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Research article

Hedgehog signal transduction proteins: contacts of the Fused kinase and Ci transcription factor with the Kinesin-related protein Costal2

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Abstract

Background: Hedgehog signaling proteins play important roles in development by controlling growth and patterning in various animals including Drosophila and mammals. Hedgehog signaling triggers changes in responsive cells through a novel transduction mechanism that ultimately controls the transcription of specific target genes via the activity of zinc finger transcription factors of the Cubitus interruptus /GLI family. In flies, key Hedgehog signal transduction components have been identified including the kinesin-related protein Costal2, the serinethreonine kinase Fused, and the PEST-containing protein Suppressor of Fused. These proteins control Cubitus interruptus cleavage, nucleo-cytoplasmic localization and activation. In fly embryos, Costal2, Fused, Suppressor of Fused and Cubitus interruptus are associated in at least one cytoplasmic complex, which interacts with the microtubules in a Hedgehog-dependent manner.

Results: Here we identified and mapped direct interactions between Cos2, Fu, and Ci using an in vitro affinity assay and the yeast two-hybrid system.

Conclusions: Our results provide new insights into the possible mechanism of the cytosolic steps of Hedgehog transduction.

Background

The Hedgehog (Hh) proteins are evolutionarily conserved signaling molecules that control the normal growth and patterning of diverse animals including Drosophila and humans. In flies Hh is required for multiple developmental processes such as embryonic segment patterning, eye and appendage development (for reviews see [1,2]) In vertebrates, three Hh homologues are expressed in a tissue specific manner and are responsible for the morphogenesis of various organs such as the neural tube and the limbs, and for cartilage and male germinal cell differentiation [3,4]. In mammals, deregulation of the Hh pathway is responsible for cancers, especially basal cell carcinoma and medulloblastoma [5–7]. In all cases described so far, Hh initiates and/or maintains the transcription of target genes in responsive cells. Among the targets are *patched (ptc)*, which encodes a Hh receptor protein, and genes encoding signaling molecules. In Drosophila, *decapentaplegic (dpp)*, a signal of the TGFβ class, and *wingless (wg)*, a member of the Wnt family, are transcribed in response to Hh. Genes

encoding related signaling molecules such as TGFβ, FGF, and Wnt are transcriptionally induced by Hh signals in vertebrates, as are genes for a variety of transcription factors.

In Drosophila, Cubitus interruptus (Ci), a zinc finger transcription factor of the vertebrate Gli family, plays a central and complex role in the transcriptional regulation of Hh target genes. Ci acts as either transcriptional activator or repressor in a Hh-dependent manner (for reviews see [1,8,9]). In the absence of Hh signal, most of Ci is cleaved to generate a 75 kD nuclear protein (Ci75-R) consisting of the N-terminus and the zinc finger DNA binding domain of the protein. Ci75-R acts as a repressor on *hh* and *dpp* transcription [10–12]. Ci cleavage is proteasome dependent and requires Ci phosphorylation by the protein kinase A (PKA), the activity of the kinesin-related protein Costal2 (Cos2) and of Slimb (Slb), a F-box WD-40 protein (other members of this family are known to direct ubiquitin-mediated proteolysis of specific phospho-proteins) [13–21]. In the absence of Hh, Ci75-R is mainly localized into the nucleus while the uncleaved fraction of Ci (155kD, Ci-155) is retained in the cytoplasm by Cos2 and Suppressor of Fused (Su(fu)), a putative PEST motif-containing protein [14,19,22–25]. The exclusion of full-length Ci155 from the nucleus is also ensured by its constitutive export [14,24]. Hh signaling inhibits Ci proteolysis, probably by reducing Ci phosphorylation level via the action of a phosphatase [10,14,18]. This results in the accumulation of full-length Ci-155. Hedgehog reception also relieves Ci-155 cytoplasmic retention and allows Ci-155 to be translocated into the nucleus via a basic nuclear localization sequence [14,18,19,25]. Nevertheless, the persistence of its export leads to the accumulation of the vast majority of Ci-155 in the cytoplasm. Last, proper induction of Hh target genes also requires Hedgehog signaling to produce, by an unknown mechanism, an activated form of Ci called Ci-A [11,23–26]. In the absence of Hh, Su (fu) appears to prevent Ci activation. Upon Hh reception, both the ser-thr protein kinase Fused (Fu) and Cos2 counteract Su(fu) to produce Ci-A [19,23–27].

The molecular mechanism by which Hh signaling controls Ci remains poorly understood. Both full-length and truncated forms of Ci are detected in the cytoplasm as part of one or more large molecular weight protein complexes [10,28–30]. The cytoplasmic complex that has been studied also includes Fu and Cos2 and Su(fu) [28–31]. In the absence of Hh signal, Fu-Cos2-Ci ternary complex binds microtubules, probably through Cos2 [28–30]. Cultured cell experiments showed that Hh signal triggers the release of the Fu-Cos2-Ci complex from the microtubules [28,29]. Concomitantly Hh signal increases the level of phosphorylation of both Fu and Cos2 [28,32].

