Development 127, 4293-4301 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2607

Expression of the vertebrate Gli proteins in *Drosophila* reveals a distribution of activator and repressor activities

Pedro Aza-Blanc[1], He-Ying Lin[1], Ariel Ruiz i Altaba[2] and Thomas B. Kornberg[1,*]

[1]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA
[2]The Skirball Institute, Developmental Genetics Program and Department of Cell Biology, New York University School of Medicine, New York, NY 10016, USA
*Author for correspondence (e-mail: tkornberg@biochem.ucsf.edu)

Accepted 7 July; published on WWW 7 September 2000

SUMMARY

The Cubitus interruptus (Ci) and Gli proteins are transcription factors that mediate responses to Hedgehog proteins (Hh) in flies and vertebrates, respectively. During development of the *Drosophila* wing, Ci transduces the Hh signal and regulates transcription of different target genes at different locations. In vertebrates, the three Gli proteins are expressed in overlapping domains and are partially redundant. To assess how the vertebrate Glis correlate with *Drosophila* Ci, we expressed each in *Drosophila* and monitored their behaviors and activities. We found that each Gli has distinct activities that are equivalent to portions of the regulatory arsenal of Ci. Gli2 and Gli1 have activator functions that depend on Hh. Gli2 and Gli3 are proteolyzed to produce a repressor form able to inhibit *hh* expression. However, while Gli3 repressor activity is regulated by Hh, Gli2 repressor activity is not. These observations suggest that the separate activator and repressor functions of Ci are unevenly partitioned among the three Glis, yielding proteins with related yet distinct properties.

Key words: Ci, Gli, Transcription factor, *Drosophila*, Activator, Repressor

INTRODUCTION

During development of multicellular organisms, protein morphogens secreted from signaling centers provide spatial direction and coordination to neighboring cells. One of the principal morphogens is Hedgehog (Hh). Members of the Hh family of proteins are involved in organizing most organ systems in both vertebrates and invertebrates, apparently by directing a process that converts a varied landscape of Hh concentration into a variety of discrete responses. Although the consequences of Hh signaling are necessarily diverse, the Hh signal transduction pathway has been highly conserved during evolution. Remarkably, Hh signal transduction appears to funnel its information through a single output, the Ci/Gli transcription factors.

The role of Cubitus interruptus (Ci) in *Drosophila* Hh signal transduction is understood best in the wing imaginal disc. This organ is composed of an anterior (A) and a posterior (P) compartment and it is subdivided by the AP compartment border. Ci is produced exclusively in A compartment cells, where it specifies the A developmental pathway by regulating target genes such as *hh*, *patched (ptc)* and *decapentaplegic (dpp)*. Far from the AP compartment border, where the level of Hh signaling is low or absent, Ci functions as a transcriptional repressor to block *hh* expression and to keep *ptc* expression low. In A cells at the AP compartment border, where the level of Hh signaling is high, Ci functions as an activator to elevate *ptc* expression (Alexandre et al., 1996); at a distance of several cells from the border, it induces *dpp* (Maschat et al., 1998). Hh regulates this set of activities by affecting the cellular localization and structure of Ci.

The full-length, 155 kDa Ci protein accumulates in the cytoplasm, a consequence of active nuclear export and association with a microtubule-bound protein complex (Aza-Blanc et al., 1997; Chen et al., 1999; Robbins et al., 1997). In the absence of Hh signaling, PKA-dependent phosphorylation and proteolysis generates an N-terminal fragment of approximately 75 kDa (Ci^R) that concentrates in the nucleus and functions as a repressor. This is the form that predominates in A cells far from the compartment border. Hh signaling near the compartment border blocks Ci proteolysis and converts Ci to a protein with activator activity (Ci^{Act}) (Aza-Blanc et al., 1997; Méthot and Basler, 1999; Ohlmeyer and Kalderon, 1998). The form of Ci that activates transcriptional targets is not known, but the process that generates Ci^{Act} appears to be PKA-dependent (Wang et al., 1999) and to respond to the level of Hh signaling. Ci has a transcriptional activation domain in its C-terminal region that might interact with CBP/p300 (Akimaru et al., 1997).

There are three Gli proteins in vertebrates (Gli1, Gli2 and Gli3). These proteins have several regions with sequence homology, including a centrally located DNA-binding domain with five C2-H2 zinc fingers and a C-terminal transcription activation domain. These proteins have distinct activities and

are not functionally equivalent. Nevertheless, their partial redundancy and often overlapping domains of expression has made it difficult to define precisely their individual features and functions. The Glis have been characterized most extensively in the developing nervous system and limb buds. Their patterns of expression are dynamic and appear to be partly orchestrated by Shh (Hui et al., 1994; Lee et al., 1997; Marigo et al., 1996; Ruiz i Altaba, 1998; Sasaki et al., 1999), suggesting that the polarizing activity of Shh could derive from the sum of the particular distribution and functional properties of each of Gli protein. For example, during neural tube development, Shh secreted from the floor plate defines the domains of expression of each Gli protein in the ventricular zone of the neural tube (Ruiz i Altaba, 1998). It restricts *Gli1* to the most ventral regions, places *Gli2* expression in a domain that extends from the ventral region above the *Gli1* domain to the most dorsal region where it overlaps with *Gli3*, and creates a graded pattern of *Gli3* expression in the most dorsal region. Other signals are thought to positively regulate the dorsal expression of *Gli2* and *Gli3*. There is a similar arrangement of domains of Gli expression in developing limb buds (Büscher and Rüther, 1998; Marigo et al., 1996). Although it is not known how these patterns are established, *Gli1* expression can be regulated by Gli3 and possibly by Gli2 as well (Dai et al., 1999; Ding et al., 1998; Matise et al., 1998), suggesting that the Gli proteins may constitute a Hh-driven regulatory network.

The Gli proteins are thought to be similar to Ci and to have transcriptional activation and repression activities. In the neural tube, Gli1 induces the expression of *ptc1* and leads to the specification of ventral neural fates (Hynes et al., 1997; Lee et al., 1997; Ruiz i Altaba, 1998). Both its C-terminal activation domain and its N-terminal region are required for these functions; there has been no indication that Gli1 functions as a repressor in these contexts. The role of Gli2 appears to be more complex, as available evidence suggests that Gli2 is both an activator and a repressor. Ectopic expression of Gli2 in the floor plate can inhibit floor plate induction by Gli1 (Ruiz i Altaba, 1998), C-terminally truncated forms act as dominant negatives (Ruiz i Altaba, 1999), and N-terminal regions of Gli2 can repress transcription when they are fused to a heterologous DNA-binding domain (Sasaki et al., 1999). Conversely, *Gli2* can be induced by Shh and can itself induce motorneuron differentiation (Ruiz i Altaba, 1998). *Gli2* deletion mutants that lack the N-terminal region can induce HNF3β, a function that is normally carried out by Gli1 (Hynes et al., 1997; Lee et al., 1997; Sasaki et al., 1997). Gli3 also has both inductive and repressive activities (Brewster et al., 1998), and its relationship to Shh is complex. Shh inhibits *Gli3* expression and Gli3 represses *Shh* expression, and the induction activity of Gli3 may be independent of Shh (Büscher et al., 1997; Marigo et al., 1996; Ruiz i Altaba, 1998). C-terminally truncated forms that mimic mutations found in human syndromes function as dominant negatives (Biesecker, 1997; Ruiz i Altaba, 1999; Shin et al., 1999). Together, these results indicate that the Gli proteins form a regulatory network that responds to Hh signaling and that the Gli proteins have distinct properties.

