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The subcellular localization and activity of *Drosophila* Cubitus interruptus are regulated at multiple levels

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SUMMARY

Cubitus interruptus (Ci), a *Drosophila* transcription factor, mediates Hedgehog (Hh) signaling during the patterning of embryonic epidermis and larval imaginal discs. In the absence of Hh signal, Ci is cleaved to generate a truncated nuclear form capable of transcriptional repression. Hh signaling stabilizes and activates the full-length Ci protein leading to strong activation of downstream target genes including *patched* and *decapentaplegic*. A number of molecules have been implicated in the regulation of Ci. Mutations in these molecules lead to changes in Ci protein level, the extent of Ci proteolysis and the expression of Ci target genes. This paper examines the regulation of Ci subcellular localization and activity. We first characterize a bipartite nuclear localization signal (NLS) within Ci. We propose that the subcellular distribution of Ci is affected by two opposing forces, the action of the NLS and that of at least two regions targeting Ci to the cytoplasm. Further our data show that loss of PKA or Costal-2 activity does not fully mimic Hh signaling, demonstrating that Ci proteolysis and Ci activation are two distinct events which are regulated through different paths. Finally, we propose that there are three levels of apparent Ci activity, corresponding to three zones along the AP axis with different sets of gene expression and different levels of Hh signaling.

Key words: *Drosophila melanogaster*, *cubitus interruptus*, *hedgehog*, NLS, *PKA*, *fused*, *costal-2*

INTRODUCTION

During development, organs and tissues must be precisely patterned. Cell-cell signaling is the primary mechanism by which the relative position and hence the identity of a cell is defined. The Hedgehog (Hh) signaling pathway is well conserved between *Drosophila* and vertebrates and is widely utilized (Hammerschmidt et al., 1997; Ingham, 1998). In *Drosophila* embryonic segments and larval imaginal discs, Hh is made/secreted by the posterior compartment cells and diffuses to the anterior compartment (Lee et al., 1992; Tabata et al., 1992; Tashiro et al., 1993; Tabata and Kornberg, 1994). Cells in the anterior compartment express the multipass transmembrane receptor Patched (Ptc), which is complexed with the serpentine protein Smoothened (Smo) and inhibits Smo activity (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). In anterior cells along the anterior-posterior (AP) compartment boundary, Hh signaling relieves Ptc repression on Smo (Marigo et al., 1996; Alcedo and Noll, 1997) and leads to stabilization/activation of the zinc finger transcription factor Cubitus interruptus (Ci) (Aza-Blanc et al., 1997), which in turn activates downstream Hh target genes (Dominguez et al., 1996; Alexandre et al., 1996; Hepker et al., 1997).

Ci contains five tandem C_2H_2 type zinc fingers and is the homologue of the Gli proteins in vertebrates (Orenic et al., 1990, Dai et al., 1999). The Ci/Gli zinc finger domain exhibits sequence-specific DNA binding (Pavletich et al., 1993), and Ci/Gli sites have been shown to be essential in several enhancers (Sasaki et al., 1997; Von Ohlen et al., 1997). One of the primary roles of Ci is to act as a transcriptional activator. The activator function of Ci is essential for *ptc* and late, anterior *engrailed* (*en*) expression and plays an important role in the regulation of *decapentaplegic* (*dpp*) expression (Methot and Basler, 1999). In the absence of Hh signal, full-length Ci (Ci-155) is proteolytically cleaved and generates a 75 kDa form (Ci-75) truncated after the zinc finger domain (Aza-Blanc et al., 1997). Ci-75 has been suggested to mediate the repressor activity of Ci, which is required for proper control of *hh* and *dpp* expression (Methot and Basler, 1999). (For the sake of simplicity, hereafter "Ci" will refer to the full-length form, Ci-155, unless otherwise specified, and "Ci activity" will refer to the activator function of Ci.)

