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

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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 (Ci^{FL}) is proteolytically processed into a truncated form, Ci^{R}, that consists of the N terminus of Ci and its zinc-finger binding domain. Ci^{R} is a repressor of *dpp* and *hh* (Aza-Blanc et al., 1997). The Hh signal opposes the proteolytic processing of Ci, which stabilizes Ci^{FL}, and causes the nuclear accumulation of Ci^{FL}, 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, Ci^{ACT}, has thus far evaded biochemical characterization, but its existence is suggested by experiments showing that expression of stabilized Ci^{FL} 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 Ci^{FL} (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 Ci^{R} (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 links 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., 1999; 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 cassette=yw actin5C < CD2 < Gal4 (Pignoni and Zipursky, 1997); $cos2^{2}$, $cos2^{3}$, $cos2^{W1}$, $cos2^{31}$ (Whittle, 1976; Grau and Simpson, 1987; Simpson and Grau, 1987; Heitzler et al., 1993; Sisson et al., 1997); FRT πMG13, FRT $cos2^{W1}$, *hsp70-flp* (Xu and Rubin, 1993; Sisson et al., 1997); $Su(fu)^{LP}$ (Bloomington Stock Center); and UAS-*Hh* (gift from Phil Beachy).

The reporter strain *H84 ptc-lacZ* (a gift from M. Fietz) is an nuclear *lacZ* enhancer trap in the *ptc* locus. *dpp-lacZ* is a transgene constructed from the dpp^{disk} promoter driving nuclear *lacZ* (Sanicola et al., 1995).

UAS-cos2, *UAS-cos2GFP*, *UAS-S182N*, *UAS-S182N-GFP*, *UAS-S182T*, *UAS-S182T-GFP*, *UAS-ΔMotor*, *UAS-ΔNeck* 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 $cos2^{-}$ 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 again 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 *Xho*I (blunted with Klenow enzyme, NEB) and *Xba*I, to pUAS-*cos2* cut with *Mam*I and *Xba*I. 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: NKIIEGTKM…, 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 *Ngo*MI 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 oligonucleotide with *Nco*I and *Aat*II compatible ends. This ΔMotor oligonucleotide encodes a 6×His tag in frame with the *cos2* ORF. Oligonucleotide sequences are as follows: 5′-CATGCACCACCAC-CACCACCACGGACGT-3′ and 5′-CCGTGGTGGTGGTGGTG-GTG-3′. A pBS-KS plasmid containing the *cos2* ORF was cut with *Nco*I and *Aat*II and the ΔMotor oligo inserted into this vector. A *Spe*I fragment from this ΔMotor construct was then inserted into pUAS*cos2-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 complementary oligonucleotides were synthesized: 5′-CCGGTGCACCACCAC-CACCACCACGAGCAGAAGCTTATATCAGAAGAAGATCTGGG-TACCTAAGC-3′ and 5′-GGCCGCTTAGGTACCCAGATCTTCT-TCTGATATAAGCTTCTGCTCGTGGTGGTGGTGGTGGTGCA-3′. These oligonucleotides were annealed and cut with *Kpn*I and ligated to pUAS-cos-GFP cut with *Sgr*AI and *Kpn*I. 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 mutagenesis strategy was used. The forward primers encode the appropriate point mutation (underlined): S182T forward primer, 5′-CCAGCGCGGCCAAGGCAAAACCTACACACTCTAC-3′; S182N forward primer, 5′-CCAGCGCGGCCAAGGCAAAAACTACACA-

