

Regulation of mammalian Gli proteins by Costal 2 and PKA in *Drosophila* reveals Hedgehog pathway conservation

Steven A. Marks* and Daniel Kalderon†

SUMMARY

Hedgehog (Hh) signaling activates full-length Ci/Gli family transcription factors and prevents Ci/Gli proteolytic processing to repressor forms. In the absence of Hh, Ci/Gli processing is initiated by direct Pka phosphorylation. Despite those fundamental similarities between *Drosophila* and mammalian Hh pathways, the differential reliance on cilia and some key signal transduction components had suggested a major divergence in the mechanisms that regulate Ci/Gli protein activities, including the role of the kinesin-family protein Costal 2 (Cos2), which directs Ci processing in *Drosophila*. Here, we show that Cos2 binds to three regions of Gli1, just as for Ci, and that Cos2 functions to silence mammalian Gli1 in *Drosophila* in a Hh-regulated manner. Cos2 and the mammalian kinesin Kif7 can also direct Gli3 and Ci processing in fly, underscoring a fundamental conserved role for Cos2 family proteins in Hh signaling. We also show that direct PKA phosphorylation regulates the activity, rather than the proteolysis of Gli in *Drosophila*, and we provide evidence for an analogous action of PKA on Ci.

KEY WORDS: Hedgehog, Costal 2, Ci, Gli, PKA, Signaling, *Drosophila*

INTRODUCTION

Extracellular Hedgehog (Hh) proteins regulate cell fates and cell proliferation in many animals by eliciting changes in gene transcription. In humans, alterations in Hh signaling are responsible for common birth defects and many types of cancer, motivating efforts to understand the biochemical basis of Hh signal transduction (Ingham and McMahon, 2001). Although early understanding of the mammalian pathway was based on conservation of molecules and interactions that were first uncovered in *Drosophila*, more recent genetic investigations in mouse have highlighted novel contributors and differences, including the central role of cilia in mammals but not in flies (Hooper and Scott, 2005; Huangfu and Anderson, 2006; Ingham and McMahon, 2001; Jiang and Hui, 2008; Wilson and Chuang, 2010). As investigations proceed independently in different organisms, it remains important to search for underlying common principles and mechanisms.

Important conserved elements of Hh signaling include the final transcriptional effectors. In *Drosophila*, *Cubitus interruptus* (Ci) can activate gene transcription as a full-length protein (Ci-155) or can generate a repressor with the same DNA-binding specificity after proteolytic processing to a truncated form (Ci-75). Hh blocks processing to Ci-75 repressor and increases the activity of full-length Ci-155 activator (Hooper and Scott, 2005; Jiang and Hui, 2008). In mammals, Gli1, Gli2 and Gli3, which bind the same DNA sequences as Ci, collectively contribute Hh-regulated repressors and activators. Gli3 is the principal source of a processed transcriptional repressor, Gli2 is the primary regulated activator,

whereas Gli1 only forms an activator and is generally expressed only in response to Hh pathway activity (Huangfu and Anderson, 2006; Wilson and Chuang, 2010). The Hh-regulated proteolytic processing of Ci, Gli2 and Gli3 is promoted by similar sets of clustered Protein Kinase A (PKA; PKA-C1 – FlyBase) sites and PKA-primed Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3; Sgg – FlyBase) sites, leading to binding of an SCF ubiquitin ligase complex containing Slimb (in flies) or β -TRCP (in mammals), ubiquitination and proteasome-mediated digestion (Jiang, 2006). Gli1 has a related, but less extensive set of PKA/CK1 sites in an analogous position, but the role of these sites has not been determined.

Mammalian and *Drosophila* Hh pathways share several other general features (Huangfu and Anderson, 2006; Wilson and Chuang, 2010). The activities of full-length Ci and Gli proteins can be inhibited by the direct binding partner, Suppressor of fused [SUFU in mouse; Su(fu) in fly]. Both *Drosophila* and mammalian pathways are silenced in the absence of ligand by the activity of the Hh receptor Patched and pathway activity requires the seven transmembrane domain protein Smoothed (Smo). Smo activation probably triggers all intracellular responses to Hh. Neither the mechanism of Smo activation nor its immediate downstream consequences are well understood but these are areas where conservation of mechanisms is in question (Ruel and Therond, 2009; Varjosalo et al., 2006). Notably, these events are localized to non-motile cilia in mouse but not in fly, and a large segment of the C terminus of *Drosophila* Smo that harbors clustered PKA and CK1 sites required for activation is absent from mammalian Smo molecules. However, subcellular localization is probably also crucial for fly Smo activation (Denef et al., 2000) and mammalian Smo appears to undergo Hh-dependent conformational changes similar to those of *Drosophila* Smo (Zhao et al., 2007). Thus, clear differences in detail between *Drosophila* and mammalian Hh signaling pathways may mask some fundamentally conserved mechanisms.

In *Drosophila*, Fused (Fu) and Costal 2 (Cos2; Cos – FlyBase) are crucial mediators downstream of Smo activation. Fu is a protein kinase that is essential for strong Hh signaling in *Drosophila*

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

*Present address: Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32306, USA

†Author for correspondence (ddk@columbia.edu)

(Hooper and Scott, 2005); Fu can bind to both Smo and Cos2, and is activated by Hh. By contrast, genetic elimination of mouse Fu does not perceptibly affect Hh signaling (Wilson and Chuang, 2010). Cos2, a kinesin-family protein, can bind to Smo, Fu, Ci, PKA, CK1 and GSK3 (Zhang et al., 2005). It is required for Ci processing in the absence of Hh and it also plays several roles in responding to Hh, most probably in the activation of Smo and Fu, as well as in blocking Ci-155 processing. The closest mammalian relatives of Cos2 by sequence are Kif7 and Kif27, which probably originated by duplication of an ancestral gene. At the time of inception of this study it was suggested, on the basis of gene knockdown and other studies, that neither Kif7 nor Kif27 contributed to Hh signaling (Varjosalo et al., 2006). It was speculated that cilia might substitute for some or all of the functions attributed to Cos2 in fly. However, it was also known that Hh could regulate Gli proteins introduced into *Drosophila* (Aza-Blanc et al., 2000; von Mering and Basler, 1999). Given the central role of Cos2 in Hh regulation of Ci, we speculated that Cos2 might also be capable of regulating Gli proteins, implying the existence of an analogous, conserved regulatory interaction in mammalian cells. We therefore investigated the regulation of the activities of Gli1 activator and Gli3 repressor in fly with particular emphasis on the potential role of Cos2. We found that Cos2 is indeed central to regulating Gli activity in *Drosophila* and that Cos2 binds to three regions of Gli1, just as for Ci. We also identified Gli1 PKA sites that are key to Gli1 regulation in fly and found that PKA primarily limits activity rather than proteolysis of Gli1, contrasting with the most prominent role for PKA-mediated phosphorylation of Ci and Gli3.

MATERIALS AND METHODS

Mutagenesis and cloning

Human *Gli1* and *Gli3* cDNAs with N-terminal Myc and C-terminal HA tags (from Konrad Basler, University of Zurich, Switzerland) were altered by mutagenesis PCR (QuikChange, Stratagene) and sub-cloned into the *pUASattB* vector (Bischof et al., 2007) between *NotI* and *XbaI* restriction sites. *pUASattB Gli1* cDNAs were inserted at cytological position 86F. Mouse Kif7 cDNA (from Kathryn Anderson, Memorial Sloan Kettering Cancer Center, USA) was cloned into the *pUAS* vector and a *UAS-Kif7* line on chromosome 3 was used for experiments. DNAs encoding Gli1 and Gli3 fragments were cloned into pGEX2T between *Sall* and *NotI* sites to produce GST-fusion proteins in bacteria. Cos2- and Kif7-coding sequences were used to make Entry clones by TOPO cloning (Gateway Technology, Invitrogen) and transferred to destination vectors with an *actin5C* promoter and N-terminal triple HA or C-terminal Myc tags for tissue culture cell transfection as described previously (Smelkinson et al., 2007).

Kc cell extracts

Kc cells were cultured in Schneider's *Drosophila* media [+ 5% FBS + 1% Penicillin-Streptomycin (Gibco)] at 25°C. Plates (10 cm) were seeded with 1×10^7 cells; the media was changed 24 hours later, 3-4 hours prior to transfection. Actin-Myc-Cos2 or Actin-Myc-Kif7 DNA (15 µg) was transfected using a calcium phosphate protocol (Invitrogen). Cells were given fresh media 24 hours later and after a further 48 hours cells were lysed in 800 µl buffer [50 mM HEPES (pH 7.5), 1.25 mM EDTA, 50 mM NaF, 0.5% NP40, 1 mM DTT, 250 mM NaCl, 500 µM NaVO₃ and protease inhibitors (complete mini, Roche)] at 4°C.

GST pull-down assay

Approximately 200 ng of GST or GST-Gli protein purified from *E. coli* was bound to glutathione sepharose beads in lysis buffer and incubated with 200 µl of Kc cell extract containing tagged Cos2 or Kif7 for 2 hours at 4°C. Beads were washed three times in lysis buffer, eluted in SDS-PAGE loading buffer (50 mM Tris-HCl, 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol) and boiled for 5 minutes. Myc-Cos2 or

Myc-Kif7 protein was visualized using western blotting with mouse monoclonal 12CA5 (HA) or 9E10 (Myc) antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) with Alexafluor-680 anti-mouse secondary antibody. Blots were visualized using a LI-COR Odyssey Infra-red Imager.

In vitro phosphorylation

Approximately 200 ng of GST-Gli1 protein purified from *E. coli* was bound to glutathione sepharose beads in Gsk3 buffer [20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT] (NEB) for 1 hour at 4°C in a volume of 150 µl, washed twice with GSK3 buffer and incubated at 30°C for 30 minutes with GSK3 buffer (adjusted to 12.5 mM MgCl₂) plus 100 µM ATP and purified protein kinase [250 U PKA, 50 U GSK3 and/or 100 U CK1 (NEB)]. Beads were washed twice with lysis buffer and GST pull-down assays were performed as above.