The parallels between Ci and the Gli proteins with respect to their primary structure, functions as transcription regulators and regulation by Hh signaling are both striking and extensive. Although significant questions remain to be answered before we understand key aspects of Ci (for instance, the form that represents Ci^{Act} remains unidentified), work in the *Drosophila* system has established the conceptual basis for the role of these proteins as the conduit of Hh signaling. Cells change both the structure and intracellular address of Ci in order to orchestrate a programmed response to different concentrations of Hh. How the functions of the three Gli proteins correspond to the multiple functions of Ci and how they mediate responses to Hh have yet to be elucidated. In order to address these issues, we expressed the three Gli proteins in *Drosophila* and monitored them in the presence or absence of Hh signaling. We found that their behavior in *Drosophila* is consistent with the properties of the Gli proteins that have been observed in their native environment. Moreover, their behavior was also remarkably similar to Ci, suggesting that the essential aspects of the mechanisms involved in Ci/Gli-mediated Hh signaling have been tightly constrained during evolution. Interestingly, the various activities of Ci appear to have been distributed among the different Glis.

MATERIALS AND METHODS

Expression constructs

UAS-Myc-Gli constructs were derived from plasmids containing frog Gli1 and Gli2 and human Gli3 cDNAs fused with six N-terminal Myc tags (Brewster et al., 1998; Lee et al., 1997). UAS-Myc-Gli2 was constructed by inserting the *Bam*HI-*Xho*I fragment into the *Bgl*II and *Xho*I sites of pUAST (Brand and Perrimon, 1993). UAS-Myc-Gli1 and UAS-Myc-Gli3 were constructed by first subcloning the 6xMyc tag into Bluescript KS+ at the *Bam*HI-*Eco*RI sites, adding the *Eco*RI fragments containing Gli1 and Gli3 sequences, and transferring the fusions into pUAST.

Expression of GLI proteins in imaginal discs

UAS-Gli transgenes were activated in the presence of *en*-GAL4 and third instar imaginal discs were stained with anti-Myc antibody to gauge levels of expression. Approximately equivalent levels of expression were achieved by adjusting the temperatures of incubation: UAS-Myc-Gli11, 25°C; UAS-Myc-Gli2 and UAS-Myc-Gli3. 20°C. To express Gli1 in a *hh* mutant background, UAS-Myc-Gli1/SM5;hh^{ts2}/TM6b was crossed with *en*-GAL4/SM5;hh^{ts2}/TM6b at 17°C until early third instar, and then shifted to 28°C. After 24 hours, larvae were genotyped by the presence or absence of TM6b and discs were dissected for analysis. Expression of Gli1 in posterior compartments of fu^1 discs was measured in discs generated by crossing fu^1/FM7c; UAS-Myc-Gli1/SM5 females with *en*-GAL4 males at 20°C. Mutant males were identified by the absence of FM7c. For the experiment shown in Fig. 1, 10-12 wing discs expressing each Gli protein under *brinker* control were cut in half, and anterior and posterior portions were lysed separately in gel loading buffer.

Other strains used: *dpp-lacZ* (Blackman et al., 1991), as a reporter for *dpp* expression, a UAS-Lamin-GFP strain, provided by N. Stuurman to outline the contour of the nuclei shown in Fig. 5a,b (Stuurman et al., 1999), and UAS-*ptc*, to lower the level of Hh in discs shown in Fig. 6c,d (Johnson et al., 1995).

Transfections and immunoprecipitation experiments

S2 cells were transfected by the calcium phosphate method, harvested 24 hours after transfection and lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl 1 mM EGTA, 10 mM NaF, 1% NP-40, 10 mM benzamidine HCl, 1 mg/ml phenantroline, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin A and 100 mM PMSF. Co-immunoprecipitation of Gli1 with Fu was with 0.5 ml of lysis buffer per 10 cm plate of confluent cells. Precleared extracts were divided in 2, and incubated

with either 2 μg of affinity-purified polyclonal anti-Fu (Thérond et al., 1996) or with 2 μg of monoclonal anti-β-galactosidase (Promega). Immunoprecipitations were essentially as described (Robbins et al., 1997). Samples were separated in a 8% SDS-polyacrylamide gel and analyzed by western blotting using anti-Myc 9E10 monoclonal antibody.

Immunohistochemistry

Imaginal discs and S2 cells were stained using standard methods. Antibodies used were: monoclonal anti-Myc 9E10 (Santa Cruz), 1:150, polyclonal AbZn (Aza-Blanc et al., 1997), monoclonal anti-Ptc, 1:100 (Capdevila et al., 1994), polyclonal anti-β-galactosidase, 1:1000 (Jackson Labs).

RESULTS

Vertebrate Gli proteins are active in *Drosophila*

Three distinct activities of Ci are known to contribute to the development of the wing disc. Ci induces Hh targets *ptc* and *dpp* at the AP compartment border and represses Hh throughout the A compartment. These roles have been revealed in two ways: (1) in clones of *ci* mutant cells in the A compartment, by the absence of *ptc* and *dpp* expression and by the ectopic expression of *hh*, and (2) by the consequences of ectopic expression in the P compartment. Both *ptc* and *dpp* are induced when Ci is present in P compartment cells (Alexandre et al., 1996; Domínguez et al., 1996; Hepker et al., 1997) and, although the presence of Hh blocks conversion of Ci to Ci^R, *hh* is repressed when a transgene encoding Ci^R is expressed in P cells (Aza-Blanc et al., 1997). To test whether vertebrate Gli proteins can mimic some of these activities in imaginal disc cells, we generated strains of flies that harbor a Gli transgene. Termed UAS-Myc-Gli1, UAS-Myc-Gli2 and UAS-Myc-Gli3, these transgenes encode frog Gli1, frog Gli2 or human Gli3, respectively, and each adds N-terminal Myc epitope tags to the proteins. These chimeric genes were placed downstream of a promoter sequence containing a *S. cerevisiae* UAS and expressed throughout the P compartment (with *en*-GAL4).

Since the level of expression of a transgene is strain-dependent, we used mAb 9E10 to stain discs that had been isolated from several different strains and identified strains with approximately equivalent levels of expression. Expression levels were also normalized by adjusting the temperature at which the flies were cultured. In this way, we determined that expression in the chosen Gli1 strain at 25°C had similar levels of expression as the strains of Gli2 and Gli3 at 20°C.