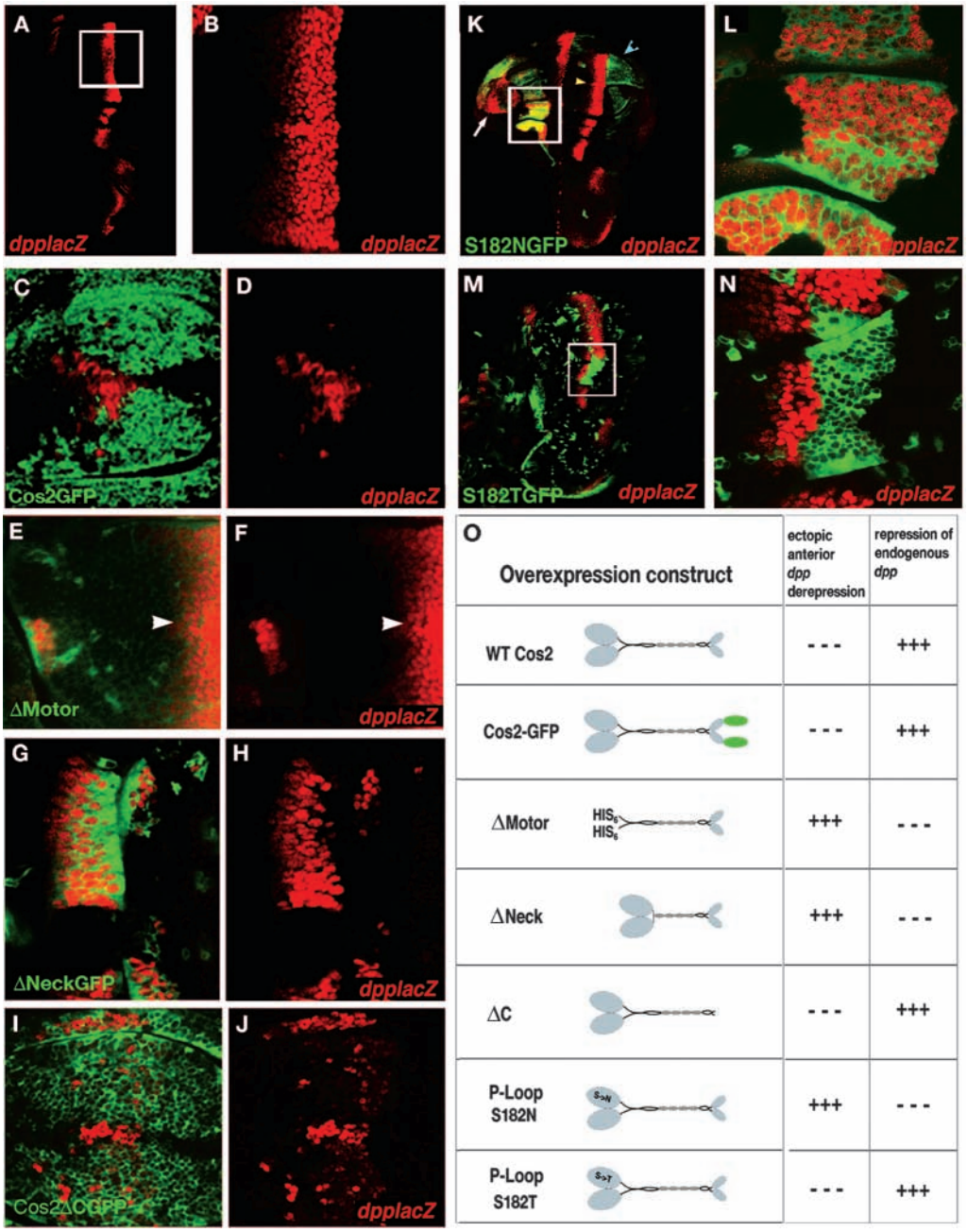

Fig. 1. Effect of different mutants of Cos2 on *dpp-lacZ* expression. (A,B) *dpp-lacZ* reporter expression at the AP boundary in wild-type third instar larval discs. Nuclear β-galactosidase is immunofluorescently labeled (red). Area in A (white box) is shown at higher magnification in B. (C,D) Overexpression 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 ΔMotor-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 ΔNeck-GFP (green) ectopically expresses *dpp-lacZ* (red) in a cell-autonomous manner. (I,J) Cos2ΔC-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). 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. Box in M indicates the area that is shown at higher magnification in N. (O) A chart summarizing the effects each mutation has on *dpp-lacZ* expression in either the anterior compartment of the disc or at the AP boundary where *dpp-lacZ* is normally expressed. +++ indicates high uniform levels of derepression or repression. Also shown are schematic drawings of the putative Cos2 homodimer with appropriate alterations to reflect each mutation or deletion. Results were the same for each construct and its C-terminally fused GFP counterpart except for ΔMotor, for which ΔMotor-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.