Our working hypothesis is that Hh signal could control Ci fate (i.e. cleavage, subcellular localization, and/or activation) by inducing changes in the activity, composition, and/or subcellular localization of the transducing cytoplasmic complex. Since Cos2 is a putative motor protein with microtubule-binding activity, it could play a central role in this process by regulating the association of the complex with the microtubules and perhaps by directing its movement to specific locations within the cell.

In order to better understand how Hh transducing complex may function, we focused on the precise relationships among its different members. The physical association of Cos2 with Fu and Ci has been previously demonstrated using gel filtration chromatography and co-immunoprecipitation from embryo and cultured cell extracts [28–30]. Here we have undertaken the identification and the mapping of the molecular interactions taking place between Cos2, Fu, Su(fu), and Ci using the yeast two-hybrid method and an *in vitro* biochemical assay. Our results show that (i) Cos2, as Su(fu), interacts with both the catalytic and regulatory domains of Fu and with the N-terminal part of Ci; (ii) Ci and Fu associate with Cos2 in the neck domain, located C-terminally to the motor domain. The precise identification of the interaction region of each protein *in vitro* provide new insights into the structure and possible mechanism of action of the complex during Hh signal transduction.

Results

Cos2 – Fu interaction

In vitro interaction assays were done between a GST-Cos2 fusion protein expressed in bacteria and S-methionine-radiolabeled Su(fu) or Fu produced by *in vitro* translation. The Glutathion-S-tranferase was fused to the N-terminus of the full-length Cos2 protein.

No interaction was detected between GST-Cos2 and Su(fu) (Figure 1A). The same negative result was obtained using GST-Su(fu) and radiolabelled Cos2 (data not shown).

In contrast, Fu binds specifically to the GST-Cos2 fusion protein but not to the GST protein alone (Figure 1A). We tested separately Fu1-305, the catalytic domain of Fu (called Fukin) and Fu306-805, the part of Fu that is not the kinase domain, referred to as the regulatory domain (called Fureg). Both domains have the capacity to specifically interact with the GST-Cos2 fusion protein (Figure 1A). Similar interactions were previously observed between Su(fu) and the two domains of Fu ([31] and data not shown).

