 Differential regulation of Hedgehog target gene transcription by Costal2 and Suppressor of Fused

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Accepted 10 January 2005
Development 132, 1401-1412
Published by The Company of Biologists 2005
doi:10.1242/dev.01689

Summary

The mechanism by which the secreted signaling molecule Hedgehog (Hh) elicits concentration-dependent transcriptional responses from cells is not well understood. In the Drosophila wing imaginal disc, Hh signaling differentially regulates the transcription of target genes decapentaplegic (dpp), patched (ptc) and engrailed (en) in a dose-responsive manner. Two key components of the Hh signal transduction machinery are the kinesin-related protein Costal2 (Cos2) and the nuclear protein trafficking regulator Suppressor of Fused [Su(fu)]. Both proteins regulate the activity of the transcription factor Cubitus interruptus (Ci) in response to the Hh signal. We have analyzed the activities of mutant forms of Cos2 in vivo and found effects on differential target gene transcription. A point mutation in the motor domain of Cos2 results in a dominant-negative form of the protein that derepresses dpp but not ptc. Repression of ptc in the presence of the dominant-negative form of Cos2 requires Su(fu), which is phosphorylated in response to Hh in vivo. Overexpression of wild-type or dominant-negative cos2 represses en. Our results indicate that differential Hh target gene regulation can be accomplished by differential sensitivity of Cos2 and Su(Fu) to Hh.

Key words: Kinesin, Morphogen, Differential gene regulation, Hedgehog, Costal2, Suppressor of Fused, Drosophila

Introduction

Hedgehog signaling proteins play wide-ranging and fundamental roles in patterning both vertebrate and invertebrate tissues throughout development (Nybakken and Perrimon, 2002; Lum and Beachy, 2004). Many of the components involved in Hh signal transduction, as well as Hh target genes, have now been identified, but how Hh proteins control appropriate target gene responses in so many different cell types and developmental contexts remains unknown.

Hh signal emanating from the posterior (P) Drosophila wing imaginal disc induces transcription of decapentaplegic (dpp), patched (ptc) and anterior engrailed (en) in stripes of cells within the anterior (A) compartment of the wing imaginal disc, adjacent to the anteroposterior (AP) boundary. dpp is induced in a 12- to 15-cell-wide stripe, ptc in a ~10-cell-wide stripe, and en in a 5- to 7-cell-wide stripe (see Fig. S1 in the supplementary material). The regulation of these target genes is differentially sensitive to Hh dose: modulating the level of Hh produced by P cells dramatically affects the presence and width of the stripes of target gene expression (Strigini and Cohen, 1997). Transcriptional activation of all three target genes by Hh is dependent on the function of the transmembrane protein Smoothened (Smo) in the responding cells, indicating that Hh acts directly on these target cells and not through a relay system involving other signaling pathways (Vincent and Briscoe, 2001).

Downstream of Smo, the zinc-finger transcription factor Ci controls the transcriptional responses to Hh (Dominguez et al., 1996; Methot and Basler, 2001). In the absence of Hh, as in anterior disc cells distant from the Hh source, full-length Ci (CiFL) is proteolytically processed into a truncated form, CiR, that consists of the N terminus of Ci and its zinc-finger binding domain. CiR is a repressor of dpp and hh (Aza-Blanc et al., 1997). The Hh signal opposes the proteolytic processing of Ci, which stabilizes CiFL, and causes the nuclear accumulation of CiFL, which is sufficient to induce dpp transcription.

Additional Hh-mediated events that further activate Ci are necessary for the transcription of ptc (Ohlmeyer and Kalderon, 1998; Chen et al., 1999a; Methot and Basler, 1999; Wang and Holmgren, 1999; Wang et al., 2000). This activated form, CiACT, has thus far evaded biochemical characterization, but its existence is suggested by experiments showing that expression of stabilized CiFL is insufficient to induce ptc and en transcription to levels comparable with wild-type levels (Jiang and Struhl, 1998; Methot and Basler, 1999; Chen et al., 1999a).

Ci exists in the cytoplasm as a component of high molecular weight protein complexes (Robbins et al., 1997; Sisson et al., 1997). Members of these complexes include Costal2 (Cos2; cos – FlyBase), which is a kinesin-related protein, Fused (Fu), which is a Ser/Thr kinase, and Suppressor of Fused [Su(fu)], which is novel PEST-motif containing protein (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; Stegman et al., 2000; Monnier et al., 2002; Stegman et al., 2004; Wang and Jiang, 2004).
Cos2 is an important negative regulator of dpp and ptc in wing imaginal discs. Loss of Cos2 from anterior cells derepresses ptc and dpp transcription (Sanchez-Herrero et al., 1996; Wang and Holmgren, 2000; Wang et al., 2000) (this paper), indicating that Cos2 inhibits the positive transcriptional activities of Ci. It may do this in several ways: Cos2 activity opposes the nuclear translocation of CiFL (Chen et al., 1999a; Stegman et al., 2000; Wang et al., 2000; Monnier et al., 2002), retaining it in the cytoplasm in a microtubule-dependent manner (Wang and Jiang, 2004). Some evidence suggests that Cos2 is also involved in proteolytic processing of Ci to CiR (Wang and Holmgren, 2000).

Cos2 can also contribute to target gene activation as well. In some cells near the AP boundary, Cos2 is required for ptc and Hh-dependent en expression (Wang and Holmgren, 2000; Wang et al., 2000). Recent work has shown that Cos2 binds to Smo and Smo to the cytoplasmic complex (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). Consistent with this, Cos2 interacts with membranes differentially in response to Hh signaling (Stegman et al., 2004). The Cos2-Smo interaction is necessary to transduce a response to a high level of Hh and to turn on en transcription in A cells (Jia et al., 2003) and the ptc-luciferase reporter in cultured cells (Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). Thus, Cos2 is both a negative and positive regulator of Hh target genes.

An intriguing feature of Cos2 is its sequence similarity to kinesins, dimeric molecular motors that bind and move along microtubules carrying organelles, vesicles, proteins and other cargo to destinations within the cell. Cos2 binds microtubules in vitro and is released from them in a Hh-dependent manner (Robbins et al., 1997; Wang and Jiang, 2004). Sequence alignments of Cos2 with other kinesins indicate that it has an N-terminal motor domain, followed by a putative ‘neck’ domain, a middle region of heptad repeats that form the coiled-coil dimerization domain, and a unique C-terminal domain that could confer cargo binding specificity (Sisson et al., 1997).

The Cos2 motor domain sequence is quite divergent (Sisson et al., 1997; Lawrence et al., 2004), indicating that its function may differ from those of classical kinesins. Despite the sequence differences, within the Cos2 motor domain is a well-conserved P-loop, a motif that is necessary for binding ATP and catalyzing the hydrolysis reaction necessary for translocation along microtubules ( Muller et al., 1997; Rice et al., 1999). The presence of a P-loop in Cos2 suggests that ATPase activity is important for Cos2 function.

To assess the importance of the putative motor, neck and cargo domains to Cos2 in Hh signaling, we made deletion constructs of Cos2 lacking each domain. In addition, we changed the Ser182 of Cos2 to Asn (S182N) in the P-loop, which in other kinesins gives rise to a dominant-negative form that lacks ATPase activity. Using these mutant forms of Cos2, we investigated the roles of Cos2 and Su(fu) in the regulation of the Hh target genes dpp and ptc. Our data indicate that differential regulation of dpp and ptc occurs by modulation of Cos2 and Su(fu) activities.