Immunohistochemistry

Larvae were heat-shocked at 2nd instar for 1 hour at 37°C to induce recombinant clones and then kept at 25°C to drive moderate expression of UAS transgenes. At late 3rd instar, larvae were dissected into cold fixing buffer (4% paraformaldehyde) and rocked at 25°C for 30 minutes. Discs were blocked for 2 hours in blocking buffer [5% goat serum, 2% BSA in PBST for *hh-lacZ*; 50 mM Tris (pH 7.0), 150 mM NaCl, 0.5% NP40, 0.5% BSA for all other antibodies]. Antibodies used for in situ staining were: rabbit anti-β-galactosidase antibody (Promega), Cy3-conjugated mouse monoclonal antibody to Myc (Amersham), rabbit anti-HA tag (Abcam) and rat anti-Ci monoclonal 2A1 (Motzny and Holmgren, 1995). Secondary antibodies used were either anti-rabbit or anti-rat Alexafluor-594 (Molecular Probes).

Fly crosses

Females of the genotype *yw hs-flp; ubi-GFP FRT40A / CyO; C765 hh-lacZ / TM6B* or *ywflp; ubi-GFP FRT40A / CyO; C765 ptc-lacZ / TM6B* were crossed to either *yw; smo² FRT40A / CyO; UAS-(Ci or Gli) / TM6B* or *yw; smo² pka-c1^{B3} FRT40A / CyO; UAS-(Ci or Gli) / TM6B* males to generate negatively marked *smo* mutant or *smo pka* mutant clones in wing discs expressing Ci, Gli1 or Gli3 ubiquitously.

Females of the genotype *yw hs-flp; smo Ubi-GFP FRT42B P[Smo⁺] / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw; smo FRT42B cos2^{W1} / CyO; UAS-Gli / TM6B* males to generate negatively marked *smo cos2* mutant clones in wing discs expressing Ci or Gli1 ubiquitously.

Females of the genotype *yw hs-flp UAS-GFP; tub-Gal80 FRT40A / CyO; C765 hh-lacZ / TM6B* were crossed to either *yw; smo² FRT40A / CyO; UAS-Gli / TM6B* or *yw; smo² pka-c1^{B3} FRT40A / CyO; UAS-Gli / TM6B* males to generate positively marked *smo* mutant and *smo pka* mutant clones expressing Gli proteins.

Females of the genotype *yw hs-flp UAS-GFP; smo² FRT42D cos2² / CyO; C765 hh-lacZ / TM6B* were crossed to *ywflp; smo² FRT42D tub-Gal80 P[Smo⁺] / CyO; UAS-Gli (+/- UAS-cos2 or UAS-Kif7) / TM6B* males to generate positively marked *smo cos2* mutant clones expressing Gli proteins with or without Cos2 or Kif7.

Females of the genotype *ywflp; Ubi-GFP FRT40A / CyO; FRT82B Su(fu)^{LP} C765 ptc-lacZ / TM6B* were crossed to *yw; smo² FRT40A / CyO; UAS-Gli Su(fu)^{LP} / TM6B* males to generate *smo* mutant clones in *Su(fu)* mutant discs expressing Gli1 ubiquitously.

Females of the genotype *ywflp fu^{mH63}; FRT42D P[y⁺] P[Fu⁺] / CyO; C765 ptc-lacZ / TM2* were crossed to *yw; FRT42D P[y⁺] ubi-GFP / CyO; UAS-Gli1 / TM6B* males to generate negatively marked *fu* mutant clones in discs expressing Gli1 ubiquitously.

Females of the genotype *ywflp; Ubi-GFP FRT40A / CyO; (FRT82B Su(fu)^{LP}) C765 ptc-lacZ / TM6B* were crossed to *yw; smo² (pka-c1^{B3}) FRT40A / CyO; UAS-Ci-S849A (Su(fu)^{LP}) / TM6B* males to generate *smo* or *smo pka* mutant clones in wild-type or *Su(fu)* mutant discs expressing Ci-S849A ubiquitously.

Females of the genotype *yw hs-flp; smo Ubi-GFP FRT42B P[Smo⁺] / CyO; (FRT82B Su(fu)^{LP}) C765 ptc-lacZ / TM6B* were crossed to *yw; smo FRT42B cos2^{W1} / CyO; (Su(fu)^{LP}) UAS-Ci-S849A / TM6B* males to generate negatively marked *smo cos2* mutant clones in wild-type or *Su(fu)* mutant wing discs expressing Ci-S849A ubiquitously.

RESULTS

Gli3 processing in *Drosophila* requires PKA and Cos2

Gli3 acts principally as a Hh-regulated repressor of transcription in mammals (Huangfu and Anderson, 2006) and was shown also to generate a processed repressor in *Drosophila*, detected by western blot and by repressor activity (Aza-Blanc et al., 2000; von Mering and Basler, 1999). Gli3 repressor production in *Drosophila* was substantially but incompletely inhibited by Hh signaling. In *Drosophila* wing discs Hh is expressed in posterior cells, which normally signal to a band of adjacent anterior cells at the anterior-posterior (AP) border to inhibit production of Ci-75 repressor and activate full-length Ci-155 (Hooper and Scott, 2005). Ci-155 is required to activate several Hh target genes at the AP border, including *ptc*, whereas Ci-75 has an important role repressing transcription of *dpp* and *hh* itself in anterior cells that are not exposed to Hh. The activities of Ci or Gli transgene products can be assayed most simply in posterior compartment cells, which do not express endogenous Ci by using *ptc-lacZ* as a reporter for activators and *hh-lacZ* as a reporter for repressors. Thus, when a wild-type Ci transgene is expressed ubiquitously in wing discs it produces strong inhibition of a *hh-lacZ* reporter in posterior *smo* mutant clones, where Hh signaling is blocked, but not outside the clone where Hh signaling blocks Ci-155 processing (Methot and Basler, 1999) (see Fig. S1C in the supplementary material). Ubiquitous Gli3 expression, unlike Ci, reduced *hh-lacZ* expression in otherwise wild-type posterior wing disc cells (see Fig. S1AB in the supplementary material) and inhibited *hh-lacZ* expression only slightly more within *smo* mutant clones (see Fig. S1D in the supplementary material). However, when UAS-Gli3 expression was restricted to *smo* mutant clones using the MARCM method (Lee and Luo, 2001) we saw strong repression of *hh-lacZ* specifically within the clone (Fig. 1B). No *hh-lacZ* repression was observed in posterior Gli3-expressing clones that retained Smo activity and hence active Hh signaling (Fig. 1A). Thus, as previously asserted (Aza-Blanc et al., 2000; von Mering and Basler, 1999), Gli3 repressor formation was substantially inhibited by Hh signaling. In addition, because some Gli3 repressor is formed even in the presence of Hh signaling, we determined that it was essential to restrict Gli3 expression to posterior *smo* mutant clones to provide a sensitive assay for factors required for Gli3 processing.

We found that Gli3 did not repress *hh-lacZ* in *smo* mutant clones when those cells also lacked PKA activity (Fig. 1C). Conversely, even in clones that retained Smo activity, excess constitutively active PKA increased Gli3 repressor activity sufficiently to see clear *hh-lacZ* repression (Fig. 1D). Thus, Gli3 processing in flies, as in mammals, is promoted by PKA and depends on PKA.

Cos2 is required for Ci processing; it is believed to promote Ci phosphorylation at essential PKA, CK1 and GSK3 sites (Zhang et al., 2005). As Gli3 and Ci processing rely on very similar sets of phosphorylation sites (Jiang, 2006; Pan and Wang, 2006; Smelkinson et al., 2007; Tempe, 2006), we tested whether efficient Gli3 phosphorylation and consequent processing in fly might, like Ci, require Cos2. Indeed, Cos2 inactivation in *smo* mutant clones prevented Gli3 from inhibiting *hh-lacZ* (Fig. 1E). The inferred ability of Cos2 to promote Gli3 processing was surprising in light of earlier suggestions that Gli3 regulation in mammals did not require a Cos2 ortholog (Varjosalo et al., 2006). Not only is dependence of processing on Cos2 conserved between Ci and Gli3, but so too is the ability for Hh to block processing of Gli3 and Ci entirely through *Drosophila* signaling components.

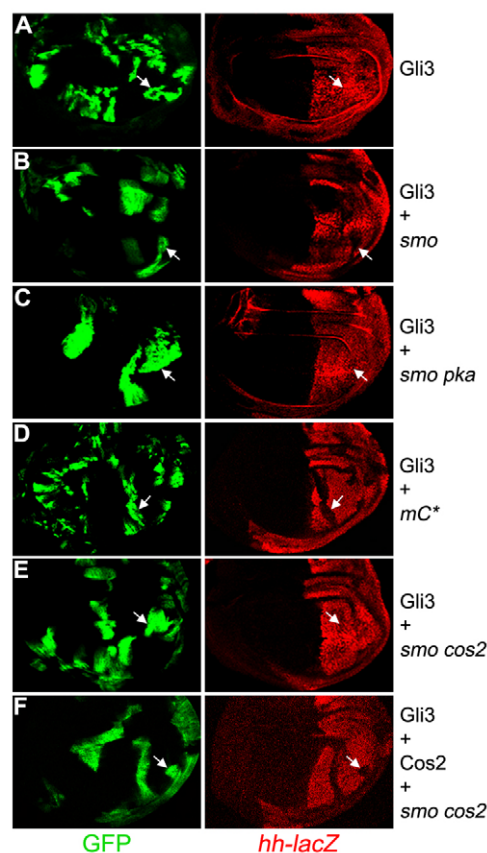


Fig. 1. Gli3 processing in *Drosophila* requires PKA and Cos2.