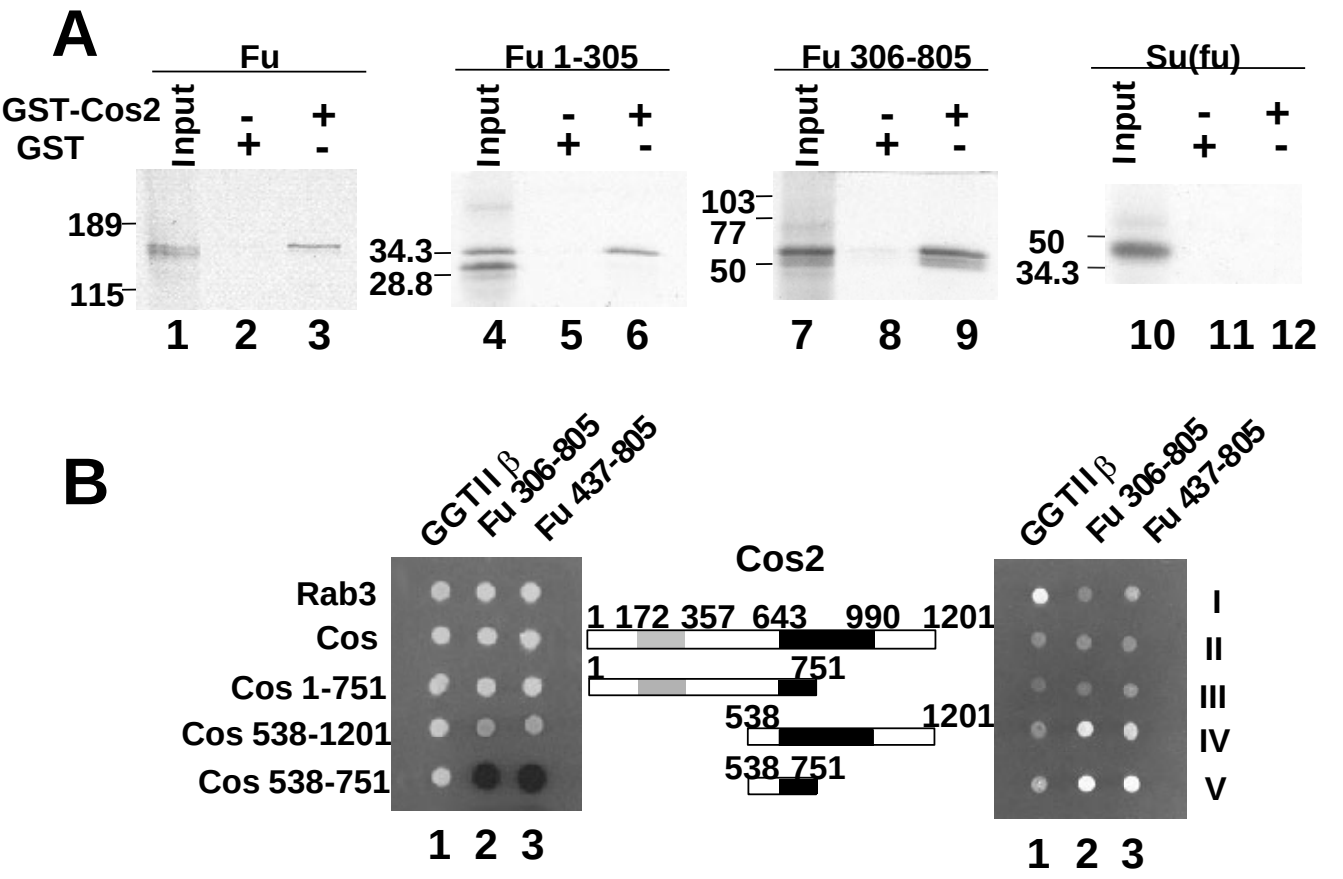

Figure 1

Direct specific interaction between Cos2 and Fu. (A). GST pull down assay: *In vitro* translated, ^{35}S-methionine-labeled Fu, Fu1-305, Fu306-805 or Su(fu) before (Input, Lanes 1, 4, 7 and 10) or after incubation with equal amount of, respectively, GST (used as a control; Lanes 2, 5, 8 and 11) or GST-Cos2 (Lanes 3, 6, 9 and 12). Input equals one third of the amount used in the pull down assay. (B). Yeast two hybrid assay: Left: β-galactosidase assay, Right: leucine assay. Each dot corresponds to a yeast diploid coexpressing, respectively, various regions of Cos2 (as shown below) or Rab3 fused to the DNA binding domain of LexA (DBD LexA) (rows I to V) and respectively Fu306-805, Fu437-805, GGTIIβ fused to the transactivation domain B42 (columns 1 to 3). Both assays were performed using RFY231-pSH18-34 (Rows I to IV) or EGY189-pSH18-34 (Row V). The negative control assays with Rab3 give the same result in both strains (Row I and data not shown). In combination with Fureg, Cos538-751 leads to even higher reporter activation than does Cos538-1201. This could be due to numerous causes as differences in stability of the protein fusion, in their nuclear targeting, and/or affinity for Fureg. Cos2: heptad repeats domain (AA643-990), in black Cos2 motor domain (AA172-357) in grey.

To confirm the Cos2-Fu interaction and to further map the interacting regions we employed the yeast two-hybrid system (Figure 1B). Neither full-length Cos2 nor full-length Fu and Fukin could be used in this assay due to interfering activities. Indeed, as we have shown previously, the presence (but not the activity) of the kinase domain of Fu prevents any protein interaction ([31] and data not shown). Using a transcription inhibition assay [33], we found that the full-length Cos2 fusion to the GAL4 DNA binding domain, and some partial Cos2 proteins (such as Cos1-751) that contain the motor domain, do not translocate efficiently to the nucleus (data not shown). The motor domain of Cos2 (AA 172–347) might tether the fusion protein in the cytoplasm, perhaps by binding to microtubules. We therefore analyzed partial Fu and Cos2 proteins.