CTCTAC-3′; reverse primer, 5′-TGCCATTAACCCCGTACATGAG-3′. PCR products were cut with *Bal*I and *Aat*II, and ligated to pRV3.9 (Sisson et al., 1997) cut with *Bal*I and *Aat*II 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 *Bst*EII and *Nhe*I and inserted the fragments to pUAS-*cos2* and pUAS-*cos2-GFP* cut with *Bst*EII and *Nhe*I. 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 *Nhe*I and *Xba*I (blunted with Klenow) and ligated this fragment to pK6.5 (Sisson et al., 1997) cut with *Nhe*I and *Mam*I. 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 Cos2ΔC-

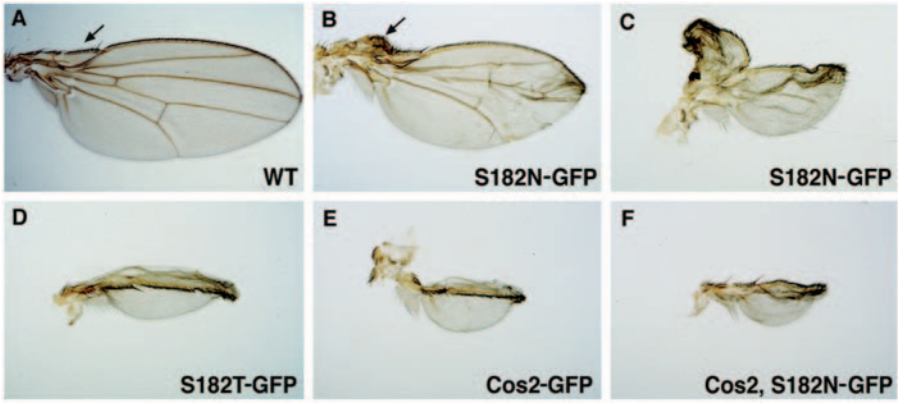

Fig. 2. Wing duplications resulting from S182N expression can be suppressed by co-expression of *cos2*. (A) Wild-type wing (normal costa indicated by arrow). (B,C) Examples of the two extremes of expressivity seen in wing blades in which *S182NGFP* is expressed using MS1096 Gal4. The same results are 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 between 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 are 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-Cos2ΔC-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/antiweb.html) using antibodies at the following dilutions: anti-Ci^{FL} 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/protdisccells.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 6× 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 Papoulos 1:1000; anti-Dsh rabbit polyclonal (a gift from K. Willert and R. Nusse) 1:1000; anti-Ci^{FL} 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 AlphaImager 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 ($cos2^{3}/cos2^{11}$) (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.

Table 1. Cos2GFP and Cos2DC-GFP rescue of post-embryonic lethality and the duplicated wing phenotype

	Genotype	*n*	% Survivors	% Duplications
Negative control (no transgene)	$cos2^{11}/cos2^{3}$	547	45.1	20.0
Original genomic rescue 6.5 kb construct	$cos2^{11}/cos2^{3}$;pK6.5 / +	698	82.2	0
Genomic construct containing *cos2GFP*	$cos2^{11}/cos2^{3}$;pK6.5-GFP / +	869	83.3	0.253
Genomic construct containing ΔC-terminus-GFP	$cos2^{11}/cos2^{3}$;pK6.5ΔC-GFP / +	310	80.0	0

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 MS1096 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 mimic 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*