Ci-155 is primarily cytoplasmic (Motzny and Holmgren, 1995) despite being required for the transactivation of target genes. Ci-75, however, is nuclear, as is the dominant negative truncation construct *ci*-NZnHA (Hepker et al., 1997; Aza-Blanc et al., 1997). Since even Ci-75 is too large for free diffusion through the nuclear pore, it is plausible that it contains a feature(s) that facilitates its nuclear translocation. Regulation of nuclear entry could provide an important means of modulating Ci activity, but the mechanisms are largely unknown and many questions remain to be answered. We find that Ci contains a

sequence that functions as a bipartite nuclear localization signal (NLS), the integrity of which is necessary for efficient nuclear import of NZnHA. A previously characterized domain is capable of counteracting the NLS and leads to cytoplasmic accumulation of a β-gal-NLS fusion protein.

In addition to inhibiting Ci cleavage, Hh signaling has been postulated to direct the transformation of Ci into an activated form (Ohlmeyer and Kalderon, 1998; Alves et al., 1998; Methot and Basler, 1999). A negative role on this transformation has been proposed for Suppressor of fused (Su(fu)), a novel PEST protein (Pham et al., 1995). It has been suggested that the activity of the Fused (Fu) kinase opposes the action of Su(fu) and promotes the activation of Ci (Ohlmeyer and Kalderon, 1998). Consistent with their roles as regulators of Ci activity, Fu, Su(fu), and the kinesin-like molecule Costal-2 (Cos2) have been found to form a complex with Ci (Sisson et al., 1997; Robbins et al., 1997; Monnier et al., 1998). However, the putative activated form of Ci has not been identified, and the mechanisms underlying such a transformation remain unknown.

Stabilization and activation of Ci are both consequences of Hh signaling. Since the activated form of Ci is as yet uncharacterized, its presence has been monitored by the activation of Ci target genes. In most cases, stabilization of Ci is accompanied by the expression of these target genes (Johnson et al., 1995; Jiang and Struhl, 1998). Are stabilization and activation the same event? Are they two events regulated by the same pathway and the same mechanism? Or are they distinct events regulated separately, either by different sets of molecules or by different activities of the same molecules? The first question was answered by a recent study which demonstrated that stabilization does not substitute for activation (Methot and Basler, 1999). The authors successfully separated activation and stabilization by using a deletion construct of Ci that cannot be cleaved and hence is automatically "stabilized". Answers to the other questions, however, remain elusive. For wild-type Ci, stabilization and target gene activation are correlative, with certain mutations either abolishing or promoting both. In this paper, using a reporter construct whose response to Ci depends on the highest level of Hh signaling, we are able to distinguish activation from stabilization with respect to wild-type Ci. We also show that Ci is stabilized but not fully activated in *PKA* or *cos2* mutant clones, suggesting that these mutations do not fully mimic Hh signaling. Finally, our data indicate that the level of apparent Ci activity is affected by both its protein levels and its state of activation.

MATERIALS AND METHODS

Fly stocks

fu^{1} and fu^{XR15} were obtained from the Bloomington stock center. $cos2^{V1}$ $Cos1^{3}$/*CyO*, $cos2^{W1}$/*CyO* were provided by R. Whittle. *FRT40A DC0* was provided by D. Kalderon. *FRT42* $cos2^{W1}$ and *FRT42* ptc^{IIW} were provided by T. Orenic.

Constructs

For Ci^{NZn} and $Ci^{NZnΔNLS}$, sequences C-terminal to amino acids L616 and A609 were deleted, respectively. Each construct also carries a triple-HA epitope tag at the N-terminus of the protein, the insertion position of which is identical to that described for *ci-NZnHA* in Hepker et al. (1997). For β-gal-NLS, Ci amino acids K581-L616 were fused to the C-terminus of β-gal encoding sequence. For β-gal-NLS-C1, Ci amino acids K581-R836 were fused to the C-terminus of β-gal encoding sequence. For $Ci^{ΔC1\text{-}A}$, amino acids L685-R836 were deleted.