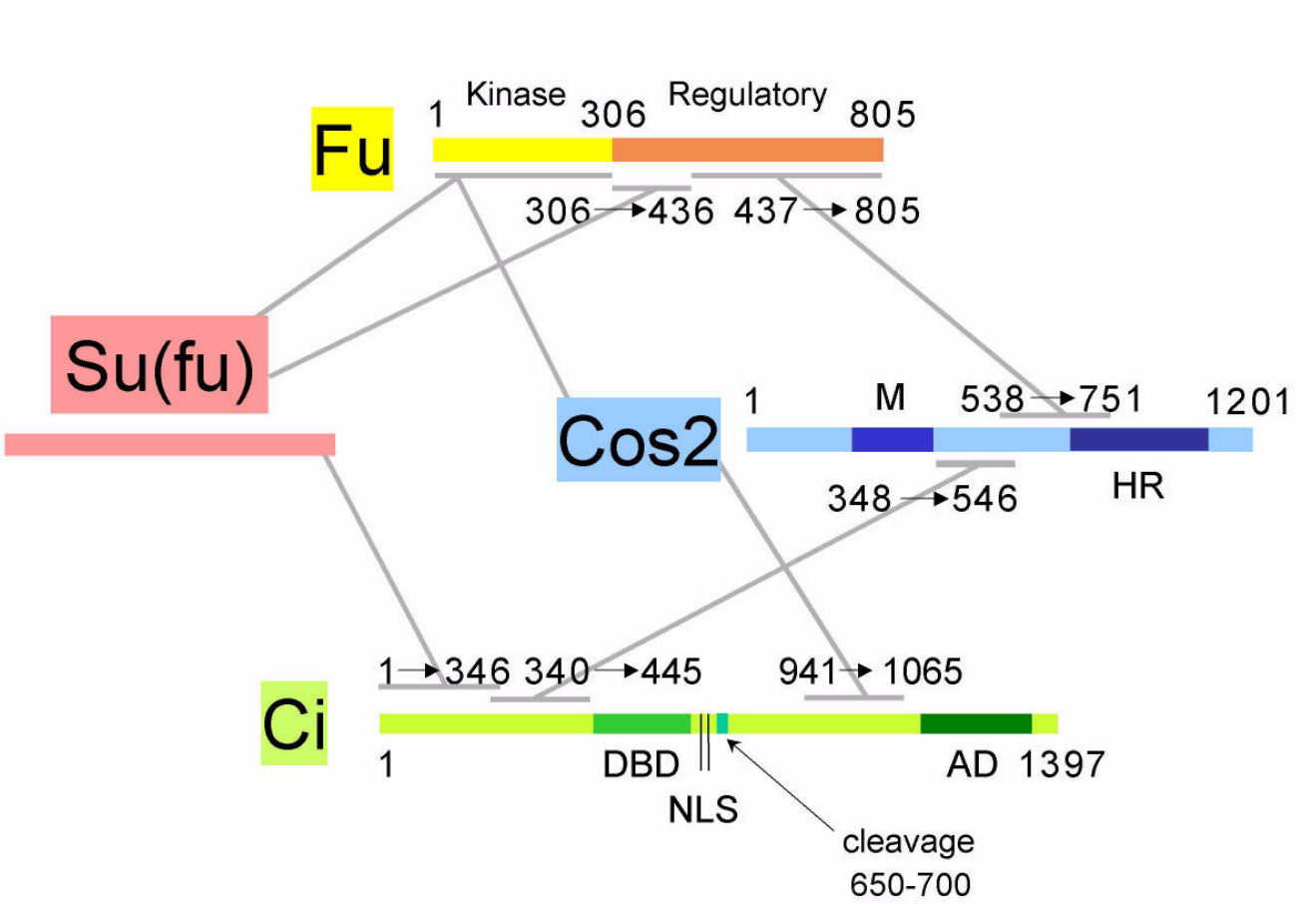

Figure 2
Direct specific interaction between Cos2 and Ci. (A). GST pull-down assay: *In vitro* translated, ^{35}S-methionine-labeled Ci or a truncated form of Ci (Ci1-430) before (Input, Lanes 1 and 4) or after incubation with equal amount of, respectively, GST (Lanes 2 and 5) or GST-Cos2 fusion (Lanes 3 and 6). Ci: zinc finger domain in grey, activation domain in black, the approximate cleavage position is around AA703. (B). Yeast two hybrid β-galactosidase assay between various regions of Cos-2 or Rab3 in fusion with the DBD lex-A (Rows I to V) and respectively GGTIIβ, Ci, Ci1-346 or Ci340-445 in fusion with B42 (Columns 1 to 4). See also legends to Figure 1B and 2A.

In the yeast two-hybrid assay, Fureg (Fu306-805) interacts specifically with the C-terminal part of Cos2 (Cos538-1201) (Figure 1B). Part of this region, Cos538-751, is sufficient to interact with Fureg. Cos538-751 encodes a region of Cos2 C-terminal to the putative motor domain (See also Figure 3) and which overlaps 14 of the 36 heptad repeats of a predicted coiled-coil motif of Cos2. In other kinesins the coiled coil repeats mediate homodimerization and belong to a flexible stalk domain involved in the movements of the motor domain along the microtubules [34–45].

We also mapped the region of Fu that interacts with Cos2 (Figures 1B and 3). The C-terminal-most region of Fu (AA 437 to 805) is sufficient to interact with both Cos538-1201 and Cos538-751. This region of Fureg is adjacent but non-overlapping to the part of Fu (AA 306 to 436) that interacts with Su(fu) [31]. No interaction between Fu and the different Cos2 constructs tested was detected with smaller pieces of Fureg (Fureg306-436, Fureg437-581, Fureg306-581, Fureg582-805; data not shown).

In summary, Cos2 interacts with both the kinase and regulatory domains of Fu, just as Su(fu) does [31]. The regulatory domain of Fu interacts with a central region of Cos2 located C-terminally to the motor domain.