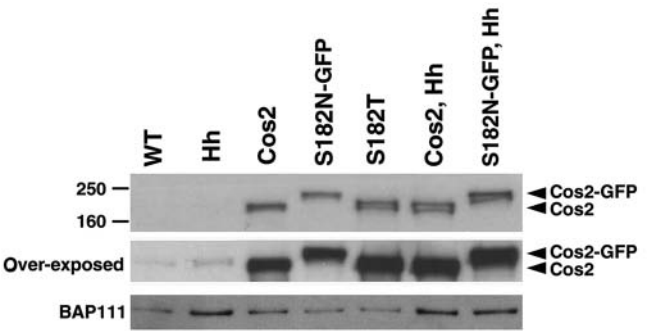

Fig. 3. The difference in S182N and Cos2 activities is not due to differences in expressed protein levels. Western blot showing overexpression of mutant and wild-type *cos2* results in production of comparable levels of each protein in imaginal wing disc lysates (30 μg protein loaded per lane). Arrows indicate migration differences between GFP-tagged proteins and their untagged counterparts. The overexpressed protein is indicated above each lane on the blot. WT, wild-type lysates; Cos2, Hh indicates overexpression of both Cos2 and Hh; S182N-GFP, Hh indicates over-expression of both Hh and S182N-GFP. All experiments were performed using independently isolated fly lines of tagged and untagged Cos2 proteins with the same results in all experiments. 71B Gal4 was the driver for all constructs. BAP111 is a ubiquitous nuclear protein used as a loading control.

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

Ci^{FL} 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 Ci^{FL} levels and stability, discs expressing S182N under the control of the 71B Gal4 driver were stained with antibodies against Ci^{FL}. The pattern of 71B Gal4-driven expression is shown in Fig. 4D.

In wild-type discs, Ci^{FL} protein is at low levels throughout the anterior compartment, with elevated levels of Ci^{FL} 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 Ci^{FL} 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 Ci^{FL}, and the S182T mutant has the same effect (not shown). S182N, by contrast, causes higher levels of Ci^{FL} 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 Ci^{FL} into Ci^{R}, thus stabilizing Ci^{FL}, protein blots of wing disc extracts were stained with an antibody that detects both Ci^{R} and Ci^{FL} (Fig. 4E,F). Multiple bands representing Ci^{R} are observed in the 75 kDa range; these are presumably isoforms of Ci^{R} (Fig. 4E). In Hh overexpressing discs (Fig. 4E, lane 2), Ci is stabilized in its full-length form and all Ci^{R} isoforms are undetectable. In S182N-expressing discs, the amount of Ci^{FL} 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, Ci^{R} 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 Ci^{FL} independently of Ci processing.

Quantification of the relative amounts of Ci^{R} versus total Ci concentration in S182N-producing discs showed a significant reduction in relative Ci^{R} concentration. Error bars show the standard deviations of three independent experiments (Fig. 4F). This altered ratio, which favors Ci^{FL}, 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).