Generation of somatic clones

Clones of mutant cells were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). *PKA* loss-of-function clones were generated in larvae of the following genotype: *y w hsp70-flp; FRT40A myc/FRT40A DC0*; *4bslacZ*/+. *cos2* loss-of-function clones were generated in larvae of the following genotype: *y w hsp70-flp; FRT42 myc/FRT42* $cos2^{W1}$; *4bslacZ*/+. *ptc* loss-of-function clones were generated in larvae of the following genotype: *y w hsp70-flp; FRT42/FRT42* ptc^{IIW} *myc; 4bslacZ*/+. Clones were induced during 48-72 hours of development by heat-shocking the larvae at 38°C for an hour.

Ubiquitous expression of Ci

Flies carrying the transgene *A5C-FC-ci* ('Flip-out' *ci*; Hepker et al., 1997) were used to ectopically express Ci. Larvae of the genotype *y w hsp70-flp; A5C-FC-ci/4bslacZ* were heat-shocked at 38°C for an hour during 48-72 hours of development. Such heat-shocks were found to cause near ubiquitous expression of Ci in the discs.

Generation of cos2 hypomorphic wings

For *cos2* hypomorphic wings, flies of the genotype *b pr* $cos2^{V1}$ *cn* $Cos1^{3}$ *bw/CyO* were crossed to flies of the genotype $cos2^{W1}$ *cn bw sp/CyO*. Larvae homozygous for *cn bw* were identified by the lack of pigment in their Malpighian tubules.

Germline transformations

All Ci constructs were cloned into the transformation plasmid pUAST (Brand and Perrimon, 1993). Transgenic flies were generated according to the method of Spradling and Rubin (1982).

Tissue culture

S2 cells were grown in standard *S2* medium at 25°C. Cells were transiently transfected (Roberts, 1986) and assayed 2 days after transfection. *cl-8* cells were grown in supplemented M3 medium at 25°C. Protein extracts for IEF gel were prepared as described in Current Protocols in Protein Science. For treatment with λ-PPase (NEB), extract was prepared by sonication in the absence of detergent. Cells were washed in ice-cold PBS and harvested in λ-PPase buffer (NEB) supplemented with protease inhibitors. The cell suspension was sonicated on ice, divided into aliquots of equal volumes, and some samples were treated with λ-PPase for 30 minutes at 30°C. The extracts were then supplemented with SDS and β-mercaptoethanol and treated with DNAse and RNAse as described in Current Protocols in Protein Science.

Preparation of IEF samples from discs

Wing discs were dissected out in ice-cold PBS and collected in Eppendorf tubes by brief centrifugation at 6,000 rpm in a benchtop centrifuge. PBS was discarded, and the discs lysed in ice-cold lysis buffer (Current Protocols in Protein Science). The remaining protocol is the same as in preparation of extract from tissue cultured cells.

IEF gel

5%T, 3.3%C acrylamide mini-gels were made according to Current Protocols in Protein Science using Ampholyte pH 5-8 (Pharmacia). 20 mM NaOH was chosen as the upper chamber buffer and 0.085% phosphoric acid as the lower buffer. Gels were run in a Mini-Protean II Electrophoresis Cell (Bio-Rad) by prefocusing at 100 V for 30 minutes and then focusing at 450 V for 4 hours.

Immunohistochemistry

Embryos were fixed and stained as described by Motzny and Holmgren (1995). Transfected tissue culture cells were fixed with