Expression of the three Gli1 proteins in the *ptc* domain (with *ptc*-GAL4) did not alter vein morphology or growth (not shown). However, expression of Gli1 and Gli2 in the P compartment generated phenotypes resembling those observed after ectopic expression of Ci – alteration of vein pattern and overgrowth in the P compartment (Fig. 1). However, there were consistent differences between the Gli1 and Gli2 strains. In wings expressing Gli2, the proximal veins 3 and 4 were invariably fused (Fig. 1C; arrow). In the Gli1 strain, portions of vein 4 were frequently missing, but proximal vein 4, usually visible, was never fused with vein 3 (Fig. 1B). Since the region between veins 3 and 4 is regulated by Hh, these slight differences may reflect different activities of the Gli proteins on Hh signaling or *hh* expression in P cells.

Gli3 expression in the P compartment did not induce growth or vein pattern alteration, and analysis of *ptc* and *dpp* expression did not reveal induction of either of these targets (data not shown). Instead, the levels of *ptc* induced by Hh in A cells was diminished (data not shown), and veins 3 and 4 were slightly fused (Fig. 1D). These effects are consistent with a slight reduction in the levels of Hh and resemble the consequences of low-level expression of the repressor form of Ci (Aza-Blanc et al., 1997; Johnson et al., 1995). These observations suggest that Gli3 does not have any Ci-like inductive functions in imaginal discs. Rather, they suggest that Gli3 reduces *hh* expression in P cells, consistent with its role in vertebrates and its efficient conversion to a processed form.

Processing of vertebrate Glis in *Drosophila*

To ask if *Drosophila* can process vertebrate Gli proteins in a Hh-regulated manner, UAS-Myc-Gli1, UAS-Myc-Gli2 and UAS-Myc-Gli3 were expressed in wing discs driven by the *brinker* GAL4 line, 3S. This GAL4 line activates transcription in both the anterior and posterior flanks of 3rd instar imaginal discs (Ramirez-Weber and Kornberg, 1999). Dissection of these discs provided A fragments containing cells with little or no Hh signal and P fragments with Hh-stimulated cells.

Western analysis was carried out with an anti-Myc antibody (mAb 9E10). Gli1-containing disc fragments revealed a single band in both A and P fragments that corresponds to the full-length protein (apparent molecular weight of approximately 175 kDa; Fig. 2A, lane 1). No evidence of a smaller form of Gli1 that might be the product of limited proteolysis was observed. In contrast, extracts from Gli2 and Gli3 fragments yielded two species of proteins, apparent full-length forms (approximately 180-200 kDa) as well as smaller forms that migrated as doublets with approximate MWs of 90 kDa for Gli2 and 100 kDa for Gli3 (Fig. 2A, lanes 3 and 5). Since these bands were detected with an N-terminal-specific probe, they represent C-terminal deletions; their size indicates that they lack sequences C-terminal of their zinc finger DNA-binding domain and have a primary structure similar to that of Ci^R. The proportion of 90 kDa Gli2 was low relative to the full-length form, and it did not change significantly with Hh signaling (Fig. 2A, lane 4). In contrast, the proportion of Gli3 in the two forms was responsive to Hh signaling (Fig. 2A, lane 6). Fragments from the A compartment contained predominantly 100 kDa forms, whereas P fragments contained forms that were more full-length than the 100 kDa form. These results suggest that *Drosophila* can subject Gli2 and Gli3 proteins to limited proteolysis in a manner similar to its processing of Ci. However, there are significant differences both in the ratio of proteolysis (less efficient proteolysis of Gli2), and in the ability of Hh to regulate the proportion between intact and proteolyzed forms.

As summarized in the Introduction, Hh signal transduction controls the proportion of different Ci forms in an A cell, and directly or indirectly regulates their nuclear/cytoplasmic distribution (Aza-Blanc et al., 1997; Chen et al., 1999). Hh signaling decreases the nuclear concentration of Ci^R, at least in part by inhibiting Ci proteolysis. Hh signaling also increases both the nuclear and cytoplasmic levels of full-length Ci. Given the importance of subcellular localization to Ci function and the remarkable activity of the vertebrate Gli proteins in fly cells, we analyzed the distribution of Gli1, Gli2 and Gli3 in wing imaginal discs. Since disc epithelial

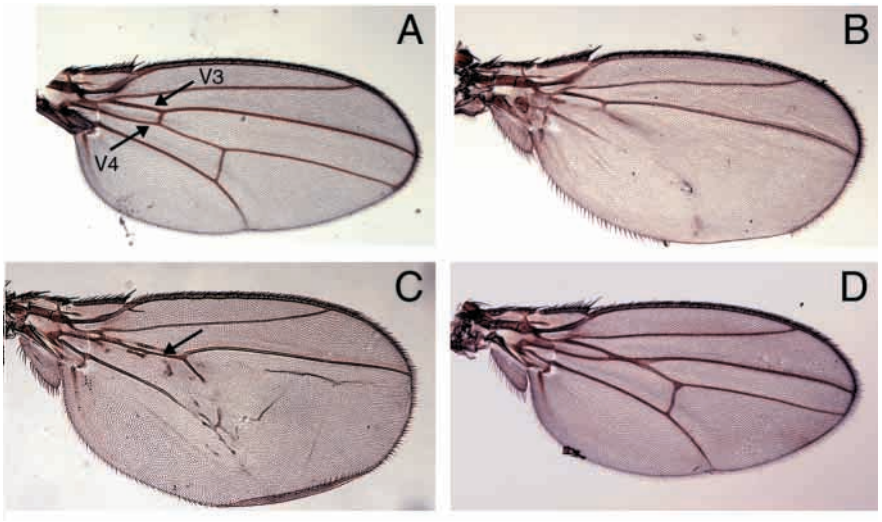

Fig. 1. Gli proteins can generate Ci-like phenotypes in *Drosophila* wings. A wild-type wing (A), and wings in which Gli1 (A), Gli2 (B) and Gli3 (C) proteins have been expressed under *en*-GAL4 control. Arrows in A indicate veins 3 and 4, and, in C, fused veins 3 and 4. Gli1 and Gli2 produced overgrowth of the posterior compartment; Gli3 did not.