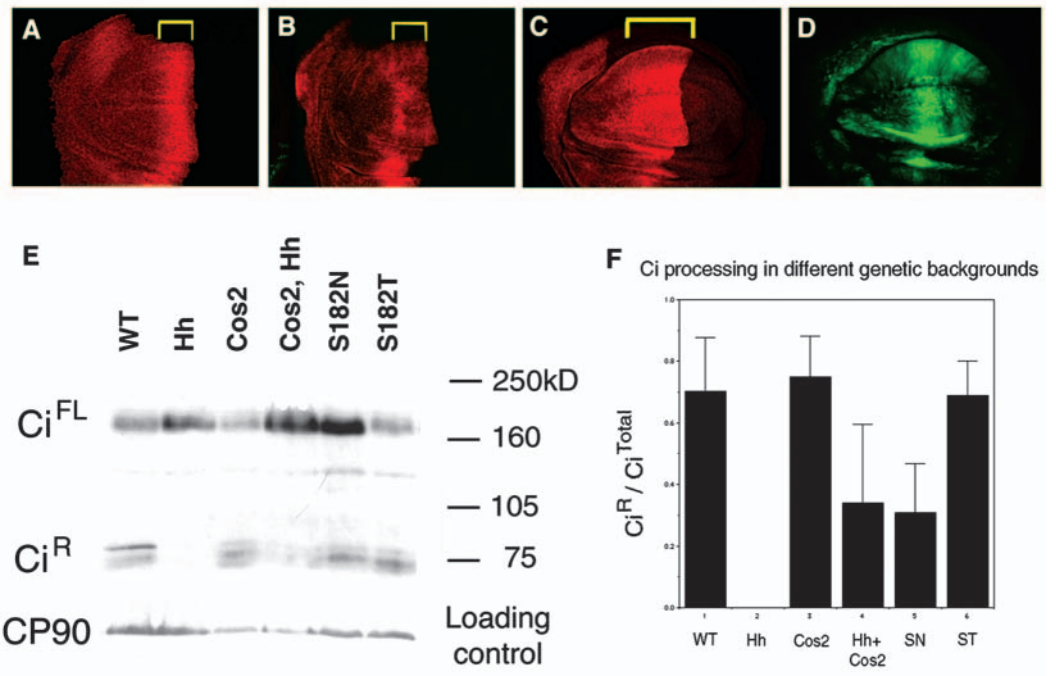

Fig. 4. Expression of S182N stabilizes Ci in its unprocessed, 175 kDa form in wing imaginal discs. (A-D) Stabilization of Ci shown by immunostaining of discs using a monoclonal antibody recognizing the unprocessed form of Ci only (Ci^{FL}). (A) A wild-type disc showing normal levels of stabilization of Ci at the anteroposterior boundary. Ci^{FL} signal is strongest along the AP boundary (bracket). (B) Over-expression of Cos2 using the 71B Gal4 driver results in reduced levels of Ci^{FL} 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 Ci^{FL} 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 Ci^{R} to Ci^{TOTAL} (*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 from a *ptc* 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 Ci^{FL} and induces *dpp* but not *ptc*. One way to stimulate the induction of *ptc* transcription by Ci^{FL} is to remove the inhibitory activity of Su(fu) (Methot and Basler, 2000). Su(fu) prevents translocation of Ci^{FL} into the nucleus (Chen et al., 1999a; Lefers et al., 2001; Methot and Basler, 2000; Wang et al., 2000) and maintains high levels of Ci^{R} and Ci^{FL} proteins (Ohlmeyer and Kalderon, 1998). The Ci^{FL} 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 Ci^{FL} 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-C,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)

