity in the absence of Hh signalling. Transgenes were expressed using the Gal4 driver *C765*, except in (e), for which the tubulin α1 promoter was used. Anterior is to the left and dorsal to the top.

monoclonal antibodies directed against the amino- and carboxy-terminal tags. For the Gli proteins, uniform expression was observed (data not shown), indicating that the transgenes are functional, their protein products are stable and that any transcriptional or translational regulatory mechanisms reported for the corresponding vertebrate genes have been eliminated successfully.

All three proteins caused characteristic wing phenotypes. Ubiquitous expression of Gli1 resulted in the interruption of cubital vein 4 (L4), closely resembling the effect of ubiquitous *ci* expression (Figure 1). In contrast, Gli3 expression resulted in small wings and in the loss of L3/L4 intervein tissue. We next monitored the expression of the two Ci target genes *patched (ptc)* and *hh*, which are specifically controlled by Ci activator and repressor function, respectively [9]. Gli1 and Ci had no effect on *hh* expression, but caused *ptc* to be expressed ectopically in the posterior wing compartment (Figure 1). Thus, they both possess activator function. In contrast, Gli3 expression reduced the levels of *hh* transcription, indicating that Gli3 has potent repressor activity.

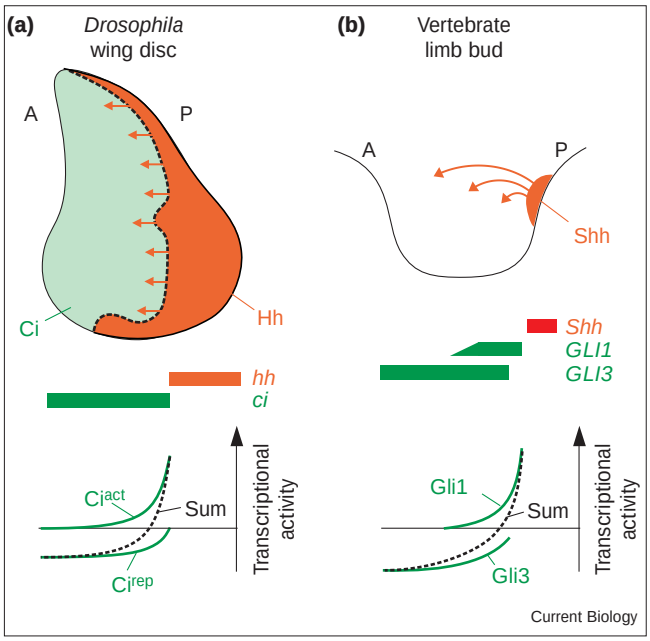
To test whether the transcriptional activator and repressor functions of Gli proteins are regulated by Hh signalling, we used posterior-compartment cells of the wing precursor tissue (imaginal discs) as an assay system. These cells are ideally suited to addressing this question because they do not express *ci*, hence the transgene-derived Gli protein can be studied in isolation; they do not normally express *ptc*, hence activator function can be readily observed (see above); they do express *hh*, simultaneously providing Hh ligand and the possibility of using an *hh* reporter gene to assay repressor activity; and finally, their ability to transduce the Hh signal can be controlled by genetically manipulating the function of *smoothened* (*smo*), which

Figure 3



Gli proteins can replace Ci during embryonic and larval development. Embryos lacking Ci die early in development with severe segmentation defects [25]. Ubiquitous expression of human Gli1 protein using the tubulin α1 promoter, however, rescues these animals to late larval stages. **(a–c)** Mutant discs were stained with an anti-Ci antibody. (b,c) No Ci protein was detected in rescued $ci^{-/-}$ larvae; note that $ci^{+/-}$ siblings were processed in parallel to control for the staining procedure (data not shown). **(d–f)** The expression of the Hh target gene *dpp* was monitored using a construct containing a fragment of the *dpp* disc enhancer (*dpp–lacZ*^{blink}; B. Müller and K. Basler, unpublished); β-galactosidase (green) was detected as in Figure 1. (e) Although Gli1 alone can rescue *ci* mutant embryos to late third instar, the wing discs of these animals are grossly enlarged and *dpp* expression is expanded anteriorly, indicative of a missing repressor function. (f) The combination of Gli1- and Gli3-expressing transgenes, however, results in discs of normal shape and size, and in a normal pattern of *dpp* expression. These animals die as pupae (or late larvae), possibly because *GLI1* and *GLI3* are expressed ubiquitously and not in the normal *ci* expression domain. Anterior is to the left and dorsal to the top.