(A-F) Wing discs with MARCM (Lee and Luo, 2001) clones (arrows) expressing Gli3 were marked by GFP expression (green) and tested for repression of *hh-lacZ* expression (red) in posterior compartment (right) cells. Clones also lacked (B) *smo*, (C) *smo* and *pka* or (E) *smo* and *cos2* activities, or additionally expressed (D) activated mouse PKA catalytic subunit (*mC**) or (F) wild-type Cos2. Repression of *hh-lacZ* in B,D,F indicates Gli3 processing to a repressor form.

Gli1 activity in *Drosophila* is regulated principally by PKA and Cos2

Gli1 activity, unlike Gli2, is substantially regulated in mammals by transcriptional induction in response to Hh pathway activity (Huangfu and Anderson, 2006; Wilson and Chuang, 2010). However, Gli1 protein expressed from the Gli2 locus can rescue Gli2-deficient mice, implying that Hh can also regulate Gli1 protein activity post-transcriptionally (Bai and Joyner, 2001). Indeed, in tissue culture cells there is evidence that the levels, activity and subcellular localization of Gli1 can respond to Hh ligands (Barnfield et al., 2005; Huntzicker et al., 2006; Sheng et al., 2006). There is, however, no evidence for Gli1 producing a transcriptional repressor in any setting. Thus, Gli1 provides an opportunity to study conserved mechanisms for regulating Ci/Gli activators in fly without complications from repressor contributions.

We confirmed the prior observation that ubiquitous expression of Gli1 in wing discs led to ectopic activation of the Hh target gene reporter *ptc-lacZ* only in posterior cells where Hh is present (von Mering and Basler, 1999). Accordingly, *ptc-lacZ* induction was also lost in posterior *smo* mutant clones, clearly confirming that Gli1 is

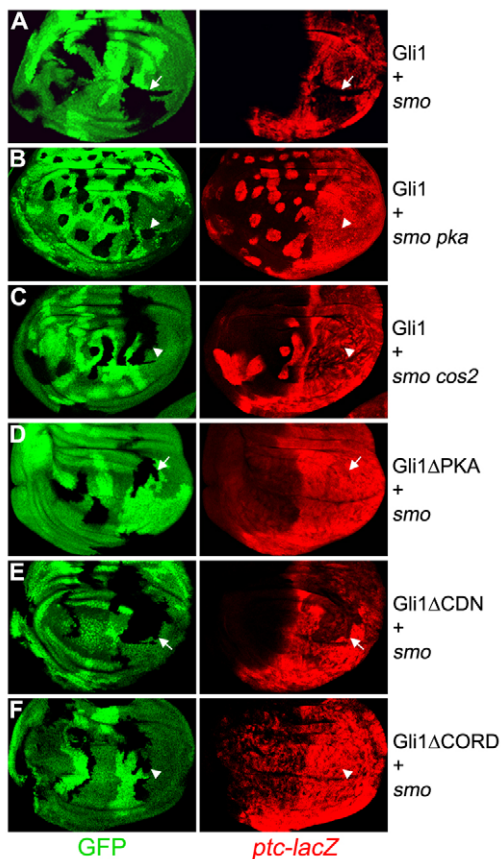


Fig. 2. PKA phosphorylation and Cos2 binding silence Gli1 in the absence of Hh signaling. (A-F) Wing discs expressing wild-type Gli1 or the indicated Gli1 variants ubiquitously using the *C765-GAL4* driver. Gli1 Δ PKA has S544A and S560A substitutions, Gli Δ CDN lacks residues 141-260, and Gli1 Δ CORD lacks residues 601-750. Activation of the Hh target gene *ptc-lacZ* (red) was measured by staining with antibody to β -galactosidase in wing discs containing clones (arrows) marked by loss of (green) GFP and lacking (A,D-F) *smo*, (B) *smo* and *pka*, or (C) *smo* and *cos2* activities. Gli1 activity is silenced within posterior (right) *smo* mutant clones (A) but is restored by loss of PKA (B), Cos2 (C), PKA sites S544 and S560 (D) or the CORD Cos2-binding domain (F).

activated by Hh signal transduction in *Drosophila* (Fig. 2A). In mouse, Gli activators are substantially inhibited by Suppressor of Fused in the absence of Hh ligands (Wilson and Chuang, 2010). We therefore expected a similar relationship in fly. However, elimination of Su(fu) did not allow Gli1 to induce *ptc-lacZ* in anterior cells or in posterior *smo* mutant clones (see Fig. S2A in the supplementary material). We also tested Gli1 variants lacking either a large N-terminal segment or a short motif, SYGH, that is known to contribute to Su(fu) association (Dunaeva et al., 2003). All Gli1 variants we tested were expressed from the same genomic location as for wild-type Gli1 to ensure equivalent expression (Bischof et al., 2007). Both Gli1 Δ 1-130 and Gli1 Δ SYGH induced *ptc-lacZ* in posterior cells but not in posterior *smo* mutant clones or in anterior cells, exactly as for wild-type Gli1 (Fig. 3A-C and see Fig. S2B,C in the supplementary material). Thus, as for Ci, factors other than Su(fu) suffice to inhibit Gli1 in the absence of Hh signaling in *Drosophila*.

In wing discs, strong induction of *ptc-lacZ* at the AP border requires Fu kinase activity, largely to antagonize repression by Su(fu) (Hooper and Scott, 2005). By contrast, Gli1 induced *ptc-*

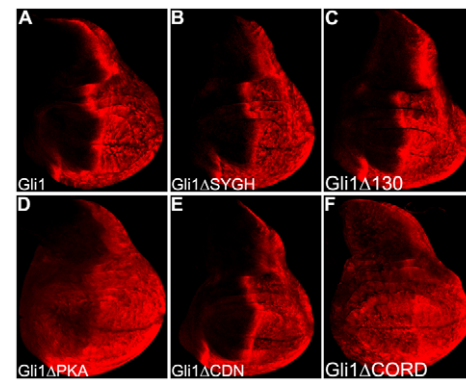


Fig. 3. Activity of Gli1 variants. (A-F) Wild-type Gli1 or variants were expressed throughout wing discs using *C765-GAL4* to test induction of the Hh target gene *ptc-lacZ* (red) in anterior cells (left) and in posterior cells (right), which produce Hh. Gli1 lacking the SYGH motif (B) or residues 1-130 (C), both of which are implicated in Su(fu) binding, or lacking CDN domain residues 141-260 (E) induced ectopic *ptc-lacZ* expression only in posterior cells. Gli1 lacking PKA sites S544 and S560 or CORD region residues 601-750 also induced *ptc-lacZ* expression weakly (D) or strongly (F) in anterior cells.

lacZ equally strongly in posterior cells in the presence or absence of Fu kinase activity, either in *fu* mutant clones or throughout *fu* mutant wing discs (see Fig. S2D-F in the supplementary material). Thus, Fu kinase activity is not required for Hh to stimulate Gli1 activity in *Drosophila*.

In the absence of Hh, full-length Ci levels and activity are restricted by PKA-dependent proteolysis (Smelkinson et al., 2007). We therefore tested whether PKA affected Gli1 activity. We found that Gli1 induced *ptc-lacZ* expression in posterior *smo* mutant clones if PKA activity was also inhibited (Fig. 2B). Thus, PKA inhibits Gli1 in the absence of Hh signaling.

A PKA site (T374) close to a Gli1 nuclear localization sequence was previously found to be necessary for a small repressive effect of PKA on Gli1 transcriptional activity, and was found to affect the nucleo-cytoplasmic Gli1 distribution in studies involving overexpressed proteins in mammalian tissue culture cells (Sheng et al., 2006). In those studies, a T374K substitution favored nuclear localization and T374D favored cytoplasmic localization of Gli1. We found that both Gli1 T374K and T374D variants induced *ptc-lacZ* in posterior but not anterior cells, and in posterior *smo pka* but not *smo* mutant clones, as observed for wild-type Gli1 (see Fig. S3A-D in the supplementary material). Thus, PKA is not regulating Gli1 activity in *Drosophila* detectably through T374.

PKA inhibition has been observed to stabilize Gli1 in NIH 3T3 cells (Huntzicker et al., 2006). In that study, Gli1 variants lacking a short putative β -TRCP binding motif, DSGVEM (residues 464-9 in mouse Gli1) or lacking sequences beyond residue 475 were also stabilized relative to wild-type Gli1 and failed to bind to β -TRCP, implying that PKA phosphorylation (probably at sites distal to residue 475) promoted β -TRCP-dependent proteolysis through, or in collaboration with, the DSGVEM motif. However, the role of specific Gli1 PKA sites was not tested. A cluster of PKA sites and surrounding PKA-primed CK1 and GSK3 sites are broadly conserved among Ci, Gli2 and Gli3, and in each case promote β -TRCP (Slimb in *Drosophila*) binding after extensive phosphorylation (Jiang, 2006). Gli1 preserves only part of this conserved region, spanning the first two conserved PKA sites (S544 and S560 in Gli1)