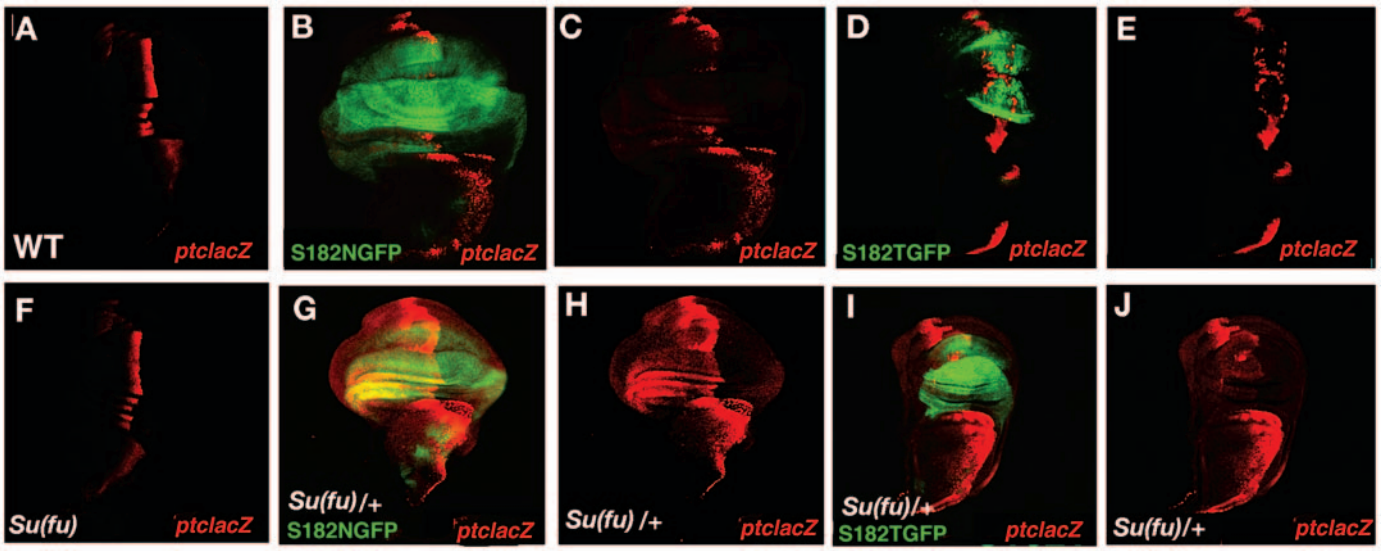

Fig. 5. Genetic removal of Su(fu) activity changes S182N Cos2 from a repressor of *ptc* transcription to an activator. (A,F) Expression of *ptc-lacZ* (red) in a wild-type (A), and *Su(fu)* homozygous mutant background. (B,C) MS1096 Gal4-driven S182N-GFP (green) represses normal *ptc-lacZ* expression at the AP border (red) in a wild-type background. (G,H) By contrast, expression of S182N-GFP (green) causes the derepression of *ptc-lacZ* (red) in anterior cells of a *Su(fu)/+* mutant wing disc. (D,E) Expression of S182T Cos2-GFP (green) by 71B Gal4 represses normal *ptc-lacZ* (red) expression at the AP boundary in a wild-type background. (I,J) Expression of S182T Cos2-GFP (green) by MS1096 Gal4 represses *ptc-lacZ* (red) expression at the AP boundary and no ectopic *ptc-lacZ* expression is observed in the anterior cells of a *Su(fu)/+* mutant wing disc.