Figure 4



Models comparing the integration of activating and repressive Gli activities in response to Hh and Shh signalling. **(a)** In *Drosophila*, *ci* is expressed uniformly throughout the entire anterior (A) compartment. Near the anterior–posterior boundary, Hh signal produced by posterior-compartment cells is transduced, causes the formation of a Ci activator form (Ci^{act} [8,9]), and prevents the formation of a repressor form (Ci^{rep} [7,9]). Together (sum), these two activities result in a spatially restricted, steep Ci activity gradient [9]. P, posterior. **(b)** In vertebrates, *GLI* genes are expressed in a more complex pattern surrounding sources of Shh [11]. In limb buds, *GLI1* expression is usually restricted to a region adjacent to the Shh source [26], with *GLI3* expression extending further to the anterior [15]. Our results indicate that Gli1 activator activity may be strongest close to the Shh source, rapidly fading as a function of the distance from the source. Moreover, Shh signalling appears to down regulate the repressor activity of Gli3; the activity with which Gli3 represses Shh target genes is therefore also a function of the distance from the source. Together (sum), the different transcriptional outputs may add up to an extended and versatile activity gradient mediating the Shh signal over a long range.

encodes an essential transducing component of the Hh pathway [16,17]. As shown in Figure 2, Gli1 expression activated *ptc* transcription in $smo^{+/-}$ posterior-compartment cells, but not in neighboring $smo^{-/-}$ cells, indicating that Gli1 possesses potent activator activity that is strictly dependent on Hh signal transduction. No effects were observed on *hh* transcription in posterior $smo^{-/-}$ cells, indicating that, unlike Ci, Gli1 does not acquire repressor activity in the absence of Hh signal transduction. Gli3, in contrast, possesses moderate repressor activity even in the presence of Hh signalling, and *hh* transcription was nearly abolished in $smo^{-/-}$ posterior-compartment cells expressing Gli3. This indicates that Gli3 repressor activity is maximal in the absence of, and is therefore negatively controlled by, Hh signalling. Together, these results indicate that the two Ci homologs differ in their ability to either repress or activate target genes: Gli1 functions as a Hh-regulated activator, and Gli3 as a Hh-regulated repressor.

One key finding of our analysis of Gli protein activities is that Hh signalling can not only tightly control the formation of an activator form ($Gli1^{act}$) but can also negatively regulate the activity of a repressor form ($Gli3^{rep}$). In *Drosophila*, these two modes of regulation operate on the product of a single gene, *ci*. It has been shown that both mechanisms are essential for Hh-mediated patterning [7–9]. The repressor function seems to be dispensable in early development, however, as a regulated 'activator-only' form of Ci, Ci^{U} [9], is sufficient to substitute for wild-type Ci during embryogenesis and early larval development. Indeed, we found that Gli1 could also provide this function, as it rescued *ci* null mutant animals to late larval stages when expressed from a tubulin α1–*GLI1* transgene (Figure 3). Thus, Gli1 is sufficient to mediate essential aspects of Hh signalling in embryos. During later stages of limb patterning, however, the repressor function of Ci is

required to repress *decapentaplegic* (*dpp)* and *hh* expression [9] and neither Ci^U nor Gli1 can provide this function. Instead, Gli3 may have taken over the important role of providing regulated repressor activity. A prediction of this assumption is that the combination of Gli1 and Gli3 activities should be able to substitute for Ci in limb patterning. Indeed, we found that proper regulation of Hh target gene expression was restored if *ci* null mutant animals were rescued by the concurrent introduction of transgenes encoding Gli1 and Gli3 (Figure 3).

Our findings indicate that, even without transcriptional regulation of *GLI* expression, the superimposition of two Gli protein activities can result in a Gli activity profile that mediates a precise Shh signalling output (Figures 3,4). The multiplication of an ancestral *GLI* gene might have enabled a more complex regulation of target genes and an increased flexibility in mediating the response to Hh. The fact that neither Gli1 nor Gli3 protein seems to have retained the entire complement of essential functions compared with its ancestor might have balanced the coexistence of their genes by rendering them functionally interdependent.

Materials and methods

Transgenes

The cDNAs encoding full-length human Gli1 and Gli3 and *Drosophila* Ci were modified to include c-Myc and triple hemagglutinin (HA) tags at the extreme amino and carboxyl termini, respectively. Constructs were inserted into pUAST [18] or into a P-element plasmid containing the promoter of the tubulin α1 gene [4]. We have also tested human Gli2 [19], and found it to have constitutive activator activity (data not shown). In contrast to *GLI1* and *GLI3*, however, none of the available human *GLI2* cDNAs appears to be full-length. Reporter genes used in this study were hh^{p30} [20], *ptc(10.8L)A* [21], and $dpp–lacZ^{blink}$ (a fragment from the *dpp* disc enhancer; B. Müller and K. Basler, unpublished). Additional transgenes were *hsp70–CD2* [22] and the Gal4 driver *C765* [23].

Marked clones of mutant cells

Flp-mediated mitotic recombination [24] was used to generate clones of cells lacking *smo* function. Genotypes and procedures were essentially as described [9].

Rescue of ci *null mutant embryos*

The ci^{94} mutation was used in conjunction with the $Dp(1;4)1021[y^+]$ chromosome [9]. This allowed the identification of homozygous mutant animals by their *yellow* mutant phenotype. Rescue was confirmed by the absence of anti-Ci antibody staining using heterozygous sibling larvae as controls.

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