Cos2 – Ci interaction

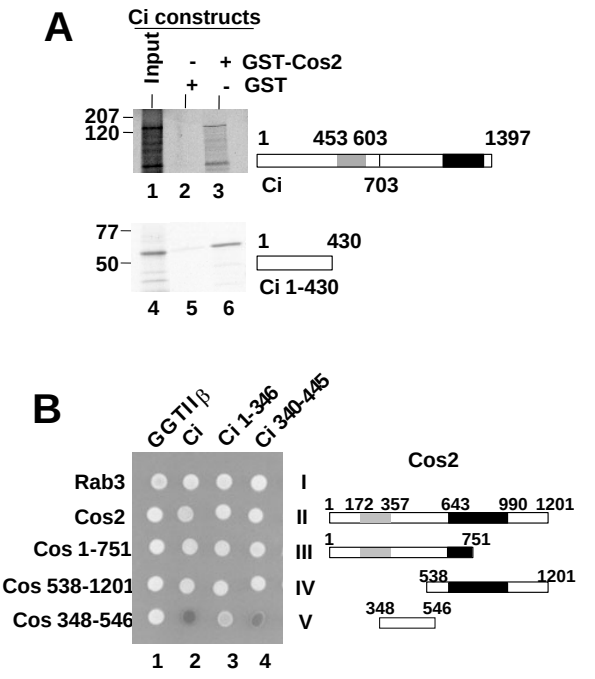

Figure 3
Summary of the interaction domains The interactions between Cos2, Ci, Fu, and Fureg described above and in former work [31] are summarized. The interaction between the region 941–1065 of Ci and Cos2 was previously shown by Wang et al. 2000 [23]. The domains of interactions are indicated in lines above or under each protein structure. No interaction could be detected between Cos2 and Sufu (data not shown). See also legends for Figures 1 and 2. M: Cos2 motor domain, HR: Cos2 heptad repeats, DBD:Ci DNA binding domain; NLS: Ci nuclear localisation signal, AD: Ci activation domain.

In vitro assays between the GST-Cos2 fusion protein and radiolabeled Ci revealed a direct specific interaction between these proteins (Figure 2A). Again, as with Fu, the yeast two-hybrid assay did not allow the detection of an interaction between full-length Cos2 or Cos1-751 and Ci. Nevertheless, interactions consistent with this GST "pull down" result were detected in yeast two-hybrid assays using full-length Ci and the region of Cos2 spanning residues 348 to 546 (Cos348-546) (Figure 2B). This part of Cos2 is adjacent to the Fureg binding site, in a region located immediately C-terminal to the motor domain at the base of the neck region. This region has been shown to regulate the directionality of the movement of kinesins along microtubules [34–45].

To identify the region of Ci that interacts with Cos2, partial deletions of Ci were generated and tested for interaction with Cos2 using a combination of *in vitro* and two-hybrid assays. The first 430 amino acids of Ci are sufficient to interact *in vitro* with GST-Cos2 (Figure 2A). In contrast the first 346 residues of Ci, which have been previously shown to bind Su (fu) [31], do not interact in yeast with Cos538-1201 nor with Cos348-546 constructs (data not shown). These results suggest that Cos2 interacts with a part of Ci between amino acids 346 and 430 (Figure 2B). Indeed, Ci340-445 is sufficient to interact in yeast with Cos348-546 (Figure 2B).

Thus, like Su(fu), Cos2 interacts within the N-terminal region of Ci, which is present in both Ci155 and Ci75-R. The region of Cos2 involved in this interaction is located at the base of its neck, which plays an important role in the activity of conventional kinesin.

Discussion

Cos2, Fu and Su(fu) play multiple and complex roles in Ci control. Cos2 is central to this control both as a negative regulator in the absence of Hh and a positive regulator in the presence of Hh. In the absence of Hh signal, Cos2 action prevents Ci target activation by favoring Ci cleavage and by cytoplasmic retention of full-length Ci [14,18,23,46]. In response to Hh, Cos2 is also required for complete Ci activation in the wing imaginal disc [19,24]. Fu is also involved in positive and negative aspects of Ci regulation. Its kinase activity is required for Ci cytoplasmic release and activation in response to Hh signal. Its C-terminal regulatory domain, although also required for Fu kinase activity, cooperates with Cos2 and Su(fu) to negatively regulate Ci in the absence of Hh signal (see bellow) [19,22,25–27,47]. Although *Su(fu)* mutants display only a very subtle phenotype, Su(fu) acts negatively in the Hh pathway. A decrease in *Su(fu)* (or cos2) dosage suppresses the effects of the loss of Fu kinase activity. *Su(fu)* loss of function aggravates cos2 phenotypes [47], so Su(fu) activity normally assists Cos2 in its negative regulation of Ci. Su(fu) seems to participate both in the retention of Ci in the cytoplasm and in preventing activation of full-length Ci [19,26,27].