Materials and methods

Stocks

The following Drosophila stocks were used: dpp-lacZ/CyO (Sanicola et al., 1995); H84ptc-lacZ (Ingham et al., 1991); 71B Gal4 (Brand and Perrimon, 1993); MS1096 Gal4 (Capdevila and Guerrero, 1994); FLP-out cassettes yw act5C < CD2 < G4 (Pignoni and Zipursky, 1997); cos2, cos2<sup>1</sup>, cos2<sup>3</sup>, cos2<sup>5</sup> (Whitele, 1976; Grau and Simpson, 1987; Simpson and Grau, 1987; Heitzler et al., 1993; Sisson et al., 1997); FRT 2M13, FRT cos2<sup>W1</sup>, hsp70-flp (Xu and Rubin, 1993; Sisson et al., 1997); Su(fu)<sup>JS</sup> (Bloomington Stock Center); and UAS-Hs (gift from Phil Beachy).

The reporter strain H84ptc-lacZ (a gift from M. Fritz) is an nuclear lacZ enhancer trap in the ptc locus. dpp-lacZ is a transgene constructed from the dpp region and the lacZ promoter driving nuclear lacZ (Sanicola et al., 1995).

UAS-cos2, UAS-cos22GFP, UAS-S182N, UAS-S182N-GFP, UAS-S182T, UAS-S182T-GFP, UAS-Neck, and UAS-Cos2ΔC were generated by P-element-mediated transformation of the constructs described below. To control for position effects, at least two different insertion lines were used to verify each result.

Generation of FLP-FRT and FLP-out clones

The heat shock regimen for making cos2Δ clones was as described (Sisson et al., 1997). For making FLP-out overexpression clones, flies were mated at 20°C, heat shocked 48 hours after egg laying (AEL) for 30 minutes, allowed to recover at room temperature for 30 minutes, and then heat shocked for 30 minutes. The heat-shock regimen was conducted on larvae twice a day (8-12 hours apart) for 3 days.

Plasmid construction

pUAS-cos2 was made by inserting a 3.4 kb fragment encoding the cos2 open reading frame (ORF) into pUAST (Brand and Perrimon, 1993). pUAS-cos2-GFP was made by fusing the GFP ORF from pBD1010 (gift from Barry Dickson), cut with Xhol (blunted with Klenow enzyme, NEB) and Xbal, to pUAS-cos2 cut with BamHI and Xbal. This fuses the GFP ORF in frame with the C-terminal end of Cos2, eight amino acids upstream of the STOP codon. The fusion amino acid sequence reads as follows: NKEGTEKTM... where M is the normal start codon of GFP and Cos2 amino acids are underlined.

To make pUAS-ΔNeck-GFP, pUAS-cos2-GFP was cut with NcoI and re-ligated. This results in a 143 amino acid in-frame deletion of the neck region of Cos2. The deletion also removes two conserved microtubule binding sites and 78 amino acids of the motor domain.

To make ΔMotor, two complimentary oligonucleotides were synthesized such that, when annealed, they form a double stranded fragment from this ΔMotor plasmid. A pBS-KS plasmid containing the Neck-GFP, pUAS-cos2 fragment from this ΔMotor construct is missing the first 313 amino acids and has a 6×His tag at the N terminus.

To make pUAS-Neck-GFP and pUAS-cos2-GFP, two complimentary oligonucleotides were annealed and cut with SpeI and HincII compatible ends. This results in a 143 amino acid in-frame deletion of the neck region of Cos2. The deletion also removes two conserved microtubule binding sites and 78 amino acids of the motor domain.

To make ΔMotor, two complimentary oligonucleotides were synthesized such that, when annealed, they form a double stranded oligonucleotide with NcoI and AarII compatible ends. ThisΔMotor oligonucleotide encodes a 6×His tag in frame with the cos2 ORF. Oligonucleotide sequences are as follows: 5′-CATGACACCCACCA
CACCAACCAGACGT-3′ and 5′-CCGTGGTTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG-3′. A pBS-KS plasmid containing the cos2 ORF was cut with NcoI and AarII and the ΔMotor oligo inserted into this vector. A SpeI fragment from this ΔMotor construct was then inserted into pUAScos2-GFP and pUAS-cos2 to create pUAS-ΔMotor-GFP and pUAS-ΔMotor constructs, respectively. The Cos2 protein encoded by ΔMotor plasmids is missing the first 313 amino acids and has a 6×His tag at the N terminus.

To make pUAS-Cos2ΔC-GFP, two complimentary oligonucleotides were synthesized: 5′-CCGGTGCACCACCA
CACCAACCAGACGT-3′ and 5′-GGCGTGGTTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG-3′. These oligonucleotides were annealed and cut with KpnI and ligated to pUAS-cos2-GFP cut with SgrAI and KpnI. The resulting construct encodes Cos2 up to amino acids 1057, after which the C-terminal end is deleted and replaced by a 6×His tag followed by fusion in frame with GFP.

To make S182T and S182N mutant insertions, a PCR-based approach was used. The forward primers encode the appropriate point mutation (underlined); S182T forward primer, 5′-CCAGCCGGCCAAAGGCAAATACACACTTAC-3′, S182N forward primer, 5′-CCAGCCGGCCAAAGGCAAAAATACACACA-
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CTCTAC-3′; reverse primer, 5′-TGCCATTAACCCCGTACATGAG-3′. PCR products were cut with BstUI and AarII, and ligated to pRV3.9 (Sisson et al., 1997) cut with BstUI and AarII to make pBS-S182T or pBS-S182N, respectively. To make pUAS-S182T, pUAS-S182N, pUAS-S182T-GFP and pUAS-S182N-GFP, pBS-S182T or pBS-S182N was cut with BstUI and Nhel and inserted the fragments to pUAS-cos2 and pUAS-cos2-GFP cut with BstUI and Nhel. The resulting plasmids encoded a single amino acid substitution at amino acids 182 to Thr or Asn, respectively. For the rescue experiment, pUAS-cos2-GFP were cut with Nhel and XbaI (blunted with Klenow) and ligated this fragment to pK6.5 (Sisson et al., 1997) cut with Nhel and Maml. This replaced the C-terminus of Cos2 with the C-terminal of Cos2-GFP from pUAS-cos2-GFP described above. Each genomic rescue fragment was inserted into pCasper4. To make the Cos2 ∆C-GFP expression (green) driven by 71B Gal4 represses dpp-lacZ expression (red) at the AP boundary. (K,L) A large FLP-out clone expressing ∆Motor-GFP (green) ectopically expresses dpp-lacZ (red) in a cell-autonomous manner. Overgrowth of anterior tissue caused by the clone is indicated (white arrow). A posterior clone, which does not ectopically express dpp-lacZ or produce overgrowth of tissue, is also indicated (blue arrowhead). Because not all nuclei in the disc lie in the same optical plane, there appear to be variations in dpp-lacZ staining in different parts of the disc. Correcting this by focusing on small local areas of the disc confirms that dpp-lacZ is expressed at uniform, high levels throughout S182N-expressing clones (data not shown). (M,N) A FLP-out clone expressing S182T-GFP at the AP boundary (green) that interrupts the normal region of dpp-lacZ expression represses dpp-lacZ expression (red) in a cell-autonomous manner. Results were the same for each construct and its C-terminally fused GFP counterpart except for ∆Motor, for which ∆Motor-GFP expressing flip-out clones could not be generated. For this and all other figures, wing discs are oriented such that anterior is leftwards and dorsal is downwards.