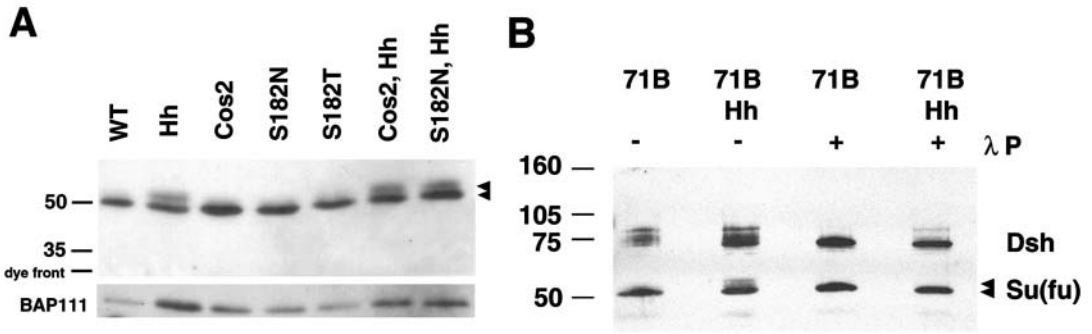

Fig. 6. Su(fu) protein is phosphorylated in response to Hh overexpression. (A) Western blot of extracts from imaginal wing discs probed with anti-Su(fu) antibody. BAP 111 was used as a loading control. Lane 1, extract from wild-type discs; lane 2, extract from Hh overexpressing discs; lane 3, extract from Cos2-overexpressing discs; lane 4, extract from discs expressing S182N; lane 5, extract from discs expressing S182T; lane 6, extract from discs overexpressing both Hh and Cos2 simultaneously; lane 7, extract from discs overexpressing both Hh and S182N simultaneously. Overexpression of proteins was achieved in each case by using the 71B Gal4 driver. Arrowheads indicate the migration of singlet and doublet bands recognized by the Su(fu) antibody. The Su(fu) doublet appears only in those lanes in which Hh has been overexpressed. (B) Treatment of wing disc lysates with the lambda phosphatase enzyme, which removes phosphates from both Ser/Thr and Tyr, shifts the doublet Su(fu) band to a single band that co-migrates with Su(fu) from wild-type lysates. Lane 1, extract from 71B Gal4 wild-type discs, showing a single band for Su(fu); lane 2, the Su(fu) doublet is seen in lysates from Hh-overexpressing discs; lane 3, treatment of 71B Gal4 wild-type lysates with lambda phosphatase does not change the migration properties of Su(fu), compare lane 3 with lane 1, suggesting that the majority of Su(fu) does not exist in a phosphorylated state in wing imaginal discs. Lane 4, treatment of Hh-over-expressing lysates with lambda phosphatase shifts the Su(fu) doublet (Lane 2) to a singlet (arrowheads). Dsh, Dishevelled protein, recognized by Rabbit anti-Dsh (gift from Karl Willert and Roel Nusse), was used as a control as it normally exists as a hyperphosphorylated protein (lane 1). The successful dephosphorylation of Dsh can be seen by a change in its migration as multiple bands (lanes 1 and 2) to a single major band (lanes 3 and 4).

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

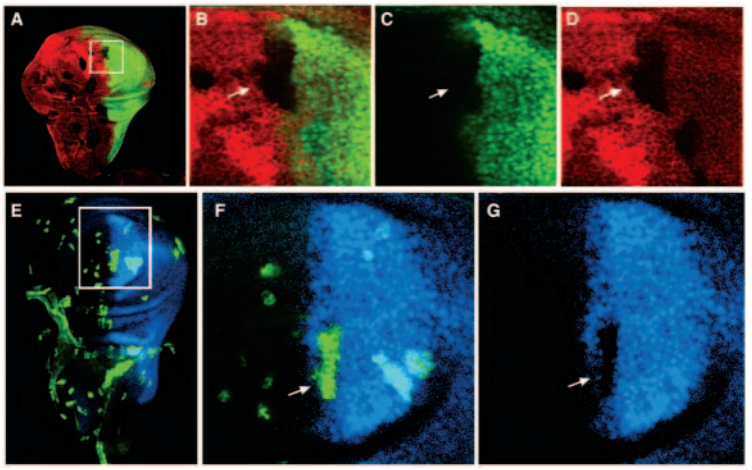

Fig. 7. *cos2* mutant clones and S182N-expressing clones at the AP boundary do not express anterior *en*. (A-D) *cos2*$^{-}$ cells in a clone at the AP boundary lacks anterior *en* expression. A disc stained with anti-Myc (red) to reveal *cos2*$^{-}$ clones, which are not Myc-positive and are therefore 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.

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. How do the components arrive at the appropriate places to affect the appropriate response? As a binding partner for all of these components and as a kinesin-related protein, Cos2 is in a unique position to orchestrate some of these events. We propose ideas for how it may accomplish this below.

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 Ci^{FL} 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 Ci^{FL}. The stabilization of Ci^{FL} 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.

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 Ci^{FL}. In addition to the constitutive nuclear export of Ci, there are two ways Ci^{FL} concentration is reduced: full-length Ci is proteolytically processed into a repressor form; and Ci^{FL} is degraded by a lysosome-mediated process involving a novel protein called Debra (Dai et al., 2003). In our experiments, the stabilization of Ci^{FL} was accomplished by expressing S182N in responsive cells, which antagonizes Cos2 repressor activity and results in the accumulation of high levels of Ci^{FL} (Fig. 4C,E), with minimal effects on the levels of Ci^{R} (Fig. 4E,F). This same type of differential effect on Ci^{R} and Ci^{FL} is accomplished by Debra, which causes the lysosomal degradation of Ci^{FL} without affecting the production of Ci^{R}. Cos2 and Debra may act in concert to destabilize Ci^{FL}, while Cos2 may also aid in the production of Ci^{R} via a Debra-independent mechanism. This would involve presenting Ci to the kinases, PKA, CKI and GSKβ (Shaggy) for