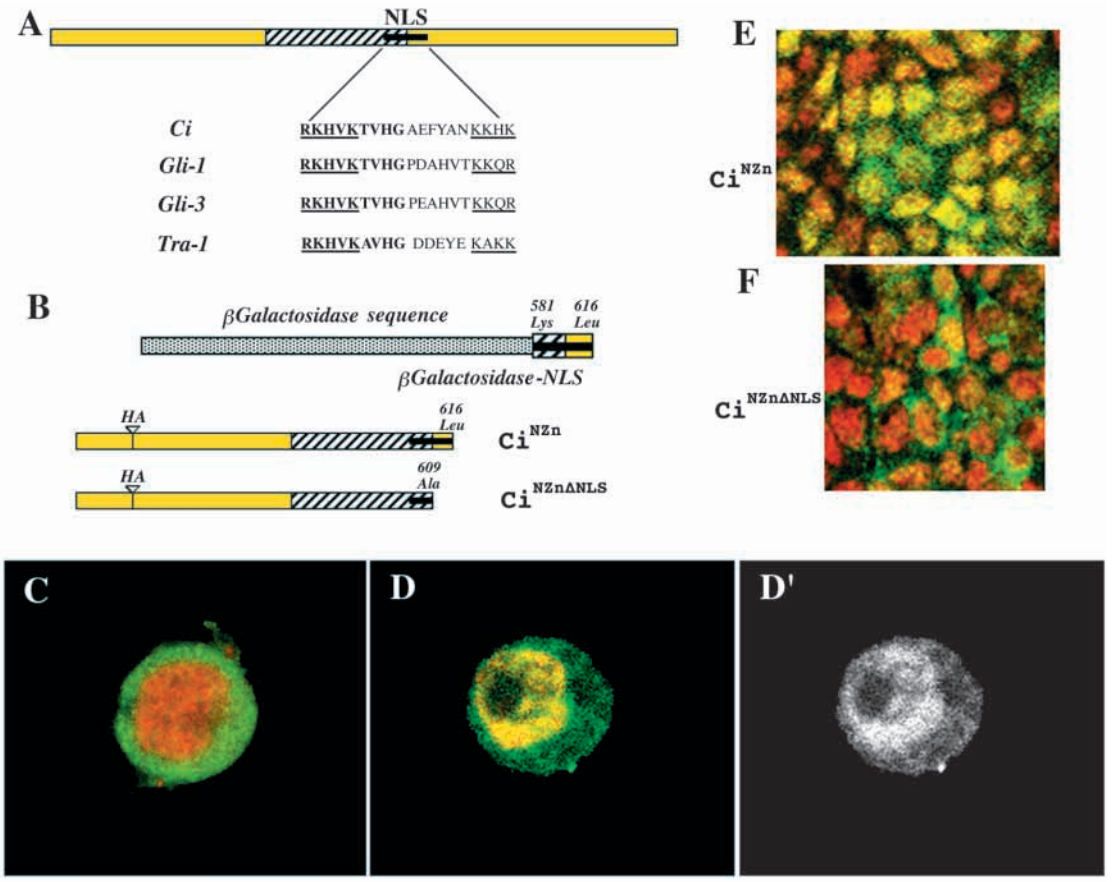

Fig. 1. A bipartite NLS sequence on Ci is necessary and sufficient to direct active nuclear import. (A) The bipartite NLS sequence is conserved between *Drosophila* Ci, vertebrate Gli and nematode Tra-1. The hatched box represents the zinc finger domain of Ci. The black bar indicates the position of the NLS. Amino acids within the zinc finger domain are in bold. The two basic clusters are underlined. (B) Constructs used in the study of the NLS. The sequence encoding β-gal is represented by a stippled box. (C) β-gal staining (green) is cytoplasmic in *S2* cells transfected with A5C-β-gal. The staining of *Drosophila* heat shock factor (dHSF) marks the nucleus in red. (D,D′) β-gal staining is primarily nuclear in cells transfected with A5C-β-gal-NLS. β-gal staining is in green and dHSF staining in red. The overlap of the two produces yellow (D). The β-gal channel alone is shown in D′. (E,F) Comparison of the subcellular distribution of Ci^{NZn} (E) and $Ci^{NZnΔNLS}$ (F). Expression of Ci^{NZn} or $Ci^{NZnΔNLS}$ in the embryos was driven by *ptcGAL4*, and the proteins detected by HA staining (green). The nuclei were marked by dHSF staining (red).