Fig. 1. Effect of different mutants of Cos2 on dpp-lacZ reporter expression at the AP boundary in wild-type third instar larval discs. Nuclear β-galactosidase is immunofluorescently labeled (red). Area in A (white box) is shown at higher magnification in B. (C,D) Overexpression of cos2GFP using the 71B Gal4 driver (green) represses dpp-lacZ expression (red) at the AP boundary. (E,F) A small FLP-out clone expressing ∆Motor-GFP (green) in the anterior compartment ectopically expresses dpp-lacZ (red) in a cell-autonomous manner. A region of normal dpp-lacZ expression at the AP boundary is shown (arrowhead). (G,H) An anterior FLP-out clone of cells expressing ∆Neck-GFP (green) ectopically expresses dpp-lacZ (red) in a cell-autonomous manner. (I,J) Cos2∆C-GFP expression (green) driven by 71B Gal4 represses dpp-lacZ expression (red) at the AP boundary. (K,L) A large FLP-out clone expressing S182N-GFP derepresses dpp-lacZ expression within the clone (red) in a cell-autonomous manner. Box in K indicates the area that is shown at higher magnification in L. The normal expression of dpp-lacZ at the AP boundary is shown in K (yellow arrowhead). Overgrowth of anterior tissue caused by the clone is indicated (white arrow). A posterior clone, which does not ectopically express dpp-lacZ or produce overgrowth of tissue, is also indicated (blue arrowhead).
Fig. 2. Wing duplications resulting from S182N expression can be suppressed by co-expression of cos2. (A) Wild-type wing (normal costa indicated by arrow). (B,C) Examples of the two extremes of expressivity seen in wing blades in which S182NGFP is expressed using MS1096 Gal4. The same results are seen in wings expressing S182N. The outgrowth of the costa is shown in B by an arrow. B represents the very mild phenotypes seen, while C represents the extreme phenotypes seen. (D) Overexpression phenotype of MS1096 Gal4; UAS S182T/+ flies. Wing blades are reduced in size and third and fourth wing veins are missing. (E) Phenotype of MS1096 Gal4; UAS cos2GFP/+ flies. There are no signs of anterior wing outgrowth or duplications, but there is the similarity seen in the extreme phenotypes of E and D. (F) Phenotype of MS1096 Gal4; UAS S182N/+; UAS cos2/+ flies. Note there are no signs of anterior wing outgrowth or duplications, and the phenotype is similar to E and D. All flies were grown at 29°C to maximize levels of expression using the MS1906 Gal4 driver, which strongly drives expression throughout the wing pouch. All wings were photographed at the same magnification to show the differences in wing size between each genotype.

GFP rescue construct, the same strategy was used, starting with pUAS-Cos2AC-GFP instead of pUAS-cos2-GFP.

Immunohistochemistry and in situ hybridization

Staining was performed using Brower’s Fix as described (http://bender.zoology.wisc.edu/antiview.html) using antibodies at the following dilutions: anti-CICL 2A1 (1:5) (a gift from Robert Holmgren); anti-βGal, mouse (Promega) 1:1000; anti-βGal, rabbit (Promega) 1:1000; anti-En 4D9, mouse monoclonal (a gift from Nipam Patel); 1:1000; anti-Myc mouse monoclonal (Sigma) 1:500; anti-Cos2 0.8 rat polyclonal, prepared and used as described previously (Sisson et al., 1997). Fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:200. In situ hybridization was carried out as described by Johnson et al. (Johnson et al., 1995). dpp and ptc probes: ptc probe was a gift from Alan Zhu. dpp probe was made with the Genius 4 kit (Promega); the template was a gift from Michael Hoffman.

Imaginal disc lysates and protein blots

Third instar wing imaginal discs (100-500) were dissected in Clone 8 cell culture media, made as described (http://www.stanford.edu/~rnusse/ownpage/protdiscscells.html), supplemented with a cocktail of protease inhibitors: PMSF, benzamidine, aprotinin, pepstatin, chymostatin, leupeptin (PIs). Discs were rinsed once in phosphate-buffered saline (PBS) supplemented with PIs and pelleted for 30 seconds in a microfuge at 4°C. Excess PBS was removed, the pellet of discs was resuspended in 10 µl 6X SDS lysis buffer (350 mM Tris-Cl, pH 6.8, 10% SDS, 30% glycerol, 0.093 g/ml DTT) and dounce homogenized. PBS (50 µl) was added. SDS-PAGE electrophoresis was carried out using standard methods with a mini-Protean3 BioRad gel apparatus (Sambrook and Russell, 2001). Antibodies used for protein blots were as follows: anti-Su(fu) rat polyclonal 1:1000; anti-BAP111 (gift from Janet Jin, Ophelia Papoulous 1:1000; anti-Dsh rabbit polyclonal (a gift from K. Willert and R. Nusse) 1:1000; anti-CICL 2A1, anti-Ci 1C2, rat monoclonal 1:5 (gifts from Robert Holmgren); anti-Cos2 0.8 rat polyclonal, prepared and used as described previously (Sisson et al., 1997). Quantitation of protein blots was carried out using Alphalager 2000 software (AlphaInnotech) (Chen et al., 1999a). For phosphatase reactions, lysates were treated with AG1 X2 resin (BioRad) to remove SDS using the method described (Weber and Kuter, 1971; Galko and Tessier-Lavigne, 2000), spun briefly to pellet resin, and the supernatant was removed to fresh tubes. Lambda phosphatase (NEB) was added for 2 hours at 30°C, then the proteins were run on a SDS-PAGE gel and immunoblotted.

Results

The Cos2 motor and neck regions are important for its function in Hh signaling

To determine which features of the kinesin-related structure of Cos2 are important for Hh signal transduction, we made mutants of Cos2 in which either the motor, neck or C terminus is deleted. As an in vivo assay for Cos2 function, we examined the effect of each mutant protein on Hh target gene transcription in wing imaginal discs. Loss-of-function cos2 clones in A cells permit cell-autonomous ectopic transcription of dpp and ptc, while cos2 clones in P cells have no effect on dpp or ptc (Sanchez-Herrero et al., 1996; Sisson et al., 1997; Wang et al., 2000; Wang and Holmgren, 2000) (see Fig. S1 in the supplementary material). Conversely, overexpression of either cos2 or full-length cos2 fused in frame to green fluorescent protein (cos2-GFP), using the UAS-GAL4 system (Brand and Perrimon, 1993) repressed dpp-lacZ and ptc-lacZ in cells at the AP boundary in a cell-autonomous manner (see Fig. S1 in the supplementary material and Fig. 1C,D).

Each mutant protein (schematically represented in Fig. 1O) was tested for its ability to repress dpp-lacZ expression. The protein lacking the C-terminal domain (denoted Cos2ΔC) was able to repress dpp (Fig. 1I,J). To confirm that GFP-tagged full-length Cos2 and the Cos2ΔC proteins have wild-type Cos2 activity, we tested their ability to rescue the phenotypes of cos2 mutants. Genomic DNA fragments encoding full-length cos2-GFP or cos2-ΔC-GFP inserted in place of the wild-type cos2 gene rescued the larval lethality of cos2 mutants and also suppressed the duplicated wing phenotypes of trans-heterozygotes of cos2 loss-of-function and null alleles (cos22/cos21) (Table 1). As the GFP fusion transgenes rescued both phenotypes as well as the genomic fragment encoding wild-type cos2 (Table 1) the C-terminal GFP fusion did not interfere with Cos2 function. Thus, data from either or both GFP-tagged or untagged deletion constructs are shown below.