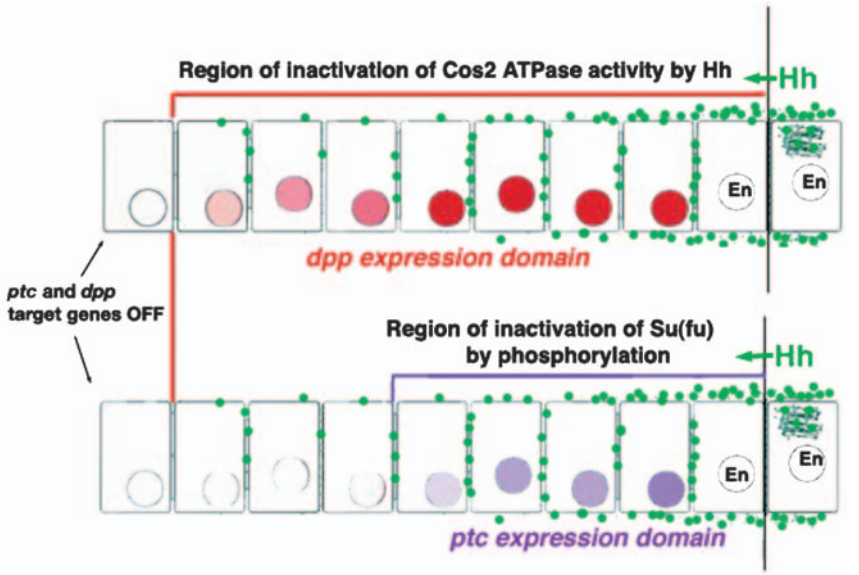

Fig. 8. Differential gene regulation by Hedgehog (Hh). Hh protein, secreted from posterior compartment cells (green), is distributed as a protein gradient in anterior compartment cells, with the highest concentration of Hh occurring at the AP boundary (black line). Cells receiving minimal amounts of Hh respond by inactivating the ATPase activity of Cos2 (red domain), perhaps by Fu-dependent phosphorylation (Nybakken et al., 2002) or other event. This results in the activation of *dpp* transcription in cells as far as 15 cell diameters away from the Hh source (red nuclei – only eight cells shown for sake of brevity). Cells receiving an intermediate amount of Hh inactivate Su(fu) (purple domain), perhaps by phosphorylation, which results in the activation of *ptc* (purple nuclei) in addition to the activation of *dpp*. *ptc* is expressed in a swath of cells 8-10 cell diameters in width (only five cells shown). Anterior *en* transcription in a 5- to 7-cell-wide stripe is Hh dependent and is located closest to the AP boundary (only one cell shown, labeled En). Posterior En protein (En) is also present but its expression is not Hh dependent. In the anterior, both *dpp* and *ptc* transcription are repressed by *en* (Sanicola et al., 1995), so lower amount of *dpp* transcription is seen in a 1- to 5-cell-wide stripe at the AP border in *en*-expressing cells (white nucleus of anterior cell labeled En).

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 Ci^{FL} 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 Ci^{FL} 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 Ci^{FL} and Ci^{R} (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: Ci^{FL} 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 relocalization of Ci^{FL}, or in further sensitizing the system to stabilized Ci^{FL}. In *Su(fu)* homozygous animals, the quantity of Ci^{FL} and Ci^{R} 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 Ci^{FL} and Ci^{R} 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 Ci^{FL} and Ci^{R} 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 Ci^{FL} to Ci^{R} toward Ci^{FL}, 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 Ci^{FL} 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 Ci^{FL} 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 $cos2^{11}$, which was used for the rescue experiment.

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Supplementary material

Supplementary material for this article is available at
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