Here, we show that Cos2 interacts directly with both Fu and Ci, as does Su(fu). This confirms and extends previous results, which showed these proteins to co-immunoprecipitate from embryo and cultured cell extracts and to be members of one or several large molecular weight cytosolic complex (es) [10,28–30]. The mapping of the regions of each protein sufficient for interaction with its partner, *in vitro* and in yeast (Figure 3), sheds new light on

the respective role of Fu, Su(fu), and Cos2 and on their relationships in the Hh pathway.

Our results demonstrate that Cos2 can bind to the N-terminus of Ci, in a region (amino acids 340–445) located between a region interacting with Su(fu) (amino acids 240–346) and the zinc finger domain (amino acids 453–603). Wang *et al*. also used the two-hybrid method to look for interactions between Ci and Cos2. In contrast to our results, they found no interaction of Cos2 with the N terminus of Ci [23]. This discrepancy can have multiple causes as the fact that their constructs lack amino acids 441 to 445 of Ci or differences in the fusion proteins tested (i.e. differences in protein folding, stability, nuclear targeting etc) or in the reporter used... Nevertheless, they also identified a second region of interaction with Cos2, located in the C-terminus of Ci (Ci 941–1065) [23]. Both the Cos2/Ci941-1065 interaction and the previously reported Su(fu)/Ci240-346 interaction have been shown to be retain Ci in the cytoplasm in the absence of Hh signal [19,23]. Strikingly, neither Su(fu) nor Cos2 interaction regions of Ci map to the amino acids 703 to 850 (Ci-cyt) region identified by Aza-Blanc *et al*. as involved in Ci cytoplasmic tethering [10]. One hypothesis is that the region 703–850 of Ci contains an export signal [14,24].

The interaction of Su(fu) and Cos2 with the N-terminal part of Ci is in agreement with the presence of both full-length Ci-155 and Ci75-R in the large complex(es). Since Fu, Su(fu) and Cos2 can also be found in complex(es) of similar size [10], Cos2 and Su(fu) may control the activity of Ci75-R *in vivo* by a so far unknown mechanism. Several roles can be proposed for the interaction between Cos2 and the Ci N-terminus. The association could reinforce the tethering of Ci in the cytoplasm by Cos2 and Su(fu). An attractive alternative is that the association could underlie the requirement for Cos2 to activate Ci155 in response to Hh. For example, Cos2 and Su(fu) could compete to bind Ci N-terminus and Hh signaling could favor the transient binding of microtubule-unbound Cos2, leading to Su(fu) release.

We find that Cos2, like Su(fu), is able to interact with two different domains of Fu: the Fu kinase and regulatory domains. No phosporylation of Cos2 nor Su(fu) by Fu has been reported so far. In S2 cells, it has been reported that Hh induces Cos2 hyperphosphorylation in a Fu independent manner. Nevertheless, the interactions of respectively Su(fu) and Cos2 with the kinase domain of Fu suggest their direct phosphorylation by Fu. Some kinesins are regulated by phosphorylation, and Fu kinase activity could control Cos2 activity [48,49]. The interaction between the regulatory domain of Fu and Cos2 is in agreement with the lack of Fu-Cos2 co-immunoprecipitation in *fu* mutants that are missing part or all of the regulatory domain ($fu^{class\ II}$ mutant, see below) [28]. Possibly, the *in vivo* interaction of the kinase domain of Fu with Cos2 requires the binding of Cos2 to Fureg, or the interaction could be too transient to be detected in $fu^{class\ II}$ mutants.

The interaction of the regulatory domain of Fu with Su(fu) and Cos2 provides a support for the complex genetic relationships previously described to occur between *Fu*, *Su(fu)*, and *Cos2*. Mutations in the catalytic (class I) and the regulatory (class II) domains of Fu are genetically distinguishable from each other in a *Su(fu)* or *cos2* mutant background, suggesting distinct roles for the two domains of Fu [22,47,50]. Thus, *Su(fu)* ; $fu^{class\ I}$ double mutants are almost wild type, while $fu^{class\ II}$; *Su(fu)* double mutants have a cos2-like phenotype associated with the activation of Ci target genes. Similarly, $fu^{class\ II}$ alleles strongly enhance the effect of a heterozygous *cos2* mutation, though alleles $fu^{class\ I}$ do not. These observations strongly suggest that the regulatory domain of Fu also plays a negative role in Hh signal transduction, this effect being detected only in association with *Su(fu)* or *cos2* mutations. We propose that, in the absence of Hh signal, Fureg could act as a scaffold to anchor Su(fu) and Cos2 in the complex, favoring their negative effect on Ci. In $fu^{class\ II}$ mutants, the negative effect of Su(fu) and Cos2 would be lessened due to their weaker anchoring in the complex. This would only be revealed in the presence of a decrease in Su(fu) or Cos2 dosage that would cause a further destabilization of the complex.