The mutant proteins lacking the motor (ΔMotor) or the neck (ΔNeck) had the unexpected and dramatic effect of inducing dpp expression. This occurred exclusively in anterior disc cells in a cell-autonomous manner (Fig. 1E-H). These mutant proteins thus appear to interfere with the repressive action of endogenous Cos2 protein.
Expression of S182N in wing discs derepresses dpp expression in A cells

Because the ΔMotor and ΔNeck deletion constructs had altered Cos2 activity, we focused our attention on this N-terminal region. In other kinesin family proteins, the motor and neck domains are necessary for ATP hydrolysis and the conformational changes necessary for microtubule-based movement (Woehlke and Schliwa, 2000). Within the motor domain of Cos2 is a conserved P-loop motif, which in conventional kinesins is necessary for ATP hydrolysis. In order to determine whether an intact P-loop is important for normal Cos2 function, we used site-directed mutagenesis to change the conserved Ser at position 182 to Asn, generating a form of Cos2 designated S182N. Mutation of a conserved Ser or Thr to Asn at that position in the P-loop has been shown to give rise to dominant-negative kinesins that dimerizes with their endogenous kinesin partners, irreversibly bind microtubules, lack ATPase activity and cannot move (Meluh and Rose, 1990; Rasooly et al., 1991; Blangy et al., 1998). These mutant kinesins decorate microtubules in mammalian cultured cells and inhibit the movements of their endogenous kinesin partners (Blangy et al., 1998; Nakata and Hirokawa, 1995).

S182T was generated as a control for the S182N mutant protein. This conservative Ser to Thr change is not expected to alter normal ATPase activity (Nakata and Hirokawa, 1995).

To determine the effect of S182N production on dpp-lacZ activation, the FLP-out system (Pignoni and Zipursky, 1997) was used to generate clones of cells that express S182N in either the anterior or posterior wing disc compartments. Strikingly, dpp-lacZ was consistently derepressed in anterior S182N-expressing clones in a cell-autonomous manner (Fig. 1K,L). The derepression of dpp-lacZ occurred regardless of the position of the anterior clone with respect to the AP boundary (Fig. 1K). The dpp-lacZ expression level in S182N-expressing clones (Fig. 1K, white arrowhead) is comparable with its Hh-dependent expression level at the AP boundary (Fig. 1K, yellow arrowhead). Discs containing large S182N-expressing clones frequently had dramatic overgrowths of anterior tissue (Fig. 1K, white arrow). As with cos2 loss-of-function clones, no ectopic dpp expression, or disc outgrowth, was observed in posterior compartment clones (Fig. 1K, blue arrowhead). By contrast, producing the control S182T protein in clones repressed dpp expression in a manner comparable to overexpression of wild-type cos2 (Fig. 1M,N).

Anterior wing duplications in cos2 loss-of-function clones arise because of ectopic dpp expression within those clones (Capdevila and Guerrero, 1994). To test whether dpp derepression in S182N-expressing cells gives rise to adult wing duplications, S182N and S182T mutant proteins were produced throughout the wing pouch using the MS109-Gal4 driver. In contrast to overexpression of wild-type cos2 and cos2GFP, expression of S182N or S182N-GFP gave rise to anterior wing duplications, with 100% penetrance and variable expressivity. The range of severity of the phenotypes generated by S182N expression is shown in Fig. 2B,C. The anterior wing duplications observed mimicked the phenotypes of hypomorphic alleles of cos2, which are large duplications, and sometimes triplications, of a proximal anterior wing structure called the costa (arrow, Fig. 2A,B), from which costal2 gets its name (Whittle, 1976). By contrast, no wing duplications occurred when S182T-GFP was overexpressed in the wing pouch using the MS109-Gal4 driver (Fig. 2D). Instead, the phenotype of S182T expression mimicked overexpression of wild-type cos2 and cos2GFP (Fig. 2E).

In order to ensure that the cause of these striking differences in activity between S182N and S182T mutants was not due to different protein levels, protein blots of wing disc lysates were stained for tagged and untagged proteins used in these experiments. The levels of wild-type and mutant Cos2 proteins produced in discs are similar (Fig. 3). The derepression of dpp transcription brought about by S182N, but not the control S182T or wild-type cos2, is due to differences in activity, not different expression levels.

S182N is a dominant inhibitor of wild-type Cos2 activity

S182N behaves like a dominant-negative protein. As many kinesins work as homodimers, a non-functional S182N-Cos2 heterodimer may inactivate endogenous Cos2, leading to a loss-of-function cos2 phenotype and ectopic expression of dpp. In this case, simultaneous expression of wild-type cos2...
along with S182N should ameliorate the effect of S182N, reducing or abolishing the wing duplications seen with S182N alone. To test this possibility, wild-type cos2 was overexpressed together with S182N using the MS109-Gal4 driver. Co-expression completely suppresses wing duplications, while giving rise to a cos2-overexpression phenotype (Fig. 2F). Overexpression of S182N with a UAS-lacZ transgene instead of UAS-cos2 as a control for Gal4 titration had no effect on the duplication phenotype caused by S182N expression (data not shown).

**S182N expression results in the production and stabilization of Ci in its full-length form**

CiFL stabilization is sufficient to induce dpp transcription in the wing (Methot and Basler, 1999; Chen et al., 1999b; Wang et al., 2000). To see how S182N expression affects CiFL levels and stability, discs expressing S182N under the control of the 71B Gal4 driver were stained with antibodies against CiFL. The pattern of 71B Gal4-driven expression is shown in Fig. 4D.

In wild-type discs, CiFL protein is at low levels throughout the anterior compartment, with elevated levels of CiFL-protein at the AP boundary (Fig. 4A, bracketed region). This elevated level of Ci is dependent on Hh signaling and reflects an inhibition of Ci cleavage by Hh at the AP boundary (Wang and Holmgren, 1999). By contrast, overexpression of wild-type cos2 or S182T throughout the wing pouch with the 71B Gal4 driver reduces CiFL staining at the AP boundary, especially in those cells overexpressing cos2 at high levels near the AP boundary (Fig. 4B, bracketed region; see 4D). cos2 overexpression therefore opposes the stabilizing effect of Hh on CiFL, and the S182T mutant has the same effect (not shown). S182N, by contrast, causes higher levels of CiFL staining in the anterior disc. The stripe of stabilized Ci is at least eight cell diameters wider than in wild-type discs (Fig. 4C, compare bracketed regions of Fig. 4A-C).

In order to see whether S182N interferes with the proteolytic processing of CiFL into CiR, thus stabilizing CiFL, protein blots of wing disc extracts were stained with an antibody that detects both CiR and CiFL (Fig. 4E,F). Multiple bands representing CiR are observed in the 75 kDa range; these are presumably isoforms of CiR (Fig. 4E). In Hh overexpressing discs (Fig. 4E, lane 2), Ci is stabilized in its full-length form and all CiR isoforms are undetectable. In S182N-expressing discs, the amount of CiFL is increased compared with its level in wild-type, cos2-overexpressing or S182T-expressing discs (Fig. 4E, lane 5, compare with lanes 1, 3, 6). Interestingly, CiR is also present in S182N-expressing discs (Fig. 4E, lane 5 compare with lanes 1, 3, 6). This indicates that proteolytic processing of Ci may persist in the presence of S182N, and that S182N may affect the stability of CiFL independently of Ci processing.