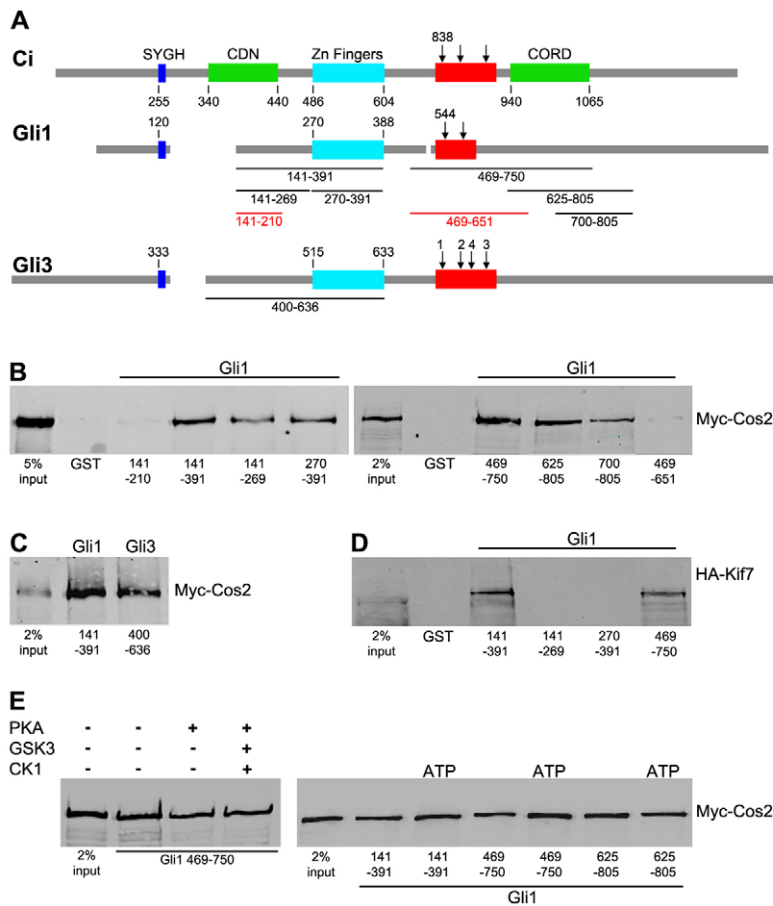


Fig. 4. Gli proteins have multiple Cos2-binding domains analogous to CDN, CORD and zinc-finger domains on Ci. (A) Schematic alignment of full-length Ci, Gli1 and Gli3 proteins using the conserved SYGH motif (blue), zinc fingers (turquoise) and PKA-nucleated phosphorylation region (red) as points of registration. Other elements, including CDN and CORD regions (green), cannot be aligned through primary amino acid sequence similarities. Lines indicate protein fragments fused to GST and found to bind Cos2 (black) or not to bind Cos2 (red). (B-E) Equal amounts of GST, GST-Gli1 or GST-Gli3 fragments (labeled by Gli residues included) were used to pull-down proteins from extracts of Kc cells expressing either Myc-tagged Cos2 or HA-tagged Kif7, and associated proteins were visualized on western blots with epitope tag antibodies. Either 2% or 5% of the extract used in each binding assay was also included on the western blots. (E) GST-Gli1 fusion protein was phosphorylated with the indicated kinase, or ATP was added to cell extracts (to 1 mM) where indicated, prior to mixing Kc cell extracts and GST proteins.

and including one inferred β -TRCP-binding site (Smelkinson et al., 2007). We found that Gli1 lacking PKA sites at 544 and 560 induced *ptc-lacZ* equally in posterior cells whether Smo was active or not (Fig. 2D). This variant, Gli1 Δ PKA, also induced ectopic *ptc-lacZ* in anterior cells, but to lower levels (Fig. 3D). We suspect that the different levels of *ptc-lacZ* in anterior cells compared with posterior *smo* mutant clones may result from Ci repressor in anterior cells partially countering the effects of Gli1 activator, as both proteins have the same DNA-binding specificity. In contrast to the activity of Gli1 Δ PKA (S544A/S560A) in the absence of Hh signaling, Gli1 with an S463A substitution in the DSGVEM motif behaved exactly like wild-type Gli1, inducing *ptc-lacZ* only in posterior cells in a Smo and PKA-regulated manner (see Fig. S3E-F in the supplementary material). Thus, PKA phosphorylation of Gli1 at S544 and S560 inhibits transcriptional activation by Gli1 independent of the DSGVEM motif.

As PKA phosphorylation of Ci is promoted by Cos2 (Zhang et al., 2005), we wondered whether Gli1 activity was influenced by Cos2. We found that the loss of Cos2 activity in posterior *smo* mutant clones allowed Gli1 to induce *ptc-lacZ* to the same level as observed in wild-type posterior cells (Fig. 2C). Thus, in the absence of Hh signaling, Cos2, like PKA, restricts Gli1 activity.

Cos2 binds to Gli proteins to regulate Gli activity

To explore how directly Cos2 regulates Gli1 activity in *Drosophila* we asked whether Gli1, like Ci, can bind to Cos2. Two Cos2-binding regions on Ci were initially defined, mainly through yeast two hybrid interaction assays (Wang et al., 2000; Wang and Jiang, 2004). One region immediately preceding the zinc-finger DNA-binding domain

was named CDN (residues 346-440 in Ci), and another immediately following the PKA-primed Slimb-binding region was named CORD (residues 940-1065). More recently, binding of tagged Cos2 from *Drosophila* Kc cell extracts to GST-Ci fusion proteins showed that the last three zinc fingers of Ci also can bind to Cos2 (Zhou and Kalderon, 2010). In contrast to the highly conserved zinc fingers, primary amino acid sequence within CDN and CORD domains is not recognizably similar among Ci and Gli proteins. We therefore tested a variety of Gli1 fragments as GST fusion proteins for their ability to pull down tagged Cos2 from Kc tissue culture cell extracts (Fig. 4A). Gli1 regions 141-269, 270-391, 469-750 and 625-805 bound well to Cos2, whereas 141-210 and 469-651 did not, implying binding domains in the same locations as the CDN, zinc-finger and CORD regions of Ci (Fig. 4B). A GST-Gli3 fusion encompassing CDN and the zinc fingers also bound well to Cos2 (Fig. 4C), although the region of Gli3 equivalent to the Ci CORD domain was not tested for lack of soluble fusion proteins. Thus, both Gli1 and Gli3 can bind Cos2. Moreover, three distinct binding regions are conserved in their location between Gli1 and Ci, despite an absence of sequence conservation in the CDN- and CORD-binding sites.

There is evidence that binding of Cos2 to the CORD domain of Ci can be regulated, at least in vitro. First, phosphorylation of Ci at a PKA site in the CORD domain and at residues primed by PKA sites in the adjacent Slimb-binding region reduce binding to Ci (Zhou and Kalderon, 2010). By contrast, the binding of GST-Gli1 fragments containing the equivalent phosphorylation sites was not altered by phosphorylation with PKA alone or together with CK1 and GSK3 (Fig. 4E). Second, binding of Cos2 to Ci-CORD is increased more than fivefold by adding ATP to 1 mM (Zhou and

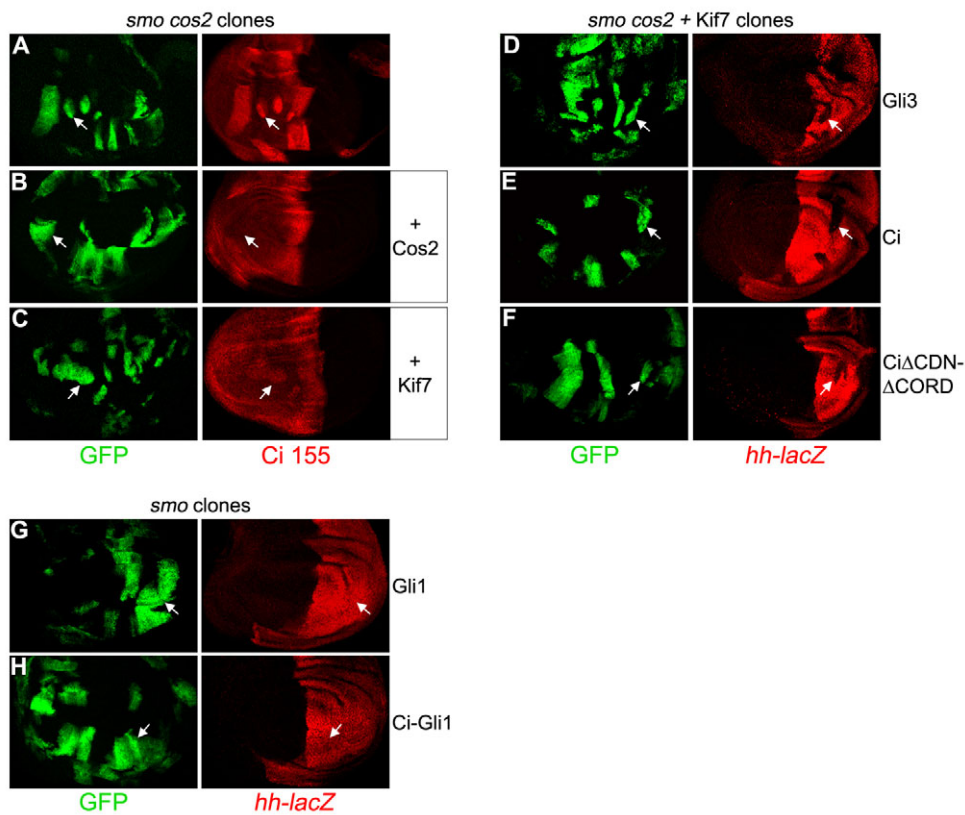


Fig. 5. Kif7 can promote Ci and Gli3 processing but Gli1 proteins are not processed in *Drosophila*. (A-C) Wing discs with MARCM (Lee and Luo, 2001) *smo cos2* mutant clones (arrows) expressing either (B) *UAS-cos2* or (C) *UAS-Kif7* were marked by GFP expression (green). Ci-155 levels, revealed by 2A1 antibody staining (red) were increased roughly to AP border levels in *smo cos2* mutant clones unless rescued by *UAS-cos2*. (D-H) Wing discs with MARCM clones (arrows) expressing Ci or Gli proteins in the absence of *smo* activity were marked by GFP expression (green) and tested for repression of *hh-lacZ* (red) in posterior compartment (right) cells. (D-F) Clones also lacked *cos2* activity but expressed Kif7. Kif7 allowed processing of (D) Gli3, (E) wild-type Ci and (F) Ci lacking CDN and CORD Cos2-binding domains, eliciting *hh-lacZ* repression in clones. (G) Wild-type Gli1 or (H) Gli1 with residues 1-524 substituted by residues 1-829 of Ci (Ci-Gli1) did not produce a repressor form.