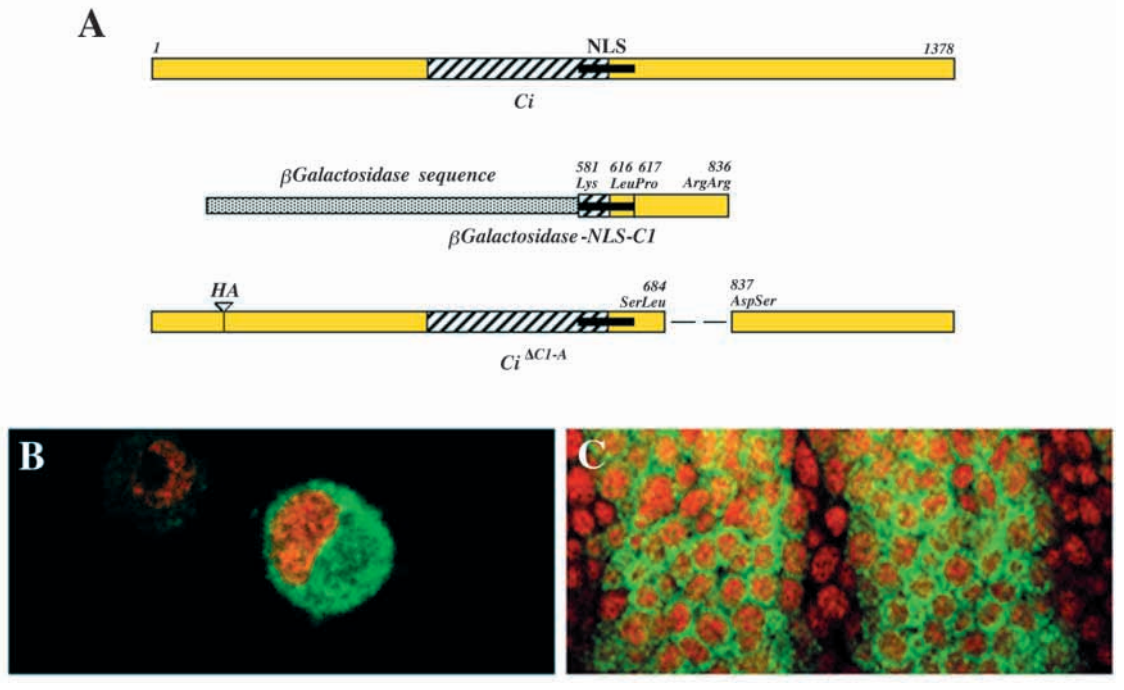

Fig. 2. Cytoplasmic targeting force is provided by more than one region. (A) Constructs used in the study of cytoplasmic targeting. Sequence encoding β-gal is represented by a stippled box. Hatched boxes indicate sequences from the zinc finger domain of Ci. NLS sequence is represented by a black bar. (B) β-gal staining (green) for β-gal-NLS-C1 is cytoplasmic in transfected *S2* cells, suggesting that C1 is able to counteract the NLS and target the fusion protein to the cytoplasm. The nucleus is marked by dHSF staining (red). (C) An embryo in which expression of $Ci^{ΔC1\text{-}A}$ was driven by *ptcGAL4*. HA staining of $Ci^{ΔC1\text{-}A}$ (green) is primarily cytoplasmic, suggesting that a region(s) other than C1 continues to provide cytoplasmic targeting force. The nuclei are marked by dHSF staining (red).

formaldehyde and stained similarly. Third instar larval imaginal discs were prepared for indirect immunofluorescence as described by Carrol and Whyte (1989). Stained embryos, cells, or discs were examined by confocal microscopy and deconvoluting microscopy. Antibodies used were as follows: anti-Ci, rat monoclonal 2A1 (1:2); anti-HA, mouse monoclonal ascites (1:1,000); anti-dHSF, rabbit polyclonal (1:1,000, gift from R. Morimoto); anti-β-galactosidase, rabbit polyclonal (1:2,000); anti-Ptc, mouse monoclonal ascites (1:1,000, gift from I. Guererro); and all fluorescent secondary antibodies (1:200, Jackson ImmunoResearch Labs).