Quantification of the relative amounts of CiR versus total Ci concentration in S182N-producing discs showed a significant reduction in relative CiR concentration. Error bars show the standard deviations of three independent experiments (Fig. 4F). This altered ratio, which favors CiFL, is likely to account for the ectopic activation of dpp in anterior cells where S182N is expressed, as stabilization of Ci in its full-length form is sufficient to activate dpp transcription (Methot and Basler, 1999).

**Fig. 4.** Expression of S182N stabilizes Ci in its unprocessed, 175 kDa form in wing imaginal discs. (A-D) Stabilization of Ci shown by immunostaining of discs using a monoclonal antibody recognizing the unprocessed form of Ci only (CiFL). (A) A wild-type disc showing normal levels of stabilization of Ci at the anteroposterior boundary. CiFL signal is strongest along the AP boundary (bracket). (B) Over-expression of Cos2 using the 71B Gal4 driver results in reduced levels of CiFL at the AP boundary when compared with wild type (bracket). (C) Expression of S182N using the 71B Gal4 driver results in a wider stripe of cells at the AP boundary positive for CiFL expression (bracket). Cos2-GFP expression driven by 71B Gal4 is shown in D. The same expression pattern is achieved for the expression of S182N driven by 71B Gal4 (not shown). (E) A protein blot of wing imaginal disc extracts probed with anti-Ci antibody 1C2 (a gift from Robert Holmgren), which recognizes both processed (75 kDa) and unprocessed (175 kDa) forms of Ci. Lane 1, wild-type imaginal disc extract; lane 2, extract from discs overexpressing Hh; lane 3, extract from discs overexpressing Cos2; lane 4, extract from discs simultaneously overexpressing Cos2 and Hh; lane 5, extract from discs overexpressing S182N; lane 6, extract from discs overexpressing S182T Cos2. 71B Gal4 was used to drive expression of all transgenes indicated. (F) Quantitation of three independent western blots is plotted as relative amounts of CiR to CiTOTAL (y-axis) in wing disc lysates corresponding to genotypes expressing the UAS transgenes indicated driven by 71B Gal4 (x-axis).
S182N represses ptc at the AP boundary and does not activate ptc in anterior cells

Cos2 is necessary to repress ptc and dpp in A cells, and overexpression of Cos2 represses both ptc and dpp in cells at the AP border (see Fig. S1 in the supplementary material), so a dominant inhibitor of Cos2 such as S182N is expected to induce ptc as well as dpp. To test this hypothesis, discs expressing S182N were stained for ptc-lacZ expression. Contrary to expectations for a dominant-negative Cos2 mutant, S182N repressed ptc-lacZ expression at the AP boundary, instead of inducing extra ptc-lacZ expression in anterior cells (Fig. 5B,C). This repressive activity of S182N may be responsible for the similarities in size and AP boundary defects in wings producing S182N and wild-type Cos2 or S182T (Fig. 2C-E). Furthermore, production of S182N, S182T or Cos2 using strong wing pouch GAL4 drivers or in FLP-out clones, does not activate ptc in the anterior compartment away from the AP boundary (Fig. 5 and data not shown). With respect to ptc regulation, then, S182N activity is similar to wild-type Cos2 and S182T activity (Fig. 5D,E and see Fig. S1I,J in the supplementary material).

Removal of Su(fu) changes S182N Cos2 from a repressor into a ptc activator

Stabilizing Ci in its full-length form, for example by inhibiting proteolysis, is sufficient to activate dpp, but not ptc, in A cells (Jiang and Struhl, 1998; Methot and Basler, 1999). S182N made in A cells also stabilizes CiFL and induces dpp but not ptc. One way to stimulate the induction of ptc transcription by CiFL is to remove the inhibitory activity of Su(fu) (Methot and Basler, 2000). Su(fu) prevents translocation of CiFL into the nucleus (Chen et al., 1999a; Lefers et al., 2001; Methot and Basler, 2000; Wang et al., 2000) and maintains high levels of CiR and CiFL proteins (Ohlmeyer and Kalderon, 1998). The CiFL observed in Su(fu) mutants, which is low in abundance and highly labile, has been proposed to consist of a highly active form of Ci, as its production correlates well with induction of high levels of ptc and anterior en transcription in discs (Ohlmeyer and Kalderon, 1998).

To see whether the transcriptional activity of stabilized CiFL in S182N-expressing cells is subject to regulation by Su(fu), we removed one copy of Su(fu) from discs producing S182N and monitored ptc-lacZ expression. The removal of one copy of Su(fu) from discs expressing S182N caused a dramatic induction of ptc-lacZ expression throughout the anterior compartment of the disc (Fig. 5G,H, compare with 5A-F,I,J). Su(fu) did not have this effect on discs in which wild-type cos2 or S182T was overexpressed. In S182N discs lacking one copy of Su(fu), the induction of ptc-lacZ consistently appears higher in the dorsal than in the ventral compartment, especially near the AP compartment boundary (Fig. 5H, arrowhead).

The dose of Su(fu) is therefore crucial in determining the transcriptional outcome of S182N expression: if two wild-type copies of Su(fu) are present, S182N represses Hh-dependent ptc-lacZ expression at the AP boundary; if one copy of Su(fu) is inactivated, then S182N activates ptc-lacZ even in A cells far from the Hh source. By contrast, S182N activates dpp-lacZ regardless of Su(fu) copy number (Fig. 1K,L; data not shown), so activation of dpp by S182N is independent of Su(fu). S182T, like Cos2, always represses both target genes, regardless of Su(fu) copy number (Fig. 5D,E,I,J and Fig. 1M,N).

Su(fu) is phosphorylated in response to Hh

In order to determine how Hh signaling might control Su(fu) activity, we examined Su(fu) protein in wild-type, hh overexpressing and S182N-expressing discs. Protein blots of wing imaginal disc lysates revealed that hh-overexpressing discs produce at least two immunoreactive bands of Su(fu)
instead of the single band seen in wild-type, cos2, S182N and S182T-expressing discs (Fig. 6A). The additional slower-migrating Su(fu) band appeared whenever hh was overexpressed, even in discs co-expressing hh with cos2 or with S182N (Fig. 6A, lanes 6,7).

Treating lysates with lambda phosphatase to remove phosphate groups on serine, threonine and tyrosine residues revealed that the shifted Su(fu) band is a phosphoisoform of Su(fu) (Fig. 6B, arrows; compare lanes 2 and 4). As a control for lambda phosphatase activity, the phosphoisoforms of Dishevelled were monitored on the same blot (Fig. 6). Dsh exists in wing discs as hyperphosphorylated isoforms, which collapse to one band after phosphatase treatment (Willert et al., 1997). Lambda phosphatase-treated Su(fu) protein co-migrated with Su(fu) from untreated wild-type lysates, suggesting that the majority of Su(fu) protein in wing imaginal discs is unphosphorylated.

We conclude that Su(fu) is phosphorylated in response to Hh and that this activity is unperturbed by the presence of excess Cos2 or by the production of S182N. Phosphorylation of Su(fu) may reduce Su(fu) activity, thus allowing Hh target gene induction at the AP border of the wing disc.