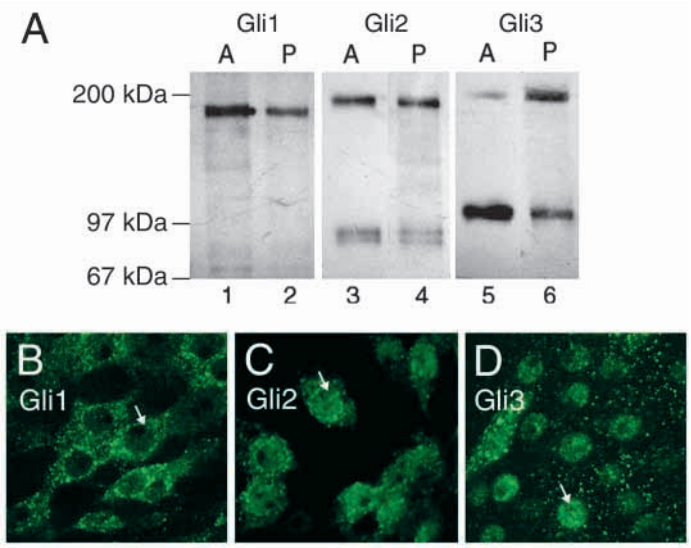

Fig. 2. (A) Gli proteins in wing imaginal disc cells respond to Hh signaling. A or P cells isolated from wing discs expressing each Gli protein under the *brinker*-GAL4 driver were subjected to western blot analysis with α-Myc antibody. The *brinker* driver activates expression in a discrete portion of the A compartment that has little or no Hh signaling (lanes 1, 3 and 5), and in P cells with active Hh signaling (lanes 2, 4 and 6). A single band of Gli1 corresponds to the full-length protein (1, 2); Gli2 (3, 4) and Gli3 (5, 6) have full-length as well as proteolyzed forms with apparent molecular weights of 90 kDa and 100 kDa, respectively. Gli3 proteolysis was reduced under Hh signaling. (B-D), subcellular distribution of Gli proteins. The nuclear/cytoplasmic disposition of Myc-Gli1, Myc-Gli2 and Myc-Gli3 was monitored in wing disc peripodial cells with α-Myc 9E10 antibody. Staining was distinctly particulate: Gli1 particles were mostly cytoplasmic, although some were nuclear (B); Gli2 particles were both nuclear and cytoplasmic (C); Gli3 particles were mostly nuclear, and some were cytoplasmic (D). Arrows point to nuclei.

cells are highly columnar and elongated, it is difficult to distinguish between their nucleus and cytoplasm. We therefore examined the Gli proteins in the larger and flatter peripodial cells. Fig. 2B-D shows peripodial cells containing low levels of Gli1, Gli2 and Gli3. For all three, anti-Myc mAb 9E10 stained discrete particles. Most Gli1 was detected in the cytoplasm, consistent with previous reports showing that Gli1, like Ci, contains a nuclear export signal that prevents its accumulation in the nucleus (Chen et al., 1999; Kogerman et al., 1999). Gli2 and Gli3 staining was detected in both cytoplasm and nucleus, in contrasting relative amounts. Gli2 was mostly cytoplasmic, while Gli3 was mostly nuclear. Since the Myc epitope tag recognized by mAb 9E10 is at the N terminus of the Gli proteins, the antibody detects both intact and proteolyzed forms. Therefore, although this data does not distinguish between intact and proteolyzed forms, the distribution in each case is consistent with the proportion of full-length and truncated protein, and with the conclusion that proteolyzed Gli products accumulate in the nucleus while intact forms accumulate in the cytoplasm.

Distinct activator functions of Gli1 and Gli2, and repressor activity of Gli2

To explore the basis for the different phenotypes produced by expression of Gli1 and Gli2 in P cells, we monitored the distribution of Ptc and Dpp proteins. The discs were the same genotype as above and were isolated from larvae grown under the same conditions. To analyze Gli regulation of *dpp*, the UAS-Myc-Gli1 and UAS-Myc-Gli2 transgenes were placed in *trans* to *en*-GAL4 and *dpp-lacZ*.

In discs with the UAS-Myc-Gli1 transgene, Ptc protein was present in P cells at levels significantly higher than the level of Ptc normally induced by endogenous Ci at the compartment border (Fig. 3A). Increasing Gli1 expression by shifting the culture temperature to 28°C led to even greater Ptc levels (Fig. 4C), suggesting that Gli1 is a potent activator of *ptc* expression. Gli1 induced expression of *dpp* to only a low level (Fig. 3C).

In contrast, the presence of Gli2 in P cells resulted in only low levels of Ptc (Fig. 3B). Moreover, when Gli2 expression was increased by raising the larvae at 28°C, the level of Ptc was reduced to an even lower level (Fig. 4E). We conclude that Gli2 has limited ability to induce *ptc* expression. Gli2 is a

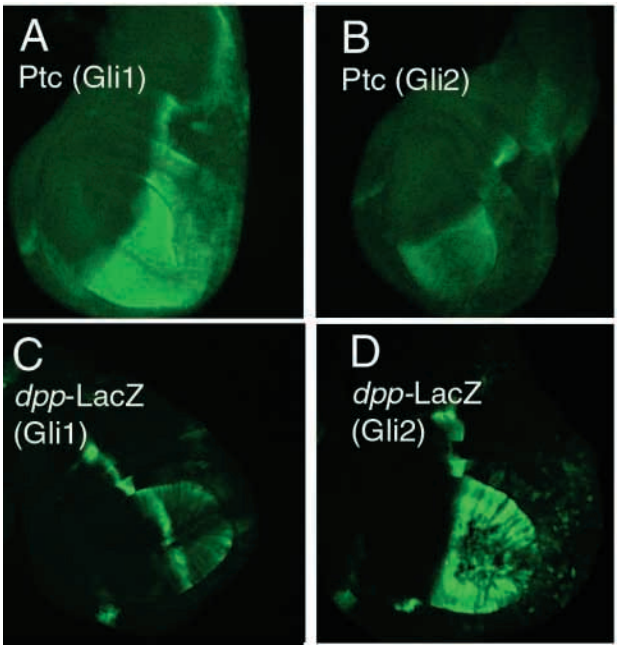

Fig. 3. Gli1 and Gli2 activate *ptc* and *dpp* differentially. In this and all subsequent figures, anterior is on the left and dorsal is uppermost. Ptc protein was abundant in P compartments expressing *en*-GAL4 and UAS-Myc-Gli1 (A), but was much less abundant in P compartments expressing UAS-Myc-Gli2 (B). *dpp* expression, monitored with a *dpp-lacZ* reporter and α-β-Galactosidase antibody, was induced to a low level by *en*-GAL4; UAS-Myc-Gli1 (C). *dpp* was induced to a uniformly high level by *en*-GAL4; UAS-Myc-Gli2 at 20°C (D).