Immunoblotting

Immunoblots were carried out as described by Harlow and Lane (1988). Mouse anti-HA ascites was used at 1:10,000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary (Jackson) was used at 1:5,000. HRP was detected with ECL reagent (NEN).

RESULTS

Ci contains a conserved bipartite NLS

Comparison of the sequences of Ci, Gli and Tra-1 drew our attention to a stretch of amino acids starting at the end of the zinc finger domain. It contains two basic clusters separated by 10 amino acids, typical features of a bipartite NLS (Fig. 1A). The first basic cluster (R596-K600) lies within the last zinc finger, while the second (K611-K614) lies completely outside the zinc finger domain and is separated from the zinc finger domain by 6 non-conserved amino acids.

To test whether this NLS-like sequence is functional, amino acids K581-L616 were fused to β-galactosidase (β-gal) (Fig. 1B) and the fusion protein was expressed in S2 cells. While β-gal is restricted entirely to the cytoplasm (Fig. 1C), β-gal-NLS (Fig. 1D,D′) shows strong nuclear staining. Therefore, the identified sequence functions as an NLS on a heterologous protein, and is sufficient to direct active nuclear transport.

Next we tested whether the identified sequence is functional in vivo. Because full-length Ci is cytoplasmic, no change in its subcellular localization would be expected if we disrupt the NLS on full-length Ci. Previous studies have shown that when the sequences C-terminal to the zinc finger domain are removed, whether as a consequence of proteolysis or a result of artificial truncations, the rest of the protein exhibits nuclear staining (Hepker et al., 1997; Aza-Blanc et al., 1997). Therefore we constructed Ci^{NZn} and $Ci^{NZn\Delta NLS}$ (Fig. 1B), which differ by only 7 amino acids, the former containing while the latter lacking the second basic cluster. We chose to remove only the second basic cluster, because mutation of the first basic cluster would result in disruption of the last zinc finger. It has been postulated that for proteins containing bipartite NLSs, the functional NLS is reconstituted from the two basic clusters by protein folding (Boulikas, 1993). Removal of either one of the clusters, therefore, should disrupt the function of the whole NLS.

When expressed in vivo, $Ci^{NZn\Delta NLS}$ (Fig. 1F) exhibited substantially reduced nuclear staining compared to Ci^{NZn}, which was predominantly nuclear (Fig. 1E). This result suggested that the second basic cluster is functional in vivo and that its presence is necessary for full activity of the NLS. Some residual nuclear staining persists, suggesting that the first basic cluster on its own or some cryptic sequence on the peptide can still direct nuclear import, though the efficiency is greatly compromised.

Amino acids 703-835 are sufficient to counteract the NLS but do not account for all cytoplasmic targeting

The characterized NLS is able to direct efficient nuclear transport of Ci^{NZn} and β-gal-NLS, yet full-length Ci is cytoplasmic despite containing this NLS. These results suggest that sequences C-terminal to the zinc finger domain target Ci to the cytoplasm, thereby overcoming the function of the NLS. It has been previously shown that a C-terminal domain encompassing N703-M850 is capable of restricting GFP, which normally diffuses freely, to the cytoplasm (Aza-Blanc et al., 1997). We further tested the cytoplasmic targeting effect of this region by fusing P617-R836 (C1) to our β-gal-NLS construct (Fig. 2A) and analyzing the subcellular distribution of β-gal in transfected *S2* cells. We found that this region is indeed able to overcome the action of the NLS and to keep the fusion protein cytoplasmic (Fig. 2B). Such a cytoplasmic targeting force must have come from sequences C-terminal to L685, because *ci*-NZnHA, which is truncated after L685, is nuclear (Hepker et al., 1997). We subsequently deleted L685-R836 (C1-A) from full-length Ci ($Ci^{\Delta C1\text{-}A}$, see Fig. 2A) and examined its subcellular distribution in vivo. Interestingly, $Ci^{\Delta C1\text{-}A}$ is cytoplasmic (Fig. 2C). These results indicate that N703-R836 are sufficient for cytoplasmic targeting, and that other C terminal sequences can also target Ci to the cytoplasm.