### S182N inhibits activator as well as repressor functions of Cos2

Cos2 has both activator and repressor functions in Hh signaling (Wang and Holmgren, 1999; Wang et al., 2000; Lum et al., 2003; Ogden et al., 2003; Jia et al., 2003; Ruel et al., 2003). Thus far, we used the dominant-negative mutant S182N to explore the role of Cos2 in regulating dpp and ptc, target genes that require repression by Cos2. We now turn to the effect of S182N expression on the target gene engrailed (en), which requires Cos2 for activation by Hh (Wang et al., 2000). While most en expression is located in the posterior compartment of the disc, a narrow band of anterior cells, 5-7 cell diameters in width, expresses en during the late third instar stage (Blair, 1992). This en expression is dependent on high levels of Hh signaling. In cultured cells, Cos2 has been shown to be required for maximal activation of the Hh pathway (Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). To demonstrate that cos2 is required for the Hh-dependent expression of en in vivo, loss-of-function cos2 clones were generated using the FLP-FRT system, and clones falling within the normal anterior en-expressing zone were examined for en expression. Many cos2 clones in the wing pouch lacked detectable en expression, in agreement with a previous study (Fig. 7) (Wang et al., 2000).

As en expression could be used as an in vivo reporter of the activating function of Cos2, we examined en expression in S182N-expressing cells at the AP border. Remarkably, FLP-out clones expressing S182N-GFP did not show any en expression (Fig. 7D-F), indicating that S182N-GFP expression could block the induction of en by Hh in a cell-autonomous manner. Overexpression of wild-type cos2 or expression of S182T in this area of the disc could also prevent the activation of en (data not shown), suggesting that the level of Cos2 protein, as well as its state of ‘activation’, is important for en regulation.

### Discussion

How Hh differentially regulates target genes is central to understanding how one signal generates multiple downstream effects and activates different target genes at different concentrations. Using mutant forms of Cos2, we have investigated how components of the Hh signal transduction pathway form a sensitive switch that governs the difference between dpp-expressing cells and cells expressing both ptc and dpp. Furthermore, we have shown that Cos2 is required for the
activation of the target gene en, and that S182N expression or cos2-overexpression can block this activation, despite the presence of high levels of Hh. Cos2 has been proposed to act not only as a scaffold for Hh signaling components, but as a sensor of the Hh signal, playing a dual role as both an activator and a repressor of the pathway (Lum and Beachy, 2004). We have shown here that mutation of the P-loop of Cos2, which is designed to disrupt the ATPase activity of the protein, profoundly affects the activity of the protein, and through that the outcome of the pathway, in agreement with a role for Cos2 as a sensor for Hh signal.

Conventional kinesins require ATPase activity in order to move along microtubules. Studies have shown that mutation of the conserved Ser or Thr at a precise position in the P-loop causes the protein to become immobile, locking itself and its cargo along microtubules prematurely, before the final intracellular destination for the kinesin has been reached (Meluh and Rose, 1990; Rasooly et al., 1991; Blangy et al., 1998). Expression of such kinesin mutants specifically inhibits the movement of its endogenous partner, but not the movements of other kinesins or dyneins along the microtubule (Blangy et al., 1998; Nakata and Hirokawa, 1995). We used this knowledge about kinesins and the importance of their P-loops to design the equivalent mutation in Cos2. The mutation of amino acid 182 of Cos2 to a conserved Thr does not detectably alter the function of Cos2 in vivo, while mutation of the same residue to Asn clearly interferes with normal Cos2 activity. This clearly suggests that Cos2 is likely to use ATPase activity for either locomotion or conformational changes in response to Hh signaling. The movement of Cos2 along microtubules in vitro has yet to be demonstrated, but the importance of intracellular localization of various Hh signaling components has been clearly demonstrated. Among the examples: in response to Hh, Smo accumulates at the plasma membrane, and associates with Cos2 and Fu; Ci accumulates in the nucleus in response to Hh signaling; and in the absence of Hh signal, Smo is located in internal membranes in the cytoplasm of responding cells, and Ci is continually exported from the nucleus, phosphorylated by kinases, and processed into CiR by the proteasome.

**Differential gene regulation by Hh**

Our data suggest that the activities of Cos2 and Su(fu) are independently regulated by different concentrations of Hh along the gradient that forms from posterior to anterior (Fig. 8). In the anterior cells distant from the AP boundary, little or no Hh is received and target genes are silent. In these cells, Cos2 is required for proteolytic processing of Ci into its repressor form (Wang and Holmgren, 1999) and possibly for the delivery of CiFL for lysosomal degradation. Our data suggest that Cos2 requires an intact P-loop for its role these events. Cos2 ATPase activity may be inhibited in cells receiving very low levels of Hh, preventing Ci proteolysis and stabilizing CiFL. The stabilization of CiFL results in the activation of dpp. Nearer the AP border, where higher levels of Hh are received, Su(fu) becomes phosphorylated, inactivating its negative regulatory hold on Ci, while inhibition of the ATPase activity of Cos2 continues to allow stabilization of Ci. In this situation, ptc and dpp are transcribed. Finally, at the highest levels of Hh signaling adjacent to the AP border, Cos2 is required for activation of the pathway and the expression of en. S182N expression, or cos2 over-expression, inhibits the induction of en by endogenous Hh in these cells. The elements of this model are addressed below.

**Fig. 7. cos2 mutant clones and S182N-expressing clones at the AP boundary do not express anterior en. (A-D) cos2−/− cells in a clone at the AP boundary lacks anterior en expression. A disc stained with anti-Myc (red) to reveal cos2−/− clones, which are not Myc-positive and are therefore lack red signal (arrow), is co-stained with anti-En 4D9 (green). The area marked in A (white box) is shown at higher magnification in B-D. No En is detected within the clone (arrow). (E-G) Cells at the AP boundary expressing S182N-GFP (green) fail to express anterior En (blue, arrow). The area shown in E (white box) is shown at higher magnification in E and F.**

**Cos2 and the regulation of Ci**

Ci plays a central role in determining which genes are repressed or activated in response to different concentrations of Hh (reviewed by Nybakken and Perrimon, 2002). In order to activate target genes such as dpp or ptc, Ci must be stabilized in its full-length form. In wild-type discs, Hh stabilizes Ci by antagonizing molecular events that reduce the concentration of nuclear CiFL. In addition to the constitutive nuclear export of Ci, there are two ways CiFL concentration is reduced: full-length Ci is proteolytically processed into a repressor form; and CiFL is degraded by a lysosome-mediated process involving a novel protein called Debra (Dai et al., 2003). In our experiments, the stabilization of CiFL was accomplished by expressing S182N in responsive cells, which antagonizes Cos2 repressor activity and results in the accumulation of high levels of CiFL (Fig. 4C,E), with minimal effects on the levels of CiR (Fig. 4E,F). This same type of differential effect on CiR and CiFL is accomplished by Debra, which causes the lysosomal degradation of CiFL without affecting the production of CiR. Cos2 and Debra may act in concert to destabilize CiFL, while Cos2 may also aid in the production of CiR via a Debra-independent mechanism. This would involve presenting Ci to the kinases, PKA, CKI and GSKβ (Shaggy) for
phosphorylation and processing by the proteasome (Nybakken and Perrimon, 2002). As Debra regulates Ci stability in limited areas of the wing disc (Dai et al., 2003) but S182N can stabilize Ci throughout the anterior compartment, it is likely that S182N interferes with both Debra-dependent and Debra-independent mechanisms of Ci stability to achieve the observed effect: cell-autonomous stabilization of CiFL leading to derepression of dpp (Fig. 4).

These results suggest that Cos2 may use its ATPase activity to transport Ci to a location where it becomes phosphorylated in preparation for processing, or to the site of processing itself. Alternatively, the ATPase activity may be important for regulating the conformation of Cos2 and its binding to partners such as Smo, Su(fu), Fu and Ci, which would be a novel role for the P-loop in a kinesin-related protein. The S182N mutation may lock Cos2 in a conformation that changes association with binding partners. For example, S182N may decrease the ability of Cos2 to bind Ci, releasing Ci from the cytoplasm, resulting in an increased level of CiFL in the nucleus and the activation of dpp.