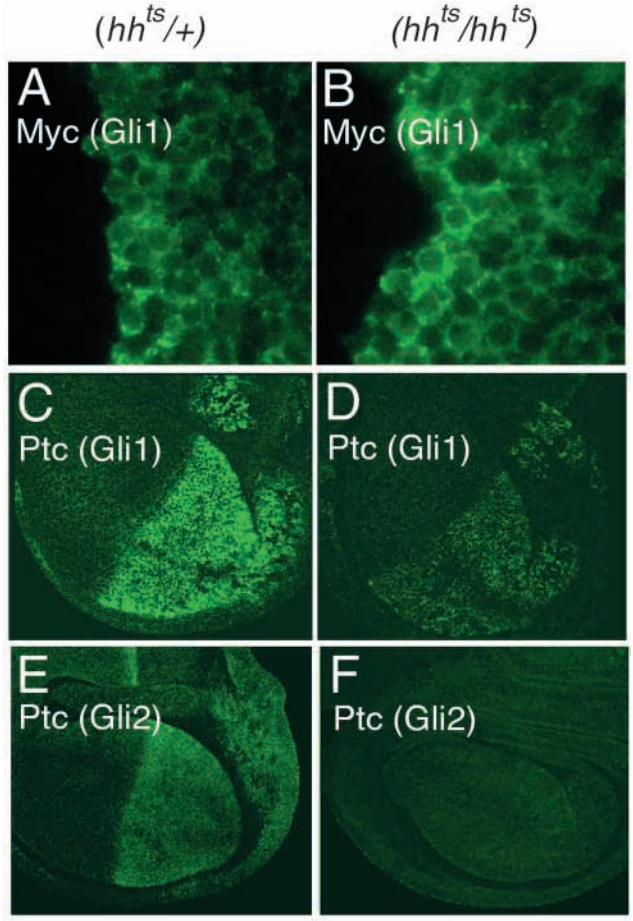

Fig. 4. Regulation of Gli1 and Gli2 activity by Hh. (A-D) To examine the role of Hh signaling in Gli1 distribution and activity, UAS-Myc-Gli1 was expressed in the P compartment with *en*-GAL4, either in the presence (hh^{ts}/+; a,c) or absence (hh^{ts}/ hh^{ts}; b,d) of Hh. Wing discs were stained with α-Myc antibody to monitor Gli1 (A,B) and α-Ptc antibody to monitor Ptc (C,D). (A,B) P cells expressing Gli1 near the compartment border are revealed by staining; the dark field to the left corresponds to the A compartment. Gli1 localizes predominantly in the cytoplasm, although, both in the presence and absence of Hh, Gli1 could be detected in the nucleus. Gli1 accumulation in the cytoplasm was slightly elevated in the absence of Hh, but its nuclear presence was unchanged. In the presence of Hh, Gli1 induced *ptc* expression to a high level (C). *ptc* induction by Gli1 was greatly reduced in the absence of Hh (D). (E,F) Expression of UAS-Myc-Gli2 with *en*-GAL4 either in the presence (hh^{ts}/+; (E) or absence (hh^{ts}/ hh^{ts}; (F) of Hh. Induction of *ptc* by high levels of Gli2 expression (at 28°C) was lower than at our standard levels (at 20°C, Fig. 3B) (E), and did not occur in the absence of Hh (F).

potent activator of *dpp*, however, and its presence in P cells led to high levels of Dpp in the wing pouch (Fig. 3D). Since wing veins 3 and 4 partially fused after ectopic expression of Gli2, we infer that Gli2 also reduced Hh levels (Fig. 1C). Such fusions can result from insufficient Hh signaling, and can be a consequence of either elevated levels of Ptc or diminished levels of Hh. The suggestion that *hh* expression is affected directly is consistent with the low level of Ptc, with the observation that Gli2 generates a Ci^R-like form and with the recent report that the N-terminal protein of the Gli2 contains repressor activity (Sasaki et al., 1999).

To summarize, both Gli1 and Gli2 have Ci-like activator functions that induce Hh targets at varied expression levels. These activator functions are distinguishable. Gli1 induces *ptc* expression strongly, but induces *dpp* expression at lower levels; Gli2 has complementary properties, inducing high levels of *dpp* expression but low levels of *ptc*. Gli2 also generates a repressor form that is able to reduce *hh* expression.

Regulation of Gli1, Gli2 and Gli3 by Hh signaling in *Drosophila*

The subcellular localization of Ci is regulated by Hh signal transduction (Aza-Blanc et al., 1997; Chen et al., 1999; Méthot and Basler, 1999). To determine if the Gli proteins are similarly affected when they are present in P cells, the influence of Hh on Gli distribution and on wing development was examined. Flies were generated that carried a UAS-Gli transgene and were either homozygous or heterozygous for a temperature-sensitive allele of *hh*. When wing discs were isolated from UAS-Gli1;hh^{ts2}/Tm6b or UAS-Gli1;hh^{ts2}/hh^{ts2} larvae that had been raised at 17°C and incubated at 28°C 24 hours prior to dissection. *ptc* expression was significantly reduced in the hh^{ts2}/hh^{ts2} discs (Fig. 4C,D), but no decrease in the nuclear Gli1 concentration was observed (Fig. 4A,B). Rather, general levels of Gli1 were slightly increased, suggesting that, in the absence of any Hh signal, Gli1 becomes more stable. Since Gli1 exists only as a full-length form, these results indicate that either nuclear staining is not a measure of Gli^{act}, the methods of histological analysis are insufficient to detect the differences in protein levels that produce such dramatic changes in target

gene expression, or there exists a different mechanism by which Hh regulates Gli function.

Gli2 activator function is also dependent on Hh. In hh^{ts2}/hh^{ts2} discs, *ptc* induction was abolished, (Fig. 4E,F), while no changes in subcellular localization of Gli2 could be observed (data not shown). In contrast, the distribution of Gli3 was strongly dependent upon Hh signaling. When UAS-Myc-Gli3 was expressed under *brinker* control (with 3S-GAL4), Gli3 in A cells was mostly in the proteolyzed form (Fig. 2A, ln5), and was mostly nuclear (Fig. 5A). In P cells, where Gli3 processing is attenuated, Gli3 did not accumulate in the nucleus, but was more evenly partitioned between nucleus and cytoplasm (Fig. 5B). An additional measure of Hh influence on Gli3 was obtained by ectopically expressing *ptc* in the P compartment along with UAS-Myc-Gli3. The presence of Ptc in the P compartment suppresses Hh function and leads to wing defects reminiscent of *fu* mutant phenotypes. Gli3 at low levels has similar effects (Figs 1C, 5C). However, as shown in Fig. 5D, expression of both *ptc* and Gli3 in the posterior compartment had an enhancing, synergistic effect, even at low levels of expression. The wings were significantly reduced in size and resembled those produced by expression of Ci^R in the posterior compartment (Aza-Blanc et al., 1997). Higher levels of expression of both transgenes were lethal. We interpret these results to indicate that a slight reduction in levels of Hh signal augmented the ability of Gli3 to repress Hh signaling. Since Hh signaling reduces Gli3 processing, we infer that Hh repression is carried out by the $Gli3^R$. Therefore, Gli3 behaves as a Hh-regulated repressor: repression of Hh expression by Gli3 can itself be inhibited by Hh, and removal of the Hh signal increases *hh* repression by Gli3.

The failure of Gli3 to induce *ptc* or *dpp* in imaginal discs is consistent with previous observations that Gli3 does not mediate Hh inductive functions in vivo (Ruiz i Altaba, 1999). However, this behavior contrasts with the demonstrated activator capability of Gli3, such as its induction of neuronal differentiation (Ruiz i Altaba, 1998), or activation of PTCH1 and Gli1 promoters in mammalian transfected cells (Dai et al., 1999; Shin et al., 1999). To explore further the behavior of Gli3, we overexpressed Gli3 in S2 cells. Co-transfection of UAS-Myc-Gli3 and actin-GAL4 resulted in accumulation of cytoplasmic particles of Ptc protein (Fig. 5E,F). Although overexpression conditions such as those in transfections might not accurately reflect in vivo function, the activator function of Gli3 in *Drosophila* cells indicates that the failure to induce Hh targets in imaginal discs was not indicative of the full potential of Gli3. It is possible that Gli3 targets genes other than those that we monitored (*ptc* and *dpp*).