In conventional kinesins, the C-terminal globular tail domain associates with vesicles. Our results show that the Cos2 C-terminus is not required to bind either to the Ci N-terminus (Ci340-445) or the Fureg domain. Instead, both interactions involve adjacent sequences located between the motor domain and the stalk homodimerisation domain. In kinesin itself, this region is called the "neck" domain and is important for efficient motor function. In at least some kinesin molecules the neck is involved in the determination of the direction of kinesin movement along the microtubule, in velocity, in processivity, and in mechanochemical coupling [34–45]. Thus, although no Cos2 motor activity has been described so far, Fu and/or Ci binding to its central region could induce structural changes and therefore modulate Cos2 motor activity. This hypothesis opens the possibility of a feedback control of Ci on Cos2 activity.

Conclusion

The present data, together with published results, suggest that the existence of direct multiple interactions between Cos2, Su(fu), Fu, and Ci, each protein interacting with at least two other partners. We propose a model based upon a complex that includes Fu, Cos2, Su(fu), and Ci (Figure 4). Changes in the composition, activity and/or subcellu-

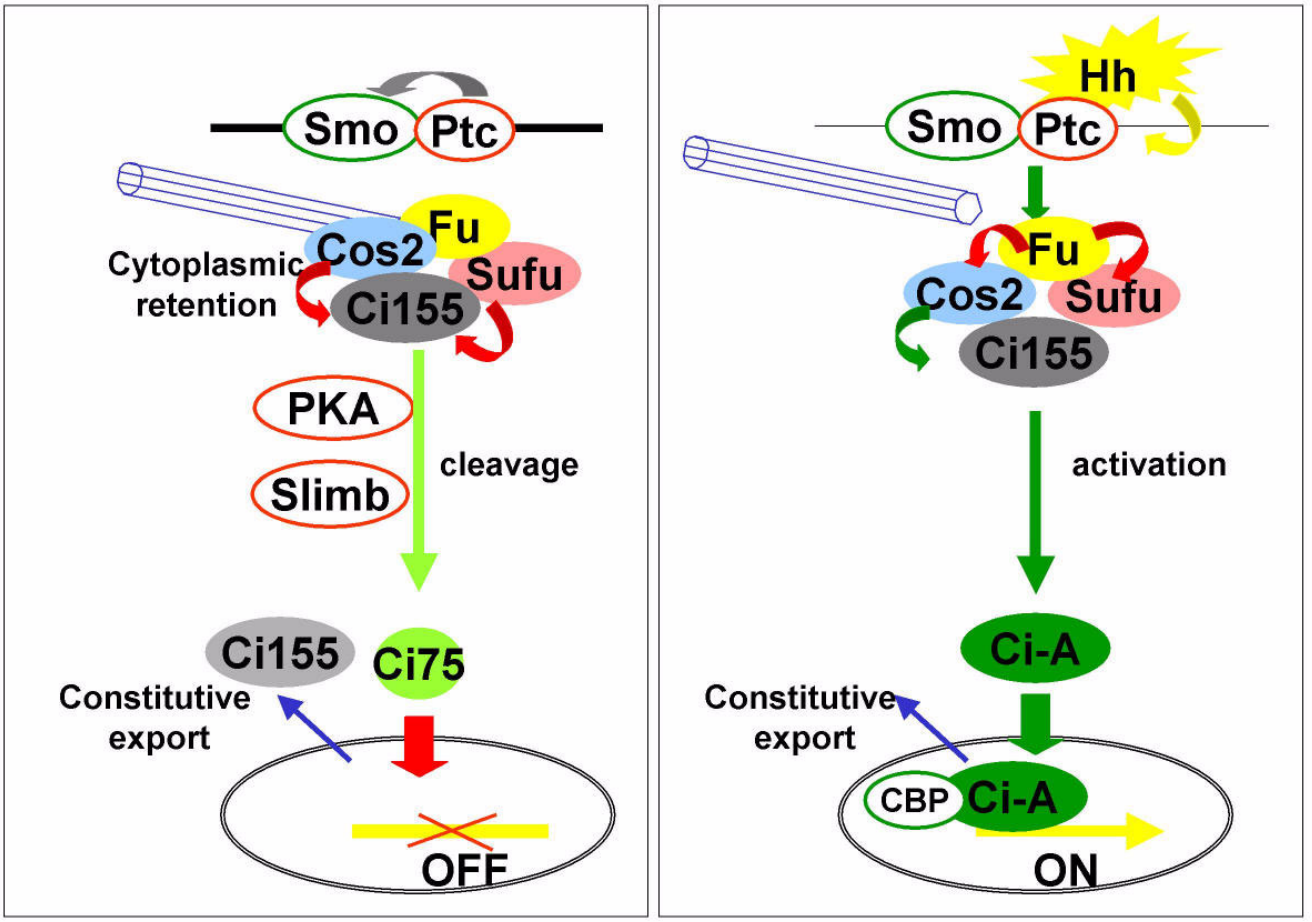

Figure 4
Model of Hedgehog signaling In the absence of Hh signal, Cos2 and Su(fu) binding to Ci prevents Ci activation and retain it in the cytoplasm. Most of Ci is available for cleavage in a process which is dependent upon its phosphorylation by the PKA and which involves Cos2 and Slimb. Uncleaved, full-length Ci, is actively exported from the nucleus. Upon Hh reception, Fused is activated and acts on Cos2 and Sufu, alleviating thus their negative effect on Ci. As a result, Ci cleavage is reduced, Ci155 nuclear import overcomes its export and Ci is activated. Ci activation requires Cos2 and Fu to antagonize Su(fu) negative effect. Activated nuclear Ci interact with the CBP to fully activate the transcription of Hh target genes.

lar localization of this complex will control Ci fate in the absence and in the presence of Hh. In the absence of Hh signal, Su(fu) and Cos2 prevent Ci activation, Ci processing into the repressor form is favored and full-length Ci is exported from the nucleus. Upon Hh reception, Fu is activated, opposing Su(fu) to allow Ci to become an activator. Simultaneously the negative effect of Cos2 is alleviated, perhaps by release from the microtubules and/or a change in subcellular location. This lowers the rate of proteolysis of Ci into its repressor form and triggers nuclear import of full-length Ci. Furthermore, Cos2 and Fu promote the conversion of Ci 155 into an activator form, ultimately resulting in the transcription of Hh target genes.