Suppressor of Fused and the regulation of patched transcription

The human ortholog of Suppressor of fused is a tumor suppressor gene (Taylor et al., 2002). Su(fu) can associate with Ci, and with the mammalian homologs of Ci, the Gli proteins, through specific protein-protein interactions (Monnier et al., 2002; Paces-Fessy et al., 2004). Through these interactions, Su(fu) controls the nuclear shuttling of Ci and Gli (Wang and Holmgren, 2000; Wang et al., 2000; Taylor et al., 2002), as well as the protein stability of CiFL and CiR (Ohlmeyer and Kalderon, 1998). Flies homozygous for Su(fu) loss-of-function mutations are normal, so the importance of Su(fu) becomes evident only when other gene functions are thrown out of balance, as in a fu mutant background (Pham et al., 1995; Alves et al., 1998; Lefers et al., 2001), with extra or diminished Hh signaling caused by ptc, slimb and protein kinase A mutations (Ohlmeyer and Kalderon, 1998; Wang et al., 1999) or, as we have shown, when altered Cos2 is produced.

We found that to activate ptc transcription in the wing disc, two conditions have to be met simultaneously: CiFL must be stabilized, and the activity of Su(fu) must be reduced. Removal of Su(fu) changes S182N from a ptc repressor into a ptc activator. Removal of Su(fu) may result in the modification, activation or relocation of CiFL, or in further sensitizing the system to stabilized CiFL. In Su(fu) homozygous animals, the quantity of CiFL and CiR proteins is greatly diminished, and Su(fu) mutant cells are more sensitized to the Hh signal (Ohlmeyer and Kalderon, 1998). The lower levels of both CiFL and CiR in mutant Su(fu) cells may contribute to the sensitivity of these cells to Hh, as a small Hh-driven change in the absolute concentration of either form of Ci would result in a significant change in the ratio between the two proteins. Both CiFL and CiR bind the same enhancer sites (Muller and Basler, 2000), so their relative ratio is likely to be important in determining target gene expression. S182N expression tips the ratio of CiFL to CiR toward CiFL, and reducing the absolute quantities of both Ci isoforms by removing Su(fu) will enhance this effect. Furthermore, Su(fu) binds Ci and sequesters it in the cytoplasm in a stoichiometric manner (Methot and Basler, 2000; Wang et al., 2000; Wang and Jiang, 2004). Reducing the amount of Su(fu) should release more CiFL to the nucleus to activate ptc.

Phosphorylation of Su(fu) in response to Hh

The activity of Su(fu) must be regulated or overcome so that target genes can be activated at the right times and places in response to Hh. We have shown that the regulation of Su(fu) activity may occur by Hh-dependent phosphorylation. A phosphoisoform of Su(fu), Su(fu)-P, was detected in discs where GAL4 was used to drive extra Hh expression (Fig. 6). At high concentrations of Hh, the phosphorylation of Su(fu) is not antagonized by overexpression of cos2 or either of the cos2 mutants, suggesting that phosphorylation of Su(fu) occurs
independently of Cos2 function. During the preparation of this manuscript, it was reported that one kinase involved in the phosphorylation of Su(fu) is the Ser/Thr kinase Fused, a well-established component of Hh signal transduction (Lum et al., 2003). It is not known whether the phosphorylation of Su(fu) by Fu is direct or indirect.

The phosphorylation state of Su(fu) may be an important factor in determining Hh target gene activity. Phosphorylation of an increasing number of Su(fu) molecules with increasing Hh signal may gradually release Ci from all of the known modes of Su(fu)-dependent inhibition, such as nuclear export and recruitment of repressors to nuclear Ci, leading to higher levels of CiFL in the nucleus and the activation of Hh target genes such as ptc.

en activation

We used anterior en expression as an in vivo reporter of high levels of Hh signaling. In agreement with a previous report, we find that cos2 mutant cells at the AP boundary fail to activate en, suggesting that Cos2 plays a positive regulatory role in en regulation. S182N, S182T and Cos2 overexpression mimics the cos2 loss-of-function condition with respect to en: en remains off in these cells. One interpretation of these data is that all the Cos2 proteins are able to associate with another pathway component, such as Smo, and overproduction of any of them inactivates some of the Smo in non-productive complexes not capable of activating en.

A Cos2 protein lacking the C-terminal region provides repressor activity

In contrast to the activity of all the other mutations we generated, deletion of the C terminal domain created a protein (Cos2ΔC) that repressed normal dpp, ptc and en expression in the wing disc (Fig. 1; data not shown). In this in vivo assay, Cos2ΔC acted just like wild-type Cos2. A similar deletion has been shown to retain function in cell culture assays (Lum et al., 2003). We further showed that this mutant, expressed under the control of its endogenous promoter, could rescue the lethality and wing duplication phenotypes of a cos2 loss-of-function allele over a cos2 deficiency. The results of the rescue experiment bring up a new possibility: that the C-terminal domain of Cos2, and the Cos2-Smo interaction via the C terminus of Cos2, is not necessary for repressor activities of Cos2. Alternatively, Cos2ΔC could complement or boost the activity of the hypomorphic allele cos211, which was used for the rescue experiment.

We thank the following people for generous gifts of reagents: Phil Beachy, Denise Busson, Barry Dickson, M. Feitz, Mike Hoffman, Bob Holmgren, Janet Jin, Tom Kornberg, Roel Nusse, Ophelia Papavolos, Nipam Patel, Karl Willert, Alan Zhu, Larry Zipursky and the Bloomington Stock Center. Michael Galko provided crucial technical information. Erin Harmon and Allan Hu provided technical assistance. We thank Rebecca Chung-hui Yang, Melicent Peck, Rob Wechsler-Reya, Ron Johnson, Ljiljana Milenkovic, Lara Collier and other members of the Scott laboratory for helpful discussions. We thank Julie Williams for comments on the manuscript and Diane Bush for excellent administrative assistance. K.S.H. was supported by a Predoctoral Fellowship from the National Science Foundation and by a Developmental Biology Departmental training grant from the National Institutes of Health. M.P.S. is an Investigator of the Howard Hughes Medical Institute.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/6/1401/DC1

References

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, E. 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 99, 1043-1053.

Blair, S. S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. Development 115, 21-33.

Blangi, A., Chausseped, P. and Nigg, E. A. (1998). Rigor-type mutation in the kinesin-related protein HsEg5 changes its subcellular localization and induces microtubule bundling. Cell Motil. Cytoskel. 40, 174-182.

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.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J. 13, 4459-4468.

Chen, C. H., von Kessler, D. P., Park, W., Wang, B., Ma, Y. and Beachy, P. A. (1999a). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. Cell 98, 305-316.

Chen, Y., Cardinaux, J. R., Goodman, R. H. and Smolik, S. M. (1999b). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. Development 126, 3607-3616.

Dai, P., Akimaru, H. and Ishii, S. (2003). A hedgehog-responsive region in the Drosophila wing disc is defined by debrera-mediated ubiquitination and lysosomal degradation of Ci. Dev. Cell. 4, 917-928.

Dominguez, M., Brunner, M., Hafem, E. and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein Cubitus interruptus. Science 272, 1621-1625.

Galko, M. J. and Tessier-Lavigne, M. (2000). Biochemical characterization of netrin-synhydring activity. J. Biol. Chem. 275, 7832-7838.