Kalderon, 2010). By contrast, ATP did not substantially affect binding of Cos2 to Gli regions equivalent to CORD or to CDN together with the zinc fingers (Fig. 4E). Thus, even though three similarly placed Cos2-binding sites are conserved in Gli1, the nucleotide and phosphorylation dependence of Cos2-CORD binding observed for Ci is not conserved.

To test the functional relevance of Cos2-binding regions of Gli1, we deleted the regions equivalent to CDN and CORD. Gli1 lacking residues 141-260 behaved like wild-type Gli1, inducing ectopic *ptc-lacZ* only in posterior cells with an intact Hh signaling pathway (Fig. 2E, Fig. 3E). Gli1 lacking either residues 601-750 or 650-750, however, induced *ptc-lacZ* additionally in anterior cells and in posterior *smo* mutant clones, indicating activity even in the absence of Hh (Fig. 2F). In fact, anterior *ptc-lacZ* induction by Gli1 Δ CORD was reproducibly higher than for Gli1 Δ PKA (Fig. 3D,F). Thus, the CORD-equivalent Cos2-binding region of Gli1, like Cos2, PKA and PKA sites 544 and 560, normally restricts Gli1 activity in *Drosophila*. We infer that Cos2 binding to the CORD region of Gli1 prevents Gli1 activation in fly.

Kif7 stimulates Gli3 and Ci processing in *Drosophila*

Kif7 and Kif27 are the closest mammalian relatives of Cos2, so we tested whether tagged Kif7 protein derived from a transgene expressed in Kc cells could bind to GST-Gli proteins *in vitro*. We found that Kif7 bound to GST-Gli1 fragments spanning the CORD region (469-750) or both the CDN and zinc-finger regions together (141-391), implying that Cos2 and Kif7 bind Gli proteins in a similar way (Fig. 4D).

We then tested whether Kif7 could affect Gli and Ci activities in *Drosophila* by substituting Kif7 for Cos2. In otherwise wild-type wing discs, *cos2* mutant clones induce elevated Ci-155 2A1

staining, which can be rescued by expression of *UAS-Cos2* in the mutant clone (Ruel et al., 2007; Zhou and Kalderon, 2010). By contrast, expression of *UAS-Kif7* in *cos2* mutant clones did not reduce Ci-155 staining to normal levels (Fig. 5A-C). Thus, Kif7 clearly did not support Ci-155 processing as effectively as Cos2. To test for lower levels of Ci processing, we expressed Kif7 in posterior *smo cos2* clones and looked for generation of the *hh-lacZ* repressor, Ci-75. Neither Ci nor Gli3 repress *hh-lacZ* in *smo cos2* clones unless rescued by expressing a *cos2* transgene (Fig. 1E,F). We found that Kif7 could substitute for Cos2 to allow strong *hh-lacZ* repression not only by wild-type Ci, but also by Ci lacking both CDN and CORD domains and by wild-type Gli3 (Fig. 5D-F). Thus, Kif7 can promote processing of Gli3 and Ci proteins in *Drosophila*, even though Ci processing was less efficient than normal when Kif7 replaced Cos2.

Gli1 proteolysis cannot account for regulation by Hh and PKA

How do direct PKA phosphorylation and Cos2 binding regulate Gli1 activity? On the one hand, Gli1 proteolysis is a likely candidate because PKA and Cos2 principally regulate Ci activity via proteolysis (Smelkinson et al., 2007) and because the key PKA sites in Gli1 correspond to essential sites in Ci, Gli2 and Gli3 that promote binding of an E3 ubiquitin ligase. Even though Gli1 is not processed to a repressor and probably lacks specific sequences found in Ci and Gli3 to arrest proteolysis by the proteasome (Pan and Wang, 2007; Wang and Price, 2008), Gli1 activator levels might be regulated by PKA-dependent full proteolysis. On the other hand, there are no studies in mammalian cells or organisms implicating PKA sites 544 and 560 in Gli1 proteolysis and Gli1 lacks phosphorylation sites beyond these two PKA sites, which are

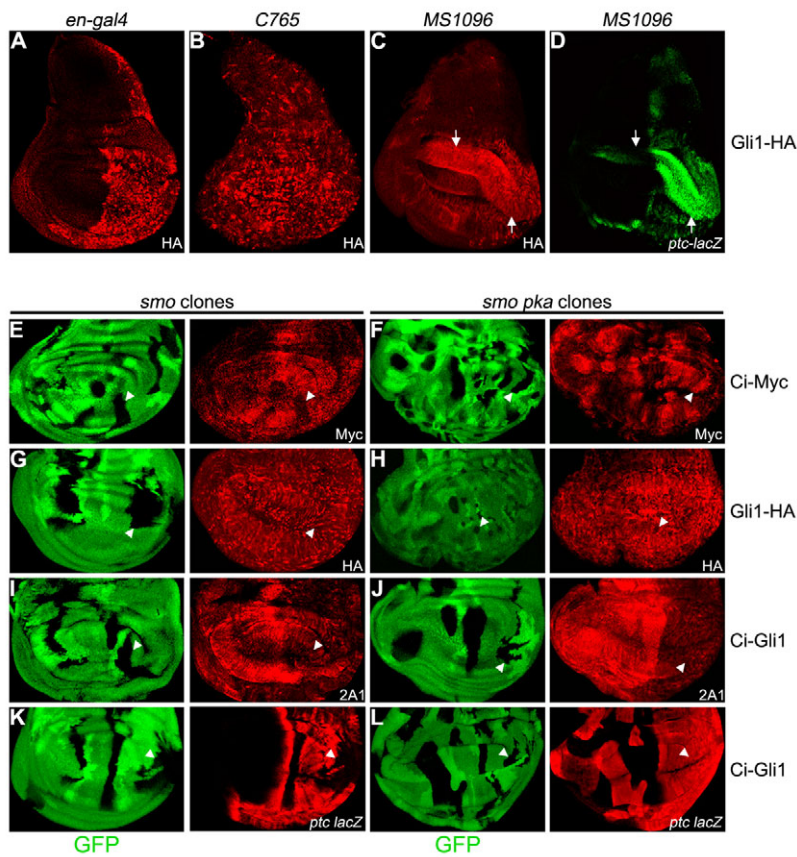


Fig. 6. Gli1 activity is regulated independently of proteolysis in *Drosophila*. (A–D) Wing discs expressing *UAS-Gli1* using (A) *en-GAL4*, (B) *C765-GAL4* or (C,D) *MS1096-GAL4*. (A–C) Gli1 protein, measured by C-terminal HA epitope tag staining (red), was confined to posterior cells (right) when expressed only in those cells (A) but was detected at similar levels in anterior (left) and posterior cells when *GAL4* was expressed evenly across the AP axis (B,C). (D) Gli1 induced *ptc-lacZ* expression (red) more strongly in posterior ventral cells (upwards arrow) than in anterior dorsal wing pouch cells (downwards arrow), even though Gli1-HA protein levels were higher in the latter region (arrows in C). (E–L) Ci, Gli1 or Ci-Gli1 proteins were expressed ubiquitously using *C765-GAL4* in wing discs containing (E,G,I,K) *smo* or (F,H,J,L) *smo pka* mutant clones (arrowheads), marked by loss of (green) GFP. (E,F) Full-length Ci protein detected by C-terminal Myc epitope staining (red) was reduced in posterior *smo* mutant clones but elevated by loss of PKA, but neither of these responses was seen for (G,H) Gli1, detected by C-terminal HA epitope staining (red) or for (I,J) Ci-Gli1, detected by 2A1 antibody (red), which also detects anterior Ci-155. (K,L) Induction of *ptc-lacZ* (red) by Ci-Gli1 was lost in posterior *smo* mutant clones and restored by loss of PKA.

essential in Gli3 and Ci for efficient E3 ubiquitin ligase binding and proteolysis. We therefore looked for evidence of regulated Gli1 proteolysis.

First, we examined C-terminal HA-epitope staining of Gli1 transgene products (Fig. 6A). We did not see differences in Gli1-HA staining consistent with stabilization by Hh, either by comparing anterior and posterior wing disc cells (Fig. 6B,C) or by comparing posterior *smo* mutant clones with surrounding cells, even though such differences are quite clear for a Ci-Myc transgene product (Fig. 6E,G). In fact, Gli1-HA levels were sometimes marginally higher in anterior than in posterior cells. In addition, Gli1-HA levels, unlike Ci-Myc levels, were not increased by inhibition of PKA; in fact, Gli1-HA levels were actually decreased in anterior *smo pka* mutant clones (Fig. 6F,H). Thus, we have no direct evidence for either Hh stimulation or PKA inhibition increasing Gli levels.

We also examined the consequences of expressing *UAS-Gli1* at varying levels. *MS1096-GAL4* drives expression of *GAL4* and *UAS-Gli1-HA* throughout wing discs, but at especially high levels in dorsal regions (Fig. 6C). In dorsal regions, Gli1 induced a little ectopic *ptc-lacZ* expression in anterior cells but not nearly as strongly as throughout the posterior compartment (Fig. 6D). Gli1-HA levels were similar in anterior and posterior cells in the dorsal wing pouch and were much higher there than in ventral regions. Thus, high dorsal anterior Gli1 levels induced *ptc-lacZ* less strongly than lower posterior ventral Gli1 levels (Fig. 6C,D), confirming that Gli1 levels are not the key determinant of Gli1 activity. Rather, the specific activity of Gli1 is clearly higher in posterior cells, under the influence of Hh, than in anterior cells. Similarly, in the absence of Hh signaling Gli1 specific activity is higher when PKA is inhibited.