Gli1 activity is regulated by the kinase Fused (Fu)

Ci is part of a microtubule-associated complex that includes the Cos2 and Fu proteins (Robbins et al., 1997; Sisson et al., 1997) and has also been observed to bind the Suppressor of Fused protein (Su(fu)) (Monnier et al., 1998). Although no evidence has been found that suggests a role for these

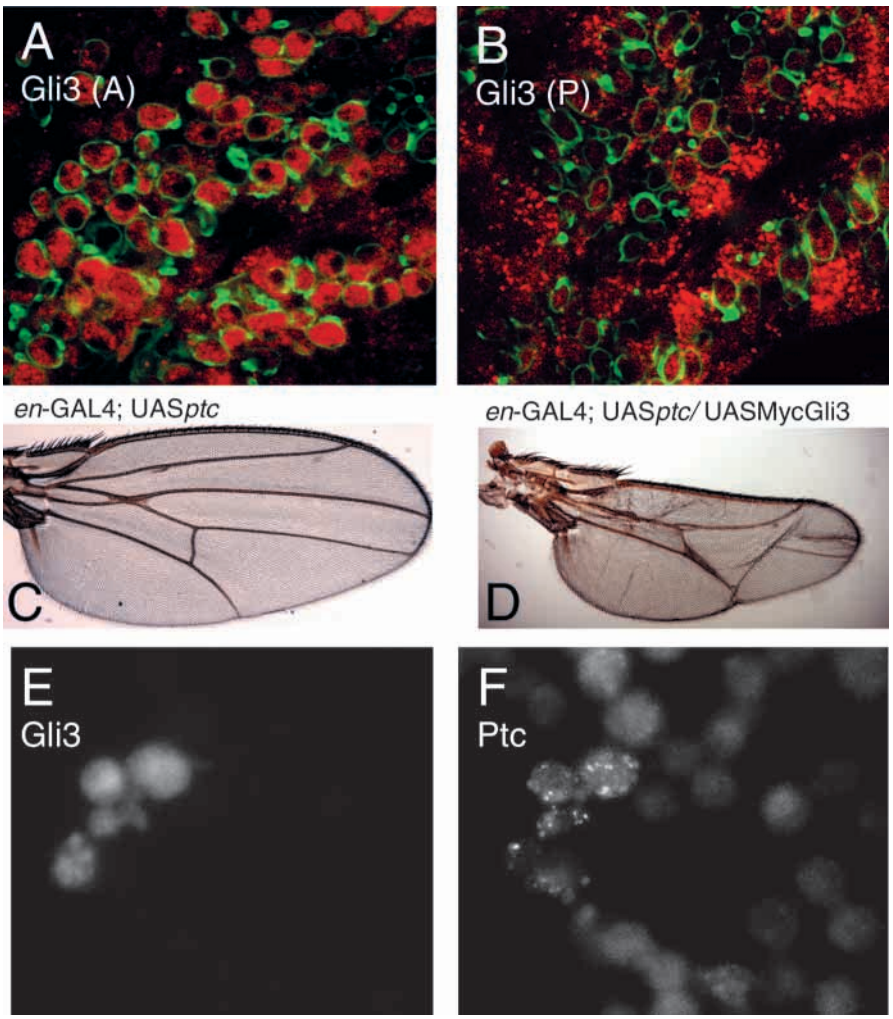

Fig. 5. Hh regulates Gli3 activity and localization. (A,B). Wing discs from larvae carrying 3S-GAL4, UAS-Lamin-GFP and UAS-Myc-Gli3 were stained with α-Myc antibody to detect Gli3 (red). GFP (green) outlines the nuclei. In A cells that have low or no Hh signaling, Gli3 particles localized primarily in nuclei (A). In P cells, Gli3 particles were both nuclear and cytoplasmic (B). (C) Adult wing phenotypes in flies with UAS-*ptc* and *en*-GAL4 raised at 17°C revealed a slight fusion between veins 3 and 4 (compare with a wild-type wing, Fig. 1A). This phenotype is similar to that produced by Gli3 expressed with *en*-GAL4 (see Fig. 1D). Flies carrying UAS-Myc-Gli3, UAS-*ptc* and *en*-GAL4 and raised at 17°C, had a much more severe phenotype (D): the central region of the wing, including veins 3 and 4 was absent, indicating a dramatic reduction of Hh signaling into the anterior compartment. (E,F) S2 cells co-transfected with UAS-Myc-Gli3 and actin-GAL4 were co-stained with α-Ci antibody (AbZn), which crossreacts with Gli3 (E) and with α-Ptc (F) to identify Gli3-expressing cells. Ptc protein was detected at low levels in nontransfected S2 cells, but was more abundant and had a particulate appearance in Gli3-expressing cells.

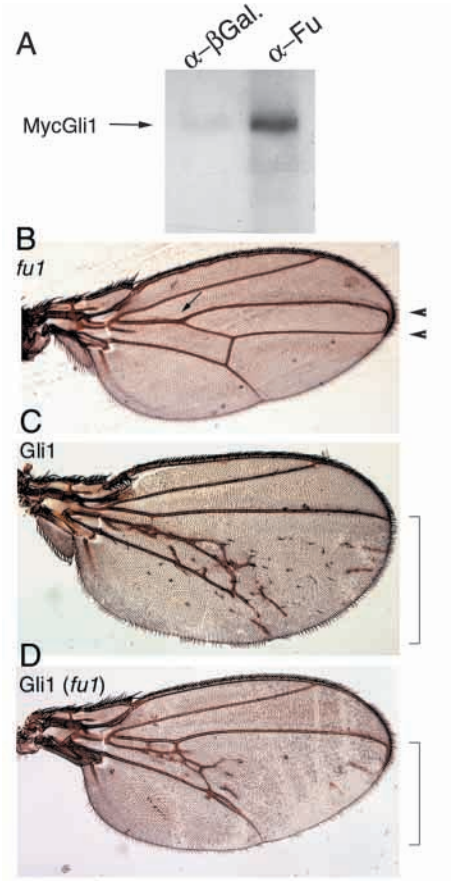

Fig. 6. Gli1 activity is regulated by the kinase Fused (Fu). (A) Extracts of S2 cells that had been co-transfected with UAS-Myc-Gli1 and Actin-GAL4 were immunoprecipitated with the α-β-galactosidase antibody (lane 1) or α-Fu (lane 2) and western analyses with α-Myc antibody were performed to detect Gli1. Gli1 was specifically co-immunoprecipitated with Fu. (B) In *fu*^{1} mutants, growth in the region between veins 3 and 4 is inadequate, seen here by the proximal fusion of veins 3 and 4 (arrow; compare with Fig. 2A). Arrowheads point to veins 3 and 4. (C) Gli1 expressed in the P compartment by *en*-GAL4 causes over-growth in the posterior region of the wing (bracket). Wings in (C) and (D) were from the same cross and, although the wings in different experiments had slight differences in venation (compare Fig. 1B and 6C), the effects on shape, size and proximal veins 3-4 were consistent aspects of the phenotype. (D) The overgrowth phenotype caused by ectopic expression of Gli1 is suppressed in males carrying the *fu*^{1} mutation.