Grau, Y. and Simpson, P. (1987). The segment polarity gene costal-2 in Drosophila. I. The organization of both primary and secondary embryonic fields may be affected. Dev. Biol. 122, 186-200.

Heitzler, P., Coulson, D., Saenz-Robles, M. T., Ashburner, M., Roote, J., Lawrence, C. J., Dawe, R. K., Christie, K. R., Cleveland, D. W., Dawson, S. C., Endow, S. A., Goldstein, L. S., Goodman, H. V., Hirokawa, N., Howard, J. et al. (2004). A standardized kinesin nomenclature. J. Cell Biol. 167, 19-22.

Ingham, P. W., Jackson, A. M. and Nakano, Y. (1991). Role of the Drosophila patched gene in positional signalling. Nature 353, 184-187.

Jia, J., Tong, C. and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. Genes Dev. 17, 2709-2720.

Jiang, J. and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature 391, 493-496.

Johnson, R. L., Grenier, J. K. and Scott, M. P. (1995). patched over-expression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. Development 121, 4161-7410.

Lawrence, C. J., Dawe, R. K., Christie, K. R., Cleveland, D. W., Dawson, S. C., Endow, S. A., Goldstein, L. S., Goodman, H. V., Hirokawa, N., Howard, J. et al. (2004). A standardized kinesin nomenclature. J. Cell Biol. 167, 19-22.

Lefers, M. A., Wang, Q. T. and Holmgren, R. A. (2001). Genetic dissection of the Drosophila Cubitus interruptus signaling complex. Dev. Biol. 236, 411-420.

Lum, L. and Beachy, P. A. (2004). The Hedgehog response network: sensors, switches, and routers. Science 304, 1755-1759.

Lum, L., Zhang, C., Oh, S., Mann, R. K., von Kessler, D. P., Taijale, J., Weiss-Garcia, E., Gong, R., Wang, B. and Beachy, P. A. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. Mol. Cell 12, 1261-1274.
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 inhibiting nuclear accumulation of the activator form of cubitus interruptus. *Development* 127, 4001-4010.

Methot, N. and Basler, K. (2001). An absolute requirement for Cubitus interruptus in Hedgehog signaling. *Development* 128, 733-742.

Monnier, V., Dussièl, F., Alves, G., Lamour-Insard, C. and Plessis, A. (1998). Suppressor of fused links fused and Cubitus interruptus on the signalling pathway. *Curr. Biol.* 8, 583-586.

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.

Muller, B. and Basler, K. (2000). The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic gli-binding sites. *Development* 127, 2999-3007.

Muller, J., Marx, A., Sack, S., Song, Y. H. and Mandelkow, E. (1999). The structure of the nucleotide-binding site of kinesin. *Biochem. Cell Biol.* 380, 981-992.

Nakata, C. and Hirokawa, N. (1995). Point mutation of adenine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. *J. Cell Biol.* 131, 1093-1053.

Nybakken, K. and Perrimon, N. (2002). Hedgehog signal transduction: recent findings. *Curr. Opin. Gen. Dev.* 12, 503-511.

Nybakken, K. E., Turek, C. W., Robbins, D. J. and Bishop, J. M. (2002). Hedgehog stimulated phosphorylation of the kinesin-related protein costal2 is mediated by the serine/threonine kinase fused. *J. Biol. Chem.* 4, 1.

Ogden, S. K., Ascano, M., Stegman, M. A., Suber, L. M., Hooper, J. E. and Robbins, D. J. (2003). Identification of a functional interaction between the transmembrane protein Smoothened and the kinesin-related protein Costal2. *Curr. Biol.* 13, 1998-2003.

Ohmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* 396, 749-753.

Paces-Fessy, M., Boucher, D., Petit, E., Paute-Briand, S. and Blanchet-Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* 396, 749-753.

Pham, A., Therond, P., Alves, G., Tournier, F. B., Busson, D., Lamour-Insard, C., Bouchon, B. L., Preat, T. and Trocoire, H. (1995). Suppressor of fused gene encodes a novel PEST protein involved in Drosophila segment polarity establishment. *Genetics* 140, 587-598.

Pignoni, F. and Zipursky, S. L. (1997). Induction of Drosophila eye development by decapentaplegic. *Development* 124, 271-278.

Rasooly, R. S., New, C. M., Zhang, F., Hawley, R. S. and Baker, B. S. (1995). The lethal(1)TW-6cs mutation of *Drosophila melanogaster* is a dominant amorphic allele of nod and is associated with a single base change in the putative ATP-binding domain. *Genetics* 129, 409-422.

Rice, S., Lin, A. W., Safer, D., Hart, C. L., Naber, N., Carragher, B. O., Cain, S. M., Pechatnikova, E., Wilson-Kubalek, E. M., Whittaker, M. et al. (1999). A structural change in the kinesin motor protein that drives motility. *Nature* 402, 778-784.

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, 225-234.

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.

Sambrook, J. and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez-Herrero, E., Couso, J. P., Capdevila, J. and Guerrero, I. (1996). The *fu* gene discriminates between pathways to control *dpp* expression in *Drosophila* imaginal discs. *Mech. Dev.* 55, 139-153.

Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W. M. (1995). Drawing a stripe in Drosophila imaginal disks: negative regulation of decapentaplegic and patched expression by engrailed. *Genetics* 139, 745-756.

Simpson, P. and Grau, Y. (1987). The segment polarity gene *costal-2* in *Drosophila*. II. The origin of imaginal pattern duplications. *Dev. Biol.* 122, 201-209.

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

Stegman, M. A., Vallance, J. E., Elangovan, G., Sosinski, J., Cheng, Y. and Robbins, D. J. (2000). Identification of a tetrameric hedgehog signaling complex. *J. Biol. Chem.* 275, 21809-21812.

Stegman, M. A., Goes, M. J., Anson, M., Jr Ogden, S. K., Nybakken, K. E. and Robbins, D. J. (2004). Kinesin-related protein Costal2 associates with membranes in a Hedgehog-sensitive, Smoothened-independent manner. *J. Biol. Chem.* 279, 7064-7071.

Strigini, M. and Cohen, S. M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. *Development* 124, 4697-4705.

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, I., Lowrance, A. et al. (2002). Mutations in Sufu predispose to medulloblastoma. *Nat. Genet.* 31, 306-310.

Vincent, J. P. and Briscoe, J. (2001). Morphogens. *Curr. Biol.* 11, R851-R854.

Wang, G. and Jiang, J. (2004). Multiple Cos2/Ci interactions regulate Ci subcellular localization through microtubule dependent and independent mechanisms. *Dev. Biol.* 268, 493-509.

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. (1999). The subcellular localization and activity of Drosophila cubitus interruptus are regulated at multiple levels. *Development* 126, 5097-5106.

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.

Weber, K. and Kuter, D. J. (1971). Reversible denaturation of enzymes by sodium dodecyl sulfate. *J. Biol. Chem.* 246, 4504-4509.

Whittle, J. R. (1976). Clonal analysis of a genetically caused duplication of the anterior wing in *Drosophila melanogaster*. *Dev. Biol.* 51, 257-268.

Willert, K., Brink, M., Wodarz, A., Varmus, H. and Nusse, R. (1997). Casein kinase 2 associates with and phosphorylates dishevelled. *EMBO J.* 16, 3089-3096.

Woolf, G. and Schliwa, M. (2000). Walking on two heads: the many talents of kinesin. *Nat. Rev. Mol. Cell Biol.* 1, 50-58.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* 117, 1223-1237.