Second, we constructed a Ci-Gli1 hybrid protein, in which residues 1-524 of Gli1 were replaced with residues 1-829 of Ci. Ci-Gli1 retains the PKA sites and Cos2-binding region of Gli1 that restrict Gli1 activity but Ci-Gli1 also includes sequences from Ci that arrest digestion by the proteasome to produce Ci-75 (Wang and Price, 2008). Hence, we might expect that if Cos2 binding to Gli1 and PKA phosphorylation of Gli1 efficiently recruits the Slimb E3 ubiquitin ligase, this should lead to processing of Ci-Gli1 and generation of Ci-75 repressor. Ci-Gli1 behaved exactly like Gli1, inducing *ptc-lacZ* in posterior but not anterior cells, and in posterior *smo pka* and *smo cos2* clones but not in *smo* mutant clones (Fig. 6K,L and data not shown). However, Ci-Gli1, like Gli1, did not repress *hh-lacZ* in posterior *smo* mutant clones, indicating that no Ci-75 repressor was formed (Fig. 5G,H). Ci-Gli1 also includes the epitope recognized by antibody 2A1, which is present in full-length Ci-155 but absent from Ci-75. If Ci-Gli1 were subject either to full proteolysis or to processing, 2A1 staining should be reduced. However, in posterior cells, where Ci-Gli1 is the only source of the 2A1 epitope, staining was not reduced in *smo* mutant clones or increased in *smo pka* mutant clones (Fig. 6I,J), indicating again no detectable effect of Hh signaling or PKA on the levels of full-length protein. We conclude that Hh, PKA and Cos2 regulate Gli1 activity in *Drosophila* principally by altering Gli1 specific activity rather than Gli1 protein levels.

PKA and Cos2 can limit the activity of processing-resistant Ci in the absence of Su(fu)

There is some evidence that PKA and Cos2 can regulate full-length Ci-155 activity as well as Ci-155 processing. For example, Cos2 is known to restrict nuclear accumulation of Ci-155 when nuclear export is inhibited and loss of PKA results in stronger activation of

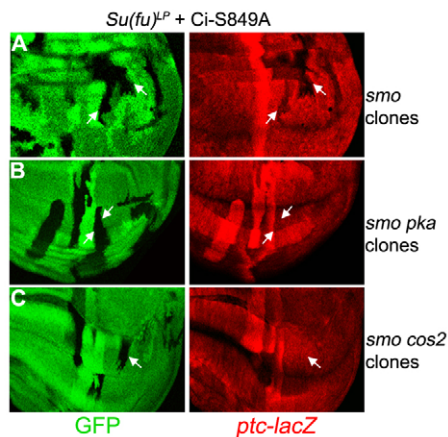


Fig. 7. PKA and Cos2 silence proteolysis-resistant Ci (Ci-S849A) in the absence of Su(fu). (A–C) *Su(fu)^{LP}* mutant wing discs ubiquitously expressing Ci-S849A, which is resistant to PKA- and Cos2-dependent processing, using the *C765-GAL4* driver. Activation of *ptc-lacZ* (red) was (A) reduced in posterior (right) *smo* clones, but was restored in posterior (B) *smo pka* and (C) *smo cos2* clones. Clones (arrows) are marked by loss of (green) GFP.

Ci-155 than other manipulations that inhibit Ci processing, including loss of Slimb or Cos2 (Smelkinson et al., 2007; Wang et al., 2000; Wang et al., 1999; Wang and Holmgren, 2000). The potential effects of PKA and Cos2 on Ci-155 specific activity were not, however, readily apparent when assaying the activity of a Ci transgene product, Ci-S849A, which is refractory to PKA-dependent proteolysis, in posterior *smo* mutant clones (see Fig. S4 in the supplementary material) (Smelkinson et al., 2007). We considered the possibility that PKA and Cos2 might limit the specific activity of both Gli1 and Ci by a common mechanism, but that these actions might be better masked for Ci-155 by a potentially redundant silencing activity of *Drosophila* Su(fu). We therefore examined whether Ci-S849A activity might be regulated by PKA or Cos2 in the absence of Su(fu). We found that the ability of Ci-S849A to induce *ptc-lacZ* in posterior cells of Su(fu) mutant discs was lost in *smo* mutant clones, provided a low temperature was used to limit the amount of Ci-S849A expressed (Fig. 7A). Under these conditions, Ci-S849A activity was increased by loss of either PKA or Cos2 (in *smo pka* or *smo cos2* mutant clones) (Fig. 7B,C), demonstrating that PKA and Cos2 can limit the activity of processing-resistant Ci-155 in the absence of Su(fu). Thus, a regulatory influence of PKA and Cos2 revealed robustly by Gli1 is also detectable for Ci.

DISCUSSION

There are clearly many conserved features of Hh signaling between *Drosophila* and mammals, and some explicit differences, most obviously with regard to the employment of cilia (Huangfu and Anderson, 2006; Wilson and Chuang, 2010). However, incomplete understanding can lead to premature conclusions as to exactly what is conserved. When we initiated our studies of Gli activity in *Drosophila* the absence of key fly Smo activation and Cos2-binding domains from mammalian Smo, coupled to studies showing no effect of Kif7 and Kif27 RNAi on the Hh pathway in tissue culture cells, had led to the suggestion that a Cos2-like molecule was not important in regulating Gli activity in mammals (Ruel and Therond, 2009; Varjosalo et al., 2006). Here, we have

shown that Cos2 is essential for Gli3 processing into a repressor form in *Drosophila*, that Cos2 prevents constitutive Gli1 activity while permitting Gli1 activation by Hh in *Drosophila* and that Cos2 binds to three distinct regions of Gli1, as observed for Ci. These results strongly indicated that a Cos2-like molecule must regulate the activity of Gli proteins in mammals. Indeed, genetic elimination of Kif7 in mouse was subsequently found to reduce processing of Gli3 and was also inferred to result in partial activation of Gli2 in the absence of a Hh ligand (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). The fundamental demonstration of conservation of Cos2 function focuses attention on more precise questions about how Cos2-family proteins regulate Ci/Gli protein function. Some of these questions have been addressed by comparing the regulation of Ci and Gli proteins in *Drosophila*, as discussed below.

How is Gli1 regulated?

Although physiological regulation of Gli1 protein activity by Shh was demonstrated by knocking Gli1 coding sequence into the mouse Gli2 locus (Bai and Joyner, 2001), those mice have not been extensively characterized further to test which specific factors regulate Gli1 activity in mouse. In mice with wild-type Gli genes, the dependence of Gli1 transcription on Hh pathway activity coupled with the ubiquitous presence of Gli2 as a major sensor of pathway activity has prevented an assessment of post-transcriptional regulation of Gli1 (Wilson and Chuang, 2010). For example, genetic loss of mouse suppressor of fused (Sufu) leads to substantial activation of Shh-target genes and transcriptional induction of Gli1, leading only to the conclusion that Sufu normally limits Gli2 activity. Similarly, the effects of mutations affecting cilia or Kif7 have been interpreted only with respect to Gli3 and Gli2 activities.

We found that silencing Gli1 in the absence of Hh signaling requires PKA, PKA sites S544 and S560, Cos2 and the Cos2-binding region equivalent to CORD in Ci, but that T374 and the DSGVEM motif, which has been previously implicated in Gli1 regulation, are not required for Gli1 silencing or activation by Hh. Although the known dependence of Ci phosphorylation on Cos2 (Zhang et al., 2005) suggests that Cos2 might be required to promote Gli1 phosphorylation by PKA, the greater activity of Gli1 lacking the CORD region compared with Gli1 lacking PKA sites in anterior wing disc cells suggests that Cos2 may do more than simply promote Gli1 phosphorylation.

How is Gli1 silenced by PKA phosphorylation in *Drosophila* Ci silencing involves equivalent PKA sites (in position and immediate sequence context) and creates a Slimb-binding site essential for Ci-155 processing (Smelkinson et al., 2007). However, additional PKA and PKA-primed phosphorylation sites important for efficient Ci and Gli3 proteolysis are absent from Gli1. Thus, it would be difficult to predict a priori whether PKA and Hh alter Gli1 activity by regulating its proteolysis.

Direct assessment of Gli1 protein levels (using a C-terminal HA tag) in wing disc cells indicated that neither Hh stimulation nor PKA inhibition increased Gli1 levels. However, Ci-155 levels can be a deceptive indicator of PKA-dependent proteolysis because Ci-155 can also be degraded by an activation-dependent mechanism that is independent of PKA (Jiang, 2006). Hence, Ci-155 levels are elevated at intermediate levels of Hh signaling, but in cells exposed to the highest levels of Hh, where PKA-dependent processing is strongly inhibited, Ci-155 levels are very similar to those in anterior cells with no Hh exposure (Strigini and Cohen, 1997). Our observation that Gli1 levels may actually be slightly reduced in

posterior cells relative to anterior cells, and in anterior clones deficient for PKA, suggests that activation may also de-stabilize Gli1 and could disguise any putative, opposite contribution from PKA-dependent proteolysis. Consequently, we looked further for evidence of PKA-dependent proteolysis by creating a Ci-Gli1 fusion protein for which PKA-dependent proteasome digestion would be expected to be arrested and produce Ci-75 repressor. The activity of the Ci-Gli1 fusion protein was regulated by PKA, Cos2 and Hh, as for Gli1 and Ci, but no repressor was formed. Hence, we found no evidence of PKA-dependent proteolysis of Gli1. We also found that weak induction of *ptc-lacZ* expression in anterior cells required much higher levels of Gli1 protein than evident for equivalent (or greater) *ptc-lacZ* induction by Gli1 lacking PKA sites 544 and 560, or by wild-type Gli1 in response to Hh. Thus, both PKA inhibition and Hh clearly increase the specific activity of Gli1 and do not appear to stabilize Gli1 protein.