associations, they are likely to help regulate Ci localization, proteolysis and/or activation. Recent reports have shown that Gli1 binds to human Su(fu) and that both this interaction and PKA antagonize Gli1 activity (Kogerman et al., 1999; Pearse et al., 1999; Ruiz i Altaba, 1999; Stone et al., 1999). In addition, recent studies have suggested that Ci regulation is a multistep process involving the independent actions of PKA and Su(fu) (Wang et al., 1999). Since Gli1 acts as a regulated transcriptional activator in our *Drosophila* assays, we wanted to determine whether its regulation also involves the interaction with known Ci partners. S2 cells were transfected with UAS-MycGli1 and Actin-GAL4 plasmids and, after 24 hours, α-Fu was used for immunoprecipitation assays. Western analysis revealed that Gli1 can be specifically co-immunoprecipitated with Fu (Fig. 6A).

To characterize further the functional interactions between Gli1 and Fu, we examined the role of *fu* in the wing phenotype caused by ectopic expression of Gli1. Gli1 induces overgrowth when expressed in the P compartment (Fig. 6C) and, in *fu* mutants, cells in the region between veins 3 and 4 fail to proliferate normally (Fig. 6B). As shown in Fig. 6D, the absence of Fu kinase activity in *fu*^{1} mutant wings partially suppresses Gli-induced overgrowth, while the 3-4 intervein region defect is partially rescued by the presence of Gli1. Since ectopic expression of Ci can also suppress the fu phenotype in the 3-4 intervein region (Alves et al., 1998), this data shows that Gli1 can function in an analogous manner.

DISCUSSION

This study was designed to ascertain whether the vertebrate Gli proteins are regulated by proteolysis in a manner analogous to their *Drosophila* homolog, Ci. The current lack of high-titer-specific antisera limits analyses of Gli proteins in vertebrate embryos, so we expressed tagged versions of the Gli proteins in *Drosophila*. This approach is predicated on the assumption that *Drosophila* can recognize the Gli proteins appropriately despite the many sequence differences that distinguish the Gli proteins and Ci. We also investigated the capacity of the Gli proteins to interact with other components of the *Drosophila* Hh signaling system and to function as transcriptional activators and repressors. We find that *Drosophila* can recognize the Gli proteins and, to a remarkable degree, the Gli proteins can transduce Hh signals in *Drosophila*.

Our results are in agreement with those of Mering et al. (1999), who showed that Gli1 and Gli3 can together rescue a Ci mutant (Mering and Basler, 1999). We have shown that Gli1 and Gli3 proteins can transduce Hh signals in *Drosophila* and that both proteins are regulated by Hh: Gli1, as an inducible activator, and Gli3 as a regulated repressor. We have also characterized Gli2, the Gli protein whose role and properties are least well understood. Gli2 shares functional properties with Gli1 and Gli3. We show here that Gli2 contains a repressor activity able to inhibit *hh* expression in vivo, as well as an activator activity that is Hh sensitive. Recent studies have shown that Gli1 function is dispensable in mice if both copies of the *Gli2* gene are present (Park et al., 2000), suggesting that Gli2 can compensate for the absence of Gli1 if it is present in sufficient amounts. Our results suggest an explanation for this interaction, since Gli2 can mimic Gli1 as a regulated activator.

We also found that both Gli2 and Gli3 can be proteolyzed in *Drosophila* in a manner similar to Ci. In addition, these experiments have allowed us to detect subtle, yet significant differences between Gli2 and Gli3 that might explain their distinct properties in vivo. First, Gli2 accumulated less proteolytic product than Gli3, and this correlates with its lower repressor activity. We believe that this difference is not an artifact of the *Drosophila* system, since transfection of Gli2 and Gli3 constructs into 10T1/2 cells generated a similar profile of proteolysis (more Gli3 proteolytic product than Gli2; not shown; P. A.-B. and T. B. K., unpublished).

Second, Hh affected Gli2 and Gli3 differently. No change in the proportion of cleaved and uncleaved Gli2 was observed in

our studies, indicating that the production of Gli2 repressor is not regulated by Hh. In contrast, Hh inhibited the accumulation of cleaved Gli3, reversing the proportion of full-length and processed forms. Consistent with this behavior in the fly system, production of Gli3 repressor is also regulated in the vertebrate limb bud, where it forms an anteroposterior gradient in response to Sonic Hh (Wang et al., 2000). Further studies on Gli2 in vertebrate systems will be required to validate our observations.

Implications of the activity profiles of Ci and Gli proteins

There are numerous examples of conserved function and regulation between vertebrate proteins and their *Drosophila* counterparts. Nevertheless, the conservation of the many aspects of Ci function and regulation seem remarkable. Ci is thought to interact with a number of different proteins, both as a resident in a protein complex tethered in the cytoplasm and as a nuclear transcription factor, and it is assumed that these interactions are critical to its ability to regulate its activities. Domains responsible for these presumed interactions that provide activities for transcriptional activation and repression, cytoplasmic and nuclear localization, DNA binding, regulated proteolysis and association with the tethered complex, map to multiple regions of the protein. We have shown that Gli1 can be co-immunoprecipitated with Fu and regulated by it, providing direct evidence for its ability to associate physically with a *Drosophila* partner. Since all three Gli proteins appear to function in a regulated manner in *Drosophila*, we presume that they retain many or most functional contacts, and it must be that the many regions responsible for these contacts are conserved. However, it seems likely that differences imposed by their particular structure delimit how they interact with other components of the pathway, since each of them retains some but not all aspects of Hh regulation.

Although in aggregate the Gli proteins appear to embody the many different attributes of Ci, only some of the Ci activities are in each. Most intriguing, perhaps, is the differential activation of *ptc* and *dpp* expression by Gli1 and Gli2, respectively. The basis for the selectivity of Gli1 for *ptc* and Gli2 for *dpp* is not understood, but it has many conceivable causes. One is that Gli2 interacts with proteins known to associate with Ci, such as CBP (Akimaru et al., 1997), but that Gli1 does not. Alternatively, the ability of Gli2 to activate *dpp* more strongly could be related to the conversion of Gli2 to a repressor form. It is formally possible that the activator and repressor forms can cooperate in some manner to enhance *dpp* transcription, or that the repressor form competes with the activator for binding sites at the *ptc* promoter. Consistent with this latter proposal, we observed that the level of *ptc* induction in wing discs was inversely related to the level of Gli2 expression: higher levels of expression produced lower levels of *ptc*. Since Ci75 is abundant in A cells that express high levels of *dpp*, but it is not in cells closer to the compartment border where *ptc* is expressed, this model may be relevant to Ci. Perhaps the most interesting possibility to consider is that the reason for the differential activation of *dpp* and *ptc* may be that Gli1 and Gli2 represent different forms of Ci^{Act}, one with a preference for *ptc* and the other for *dpp*.