So far, each protein of the complex can serve as a direct link between two other proteins, and could in this way form a scaffold for the complex. It is important to note that the interactions observed *in vitro* may not define a singular protein complex *in vivo*. A subset of the proteins could associate at any given time, or more than one type of complex may exist simultaneously. Additional interactions, may of course contribute to the stability, organization, or activity of the complex(es). Structural data and new functional tests will be required to learn more about the multiple interactions among the components, and their multiple roles in the control of Hh signaling.

Materials and Methods

Strains

RFY231: *Matα ura3-1 his3 trp1 Δ :: hisG 3lexAop-LEU2 : :leu2* was built by R. Finley (unpublished data). EGY189 *Matα ura3-1 his3 trpl-1 1lexAop-LEU2: :leu2* has a less sensitive *leu2* reporter gene than RFY231 due to a lower number of copies of *lexAop*[51]), RFY206: *Mata his3Δ200 leu2-3 lys2 Δ 201 ura3-52 trp1ΔhisG.* Yeast cultures and transformations were performed as in [31].

Plasmids

The two-hybrid pEG and pJG expression vectors were derived from pEG202 (translational fusion with the DNA binding domain of lexA) and pJG4-5 (translational fusion with the activation domain B42) [33,52]. Two numbers added after the protein name indicate the first and last residues of truncated proteins. pJGCi (full-length), pJGCi1-346 and pJGFu306-805 (pJGFureg), were described in [31]. pEGCos2 (full-length), pEGCos1-751, pEGCos348-546, pEGCos538-1201, pEGCos538-751, pJGCi340-445, pJGFu437-805 were built by in frame cloning of restriction fragments or PCR products. pSH18-34 carries the lacZ reporter gene [52]. The GST-Cos2 translational fusion was made by introducing the entire Cos2 coding sequence in frame with the glutathione S-transferase (GST) gene in pGEX-4T3. All PCR products and junctions were sequenced.

GST fusion protein binding assays

GST-Cos2 and GST protein production and purification, *in vitro* coupled transcription-translation, and *in vitro* interaction assays were performed as described in [31]. In all the figures, the amount of radiolabelled protein used in each "input" lane equals one-third the amount of protein used in the assay and equivalence in the amount of GST and GST-Cos2 was checked by Coomassie blue staining. All assays were done at least twice.

Two hybrid assays

RFY231-pSH18-34 or EGY189-pSH18-34 were transformed with pEG (lexA fusion) constructs and subsequently mated with RFY206 transformants containing the pJG constructs (B42 fusion). Geranyl-geranyl transferase II β (GGTIIβ) and Rab3 are two proteins known to interact together [53]. They are respectively used as negative controls in tests of the pEGCos2 constructs and the pJGCi or pJGFu constructs. Diploids were selected on SCglu-UHW. For each diploid strain, 3 μl of cell suspension were spotted on SCgal-UHW (for β-galactosidase assays) or SCgal-UHWL solid media (for leucine auxotrophy assays). Growth on SCgal-UHWL was observed after 24 hours of incubation at 30°C. β-galactosidase assays were performed by the overlay method as described in [54]. All assays were done at least three times.

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