Comparative effects of PKA, Cos2 and Su(fu) on Gli1 and Ci in fly

If PKA principally regulates the specific activity of Gli1 in *Drosophila*, it might be expected that the same mechanism can regulate Ci-155 activation. Direct evidence for such a role was not observed previously when comparing the activity of low levels of proteolysis-resistant Ci (Ci-S849A) in posterior *smo* and *smo pka* mutant clones, even though it is clear that endogenous Ci-155 is activated more strongly by loss of PKA than by loss of Slimb (Smelkinson et al., 2007). Likewise, loss of Cos2 activates Ci-155 more strongly than loss of Slimb but did not detectably increase the activity of Ci S849A in posterior *smo* mutant clones. Su(fu) also has the potential to limit Ci-155 activity. Here, by eliminating Su(fu), we did reveal a repressive influence of both PKA and Cos2 on Ci-S849A in posterior *smo* mutant clones. We conclude that the potentially redundant effects of PKA, Cos2 and Su(fu) limiting Ci-155 activity can each be exposed in the absence of Ci-155 processing but are probably most readily seen at high Ci-155 levels. Our experiments with Ci do not reveal the mechanism by which PKA and Cos2 can limit Ci-155 activity. An equivalent regulatory influence was much clearer for Gli1; it involves direct Cos2 binding and phosphorylation of defined PKA sites, providing an important starting point for further investigation.

Regulation of Ci and Gli activities by Cos2 and Kif7

Kif7 is known to promote the proteolysis of Gli2 and Gli3 in mouse (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Here, we showed that Kif7 can also promote Ci-155 and Gli3 processing in *Drosophila*. We also showed that Cos2 promotes Gli3 processing and limits Gli1 activity in *Drosophila*. In all of these cases, PKA and conserved PKA sites have analogous effects to Cos2, consistent with the common hypothesis that Cos2 and Kif7 can promote phosphorylation of Ci-155 and Gli proteins at specific PKA and PKA-primed sites.

Here, we have found that Cos2 effects on Gli1 required the CORD-equivalent region of Gli1. Regulation of Ci-155 by Cos2 does not require the CORD region of Ci. In fact, Ci-155 can be processed and silenced in the absence of both CDN and CORD domains, provided a third Cos2 interaction domain within its zinc fingers is present (Zhou and Kalderon, 2010). Gli1 shares all three Cos2 interaction domains. Moreover, Kif7 can promote processing of Ci lacking CDN and CORD regions, implying that a single Ci/Gli interface with a Cos2/Kif7 protein can silence Ci-155 but

not Gli1. It is possible that a single Cos2/Ci-155 interface suffices because it is stabilized by additional common binding partners, Fu and Su(fu) (Methot and Basler, 2000; Monnier et al., 2002), and that these proteins do not contribute efficiently to Cos2/Gli1 binding.

Most importantly, Hh inhibits the Cos2-dependent processing of Gli3 and Cos2-dependent silencing of Gli1 in *Drosophila*. The mechanisms by which Hh opposes Cos2 actions on Ci-155 are not yet fully resolved. One potential mechanism, the dissociation of PKA, CK1 and GSK3 from Cos2, would be expected to influence both Ci and Gli protein activities (Zhang et al., 2005). However, there is probably also a role for Hh-dependent dissociation of Cos2 from Ci (Ruel et al., 2007; Ruel et al., 2003; Zhou and Kalderon, 2010), implying that the interactions between Cos2 (or Kif7) and Gli proteins may be regulated by an analogous mechanism.

Acknowledgements

We thank Konrad Basler (University of Zurich, Switzerland), Kathryn Anderson (Memorial Sloan Kettering Cancer Center, USA), the Developmental Studies Hybridoma Bank (University of Iowa, USA) and the Bloomington Stock Center (Indiana University, USA) for supplying critical reagents. We thank lab members Pui-Leng Ip for generation of transgenic flies, Qianhe Zhou, Jamie Little and Andreia Lee for experimental contributions, and Qianhe Zhou and Cynthia Vied for discussions and advice. This work was supported by NIH grant GM41815 (D.K.). Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

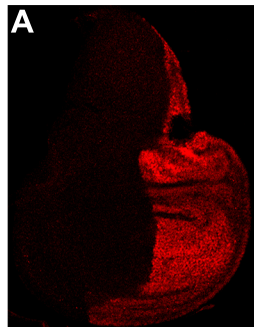
Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.063479/-DC1>

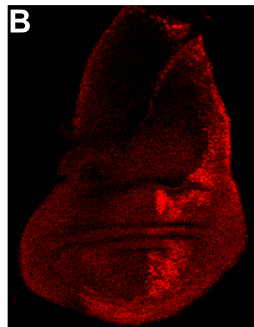
References

- Aza-Blanc, P., Lin, H. Y., Ruiz i Altaba, A. and Kornberg, T. B. (2000). Expression of the vertebrate Gli proteins in *Drosophila* reveals a distribution of activator and repressor activities. *Development* **127**, 4293-4301.
- Bai, C. B. and Joyner, A. L. (2001). Gli1 can rescue the in vivo function of Gli2. *Development* **128**, 5161-5172.
- Barnfield, P. C., Zhang, X., Thanabalasingham, V., Yoshida, M. and Hui, C. C. (2005). Negative regulation of Gli1 and Gli2 activator function by Suppressor of fused through multiple mechanisms. *Differentiation* **73**, 397-405.
- Bischof, J., Maeda, R. K., Hediger, M., Karch, F. and Basler, K. (2007). An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. USA* **104**, 3312-3317.
- Cheung, H. O., Zhang, X., Ribeiro, A., Mo, R., Makino, S., Puvion-Randall, V., Law, K. K., Briscoe, J. and Hui, C. C. (2009). The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. *Sci. Signal.* **2**, ra29.
- Denef, N., Neubuser, D., Perez, L. and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-531.
- Dunaeva, M., Michelson, P., Kogerman, P. and Toftgard, R. (2003). Characterization of the physical interaction of Gli proteins with SUFU proteins. *J. Biol. Chem.* **278**, 5116-5122.
- Endoh-Yamagami, S., Evangelista, M., Wilson, D., Wen, X., Theunissen, J. W., Phamluong, K., Davis, M., Scales, S. J., Solloway, M. J., de Sauvage, F. J. et al. (2009). The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development. *Curr. Biol.* **19**, 1320-1326.
- Hooper, J. E. and Scott, M. P. (2005). Communicating with Hedgehogs. *Nat. Rev. Mol. Cell Biol.* **6**, 306-317.
- Huangfu, D. and Anderson, K. V. (2006). Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from *Drosophila* to vertebrates. *Development* **133**, 3-14.
- Huntzicker, E. G., Estay, I. S., Zhen, H., Lokteva, L. A., Jackson, P. K. and Oro, A. E. (2006). Dual degradation signals control Gli protein stability and tumor formation. *Genes Dev.* **20**, 276-281.
- Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
- Jiang, J. (2006). Regulation of Hh/Gli signaling by dual ubiquitin pathways. *Cell Cycle* **5**, 2457-2463.
- Jiang, J. and Hui, C. C. (2008). Hedgehog signaling in development and cancer. *Dev. Cell* **15**, 801-812.

- Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development.[erratum appears in Trends Neurosci 2001 Jul;24(7):385]. *Trends Neurosci.* **24**, 251-254.
- Liem, K. F., Jr, He, M., Ocbina, P. J. and Anderson, K. V. (2009). Mouse Kif7/Costal2 is a cilia-associated protein that regulates Sonic hedgehog signaling. *Proc. Natl. Acad. Sci. USA* **106**, 13377-13382.
- Methot, 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**, 819-831.
- Methot, N. and Basler, K. (2000). Suppressor of fused opposes hedgehog signal transduction by impeding nuclear accumulation of the activator form of *Cubitus interruptus*. *Development* **127**, 4001-4010.
- Monnier, V., Ho, K. S., Sanial, M., Scott, M. P. and Plessis, A. (2002). Hedgehog signal transduction proteins: contacts of the Fused kinase and Ci transcription factor with the kinesin-related protein Costal2. *BMC Dev. Biol.* **2**, 4.
- Motzny, C. K. and Holmgren, R. (1995). The *Drosophila cubitus interruptus* protein and its role in the wingless and hedgehog signal transduction pathways. *Mech. Dev.* **52**, 137-150.
- Pan, Y. and Wang, B. (2007). A novel protein-processing domain in Gli2 and Gli3 differentially blocks complete protein degradation by the proteasome. *J. Biol. Chem.* **282**, 10846-10852.
- Ruel, L. and Therond, P. P. (2009). Variations in Hedgehog signaling: divergence and perpetuation in Sufu regulation of Gli. *Genes Dev.* **23**, 1843-1848.
- Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L. and Therond, P. P. (2003). Stability and association of Smoothened, Costal2 and Fused with *Cubitus interruptus* are regulated by Hedgehog. *Nat. Cell Biol.* **5**, 907-913.
- Ruel, L., Gallet, A., Raisin, S., Truchi, A., Staccini-Lavenant, L., Cervantes, A. and Therond, P. P. (2007). Phosphorylation of the atypical kinesin Costal2 by the kinase Fused induces the partial disassembly of the Smoothened-Fused-Costal2-*Cubitus interruptus* complex in Hedgehog signalling. *Development* **134**, 3677-3689.
- Sheng, T., Chi, S., Zhang, X. and Xie, J. (2006). Regulation of Gli1 localization by the cAMP/protein kinase A signaling axis through a site near the nuclear localization signal. *J. Biol. Chem.* **281**, 9-12.
- Smelkinson, M. G., Zhou, Q. and Kalderon, D. (2007). Regulation of Ci-SCF limb binding, Ci proteolysis, and hedgehog pathway activity by Ci phosphorylation. *Dev. Cell* **13**, 481-495.
- Strigini, M. and Cohen, S. M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* **124**, 4697-4705.
- Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M. F. and Concordet, J. P. (2006). Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. *Mol. Cell. Biol.* **26**, 4316-4326.
- Varjosalo, M., Li, S. P. and Taipale, J. (2006). Divergence of hedgehog signal transduction mechanism between *drosophila* and mammals. *Dev. Cell* **10**, 177-186.
- von Mering, C. and Basler, K. (1999). Distinct and regulated activities of human Gli proteins in *Drosophila*. *Curr. Biol.* **9**, 1319-1322.
- Wang, G. and Jiang, J. (2004). Multiple Cos2/Ci interactions regulate Ci subcellular localization through microtubule dependent and independent mechanisms. *Dev. Biol.* **268**, 493-505.
- 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.
- Wang, G., Amanai, K., Wang, B. and Jiang, J. (2000). Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of *cubitus interruptus*. *Genes Dev.* **14**, 2893-2905.
- Wang, Q. T. and Holmgren, R. A. (2000). Nuclear import of *cubitus interruptus* is regulated by hedgehog via a mechanism distinct from Ci stabilization and Ci activation. *Development* **127**, 3131-3139.
- Wang, Y. and Price, M. A. (2008). A unique protection signal in *Cubitus interruptus* prevents its complete proteasomal degradation. *Mol. Cell. Biol.* **28**, 5555-5568.
- Wilson, C. W. and Chuang, P. T. (2010). Mechanism and evolution of cytosolic Hedgehog signal transduction. *Development* **137**, 2079-2094.
- Zhang, W., Zhao, Y., Tong, C., Wang, G., Wang, B., Jia, J. and Jiang, J. (2005). Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of *Cubitus interruptus*. [see comment]. *Dev. Cell* **8**, 267-278.
- Zhao, Y., Tong, C. and Jiang, J. (2007). Hedgehog regulates smoothened activity by inducing a conformational switch. *Nature* **450**, 252-258.
- Zhou, Q. and Kalderon, D. (2010). Costal 2 interactions with *Cubitus interruptus* (Ci) underlying Hedgehog-regulated Ci processing. *Dev. Biol.* **348**, 47-57.