The finding that the individual Gli proteins contain a subset of the activities retained by Ci suggests that evolution has dispersed these functions in the course of gene duplication and diversification. It also suggests that proteins like Ci can be considered to represent composites whose multiple functions are compressed into a single polypeptide. If Ci is only one example of many such proteins, then such composite proteins might contribute significantly to the complexity of functions encoded by the *Drosophila* genome. The *Drosophila* genome is thought to be especially compact, with fewer genes even than *C. elegans* (Rubin et al., 2000). It has been assumed that splicing variants and alternative promoters account for additional complexity. The Ci paradigm suggests that composite proteins may contribute as well.

We thank K. Nybakken for anti-Fu antibody; I. Guerrero and N. Gorfinkel for anti-Ptc antibody; Nico Stuurman for the lamin-GFP strain; Shelly Halpain for microscope use; Bill McGinnis for his generous hospitality; and, for support, the California Division of the American Cancer Society for a senior fellowship (#1-32-98) to P. A.-B and the March of Dimes and the NIH for grants to T. B. K.

REFERENCES

Akimaru, H., Chen, Y., Dai, P., Hou, D. X., Nonaka, M., Smolik, S. M., Armstrong, S., Goodman, R. H. and Ishii, S. (1997). Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature* **386**, 735-8.

Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of *hedgehog* target genes in Drosophila is mediated directly by the Cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003-13.

Alves, G., Limbourg-Bouchon, B., Tricoire, H., Brissard-Zahraoui, J., Lamour-Isnard, C. and Busson, D. (1998). Modulation of Hedgehog target gene expression by the Fused serine-threonine kinase in wing imaginal discs. *Mech. Dev.* **78**, 17-31.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-53.

Biesecker, L. G. (1997). Strike three for GLI3. *Nature Genet.* **17**, 259-60.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. and Gelbart, W. M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. *Development* **111**, 657-666.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brewster, R., Lee, J. and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393**, 579-83.

Büscher, D., Bosse, B., Heymer, J. and Rüther, U. (1997). Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. *Mech. Dev.* **62**, 175-82.

Büscher, D. and Rüther, U. (1998). Expression profile of Gli family members and Shh in normal and mutant mouse limb development. *Dev. Dyn.* **211**, 88-96.

Capdevila, J., Estrada, M. P., Sanchez-Herrero, E. and Guerrero, I. (1994). The Drosophila segment polarity gene patched interacts with decapentaplegic in wing development. *EMBO J.* **13**, 71-82.

Chen, C. H., von Kessler, D. P., Park, W., Wang, B., Ma, Y. and Beachy, P. A. (1999). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. *Cell* **98**, 305-16.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M. and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* **274**, 8143-52.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2533-43.

Domínguez, M., Brunner, M., Hafen, E. and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the *Drosophila* Gli protein Cubitus interruptus. *Science* **272**, 1621-25.

Hepker, J., Wang, Q. T., Motzny, C. K., Holmgren, R. and Orenic, T. V. (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. *Development* **124**, 549-58.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-13.

Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26.

Johnson, R. L., Grenier, J. K. and Scott, M. P. (1995). patched overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. *Development* **121**, 4161-70.

Kogerman, P., Grimm, T., Kogerman, l., Krause, D., Undén, A. B., Sandstedt, B., Toftgård, R. and Zaphiropoulos, P. G. (1999). Mammalian Suppresso-of-Fused moldulates nuclear-cytoplasmic shuttling of GLI-1. *Nature Cell Biol.* **1**, 312-319.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-52.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev. Biol.* **180**, 273-83.

Maschat, F., Serrano, N., Randsholt, N. B. and Geraud, G. (1998). engrailed and polyhomeotic interactions are required to maintain the A/P boundary of the Drosophila developing wing. *Development* **125**, 2771-80.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A. and Joyner, A. L. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759-70.

Mering, C. v. and Basler, K. (1999). Distinct and regulated activities of human Gli proteins in *Drosophila*. *Current Biol.* **9**, 1319-1322.

Méthot, N. and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-31.

Monnier, V., Dussillol, F., Alves, G., Lamour-Isnard, C. and Plessis, A. (1998). Suppressor of fused links fused and Cubitus interruptus on the hedgehog signalling pathway. *Current Biology* **8**, 583-6.

Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396**, 749-53.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashiima, M. and Joyner, A. L. (2000). Mouse *Gli1* mutants are viable but have defects in SHH signaling in combination with a *Gli2* mutation. *Development* **127**, 1593-1605.

Pearse, R. V., 2nd, Collier, L. S., Scott, M. P. and Tabin, C. J. (1999). Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators. *Dev. Biol.* **212**, 323-36.

Ramirez-Weber, F. A. and Kornberg, T. B. (1999). Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* **97**, 599-607.

Robbins, D. J., Nybakken, K. E., Kobayashi, R., Sisson, J. C., Bishop, J. M. and Therond, P. P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. *Cell* **90**, 225-34.

Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W. et al. (2000). Comparative genomics of the eukaryotes. *Science* **287**, 2204-15.

Ruiz i Altaba, A. (1998). Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* **125**, 2203-12.

Ruiz i Altaba, A. (1999). Gli proteins encode context-dependent positive and negative functions: implications for development and disease. *Development* **126**, 3205-3216.

Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-22.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M. and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915-24.

Shin, S. H., Kogerman, P., Lindstrom, E., Toftgard, R. and Biesecker, L. G. (1999). GLI3 mutations in human disorders mimic Drosophila cubitus interruptus protein functions and localization. *Proc. Natl Acad. Sci. USA* **96**, 2880-4.

Sisson, J. C., Ho, K. S., Suyama, K. and Scott, M. P. (1997). Costal2, a novel kinesin-related protein in the Hedgehog/Patched signaling pathway. *Cell* **90**, 235-245.

Stone, D. M., Murone, M., Luoh, S.-M., Ye, W., Armamini, M. P., Gurney, A., Phillips, H., Grush, J., Goddard, A., de Sauvage, F. J. et al. (1999). Characterization of the human Suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. *J. Cell Sci.* **112**, 4437-4448.

Stuurman, N., Delbecque, J. P., Callaerts, P. and Aebi, U. (1999). Ectopic overexpression of Drosophila lamin C is stage-specific lethal. *Exp. Cell Res.* **248**, 350-7.

Thérond, P. P., Knight, J. D., Kornberg, T. B. and Bishop, J. M. (1996). Phosphorylation of the fused protein in response to signaling from hedgehog. *Proc. Nat. Acad. Sci. USA* **93**, 4224-8.

Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-34.

Wang, G., Wang, B. and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus. *Genes Dev.* **13**, 2828-2837.