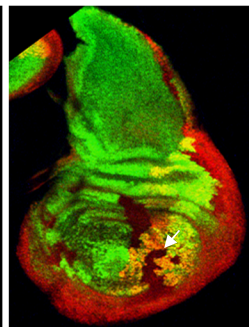
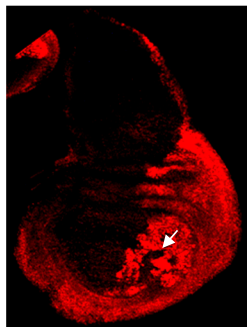
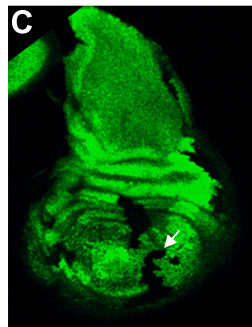


UAS
Ci

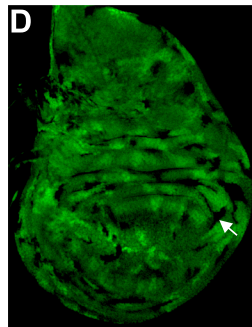


hh-lacZ

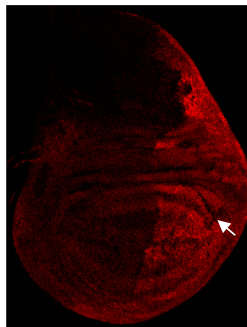
UAS
Gli3



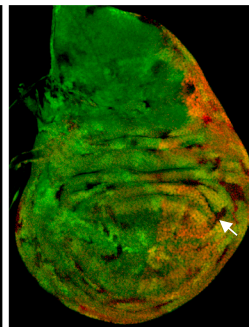
UAS
Ci



GFP



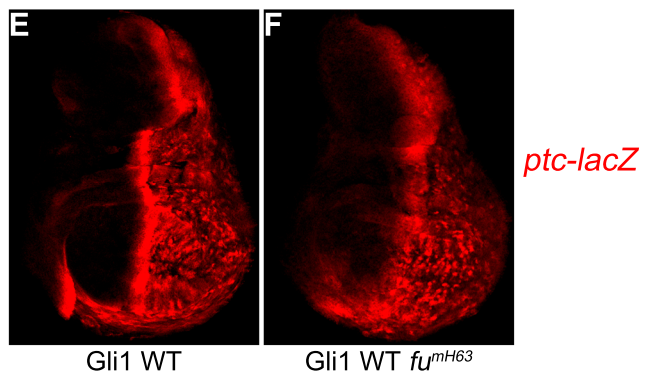
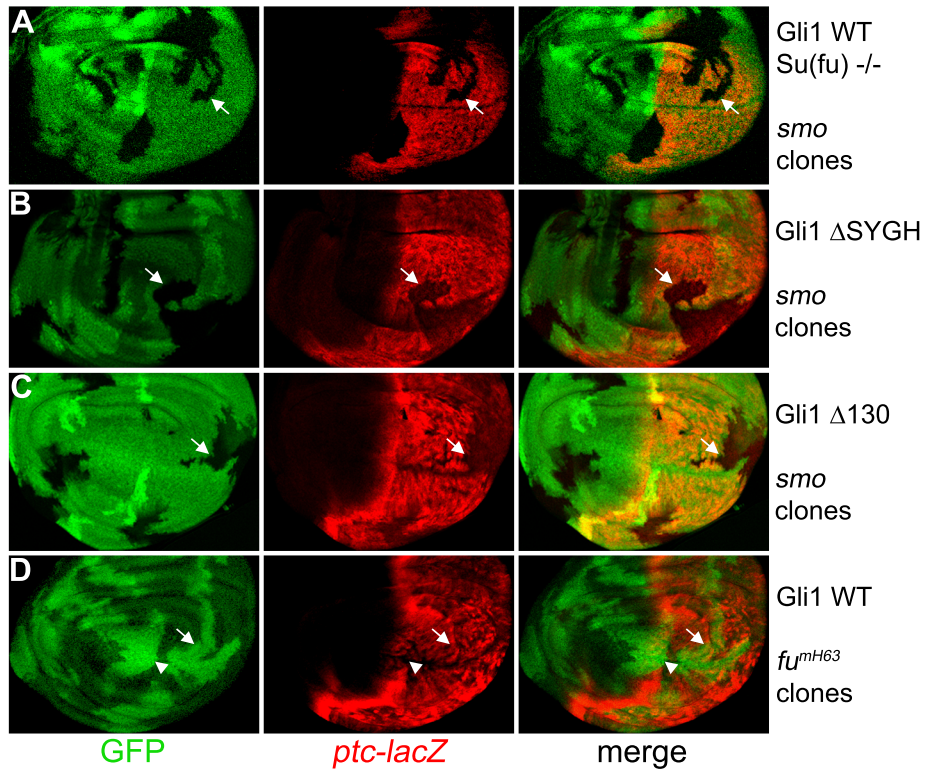
hh-lacZ

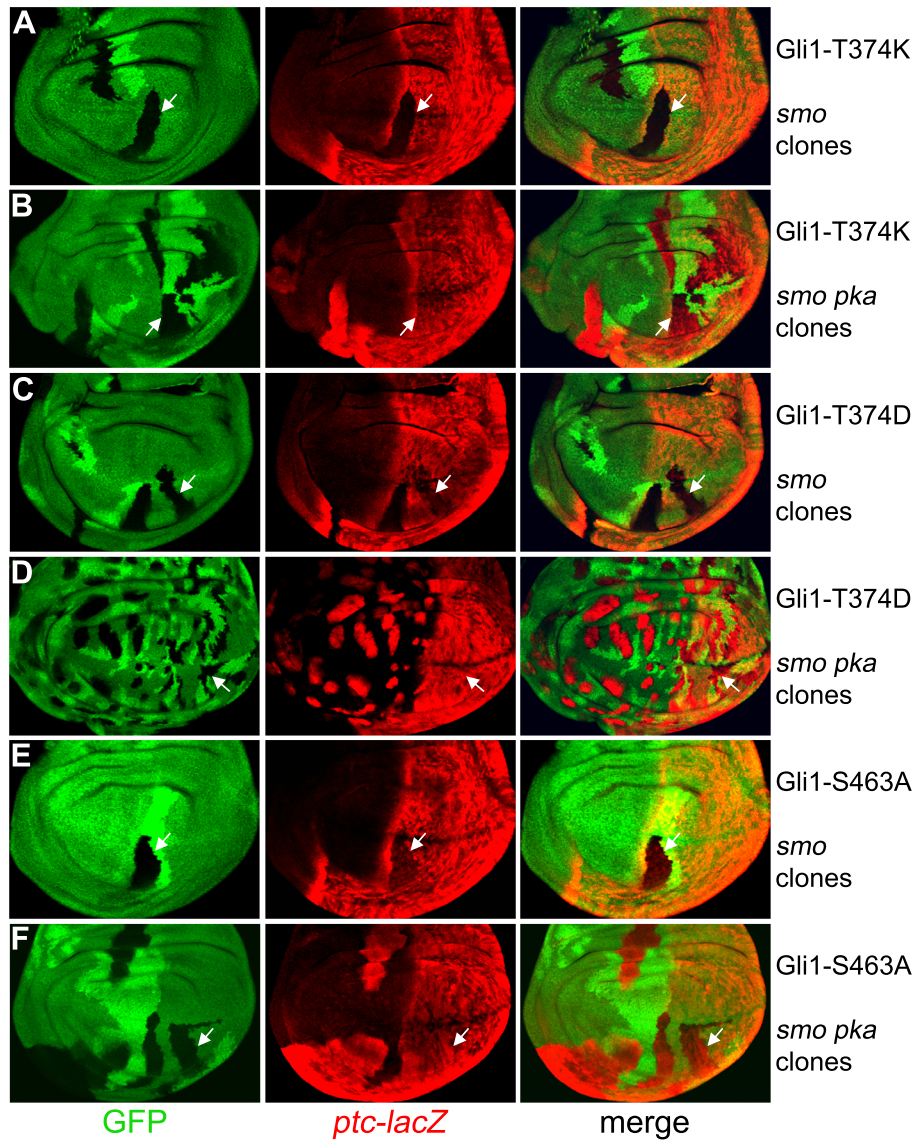


UAS
Gli3

merge

smo clones





Ci-S849A