Ci exists as a series of multiple phosphorylation isoforms

By SDS-PAGE, Ci exhibits an apparent molecular mass of >155 kDa and is not readily resolved into isoforms. When a *cl-8* cell extract is resolved by isoelectric focusing (IEF), however, Ci is detected as a group of charge isoforms with pI values around 7 (Fig. 3A, *lane 1*). At least 6 major isoforms are easily and consistently detected. Samples prepared from wild-type (OreR) wing discs gave a similar western pattern (Fig. 3B). In fact, when OreR and *cl-8* samples are resolved in the same lane, all bands superimposed and no additional species is visible (data not shown).

Phosphorylation is one of the common events that could result in charge isoforms. We tested whether the isoforms observed for Ci are due to phosphorylation. When a *cl-8* extract was treated with λ protein phosphatase (λ-PPase), the proportion of the Ci isoform with the highest pI (the unphosphorylated) increased at the expense of those with lower pI values (the more phosphorylated forms) (Fig. 3C). Therefore, the bands observed in *cl-8* extract are phosphorylation isoforms, and the band marked with an arrow in Fig. 3A represents the unphosphorylated protein.

PKA activity targets Ci for rapid proteasome degradation

Previous studies have demonstrated that protein kinase A plays an important role in Ci proteolysis. In *PKA* loss-of-function clones, Ci protein level is greatly elevated (Johnson et al., 1995). An extract from discs carrying large numbers of such clones exhibits reduced proteolysis (Jiang and Struhl, 1998). Furthermore, Ci mutated at putative PKA sites is resistant to cleavage (Chen et al., 1998). The resolution of Ci into phosphorylation isoforms enabled us to directly test the action of PKA upon Ci. When *cl-8* cells were treated with H-89, a potent PKA inhibitor, for an hour, we observed by SDS-

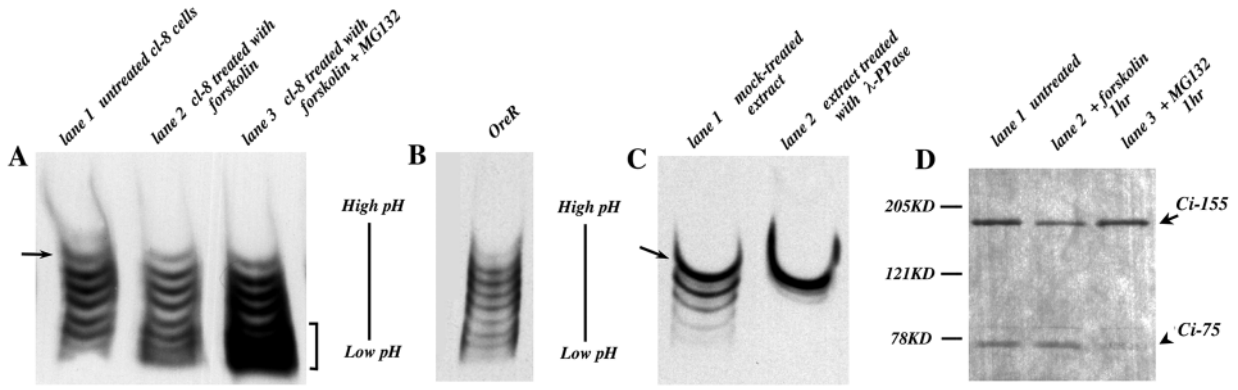

Fig. 3. Ci exists as a mixture of multiple phosphorylation isoforms. (A-C) Protein extracts were prepared from tissue culture cells or from larval imaginal discs, run on IEF gels, blotted onto membrane and probed with anti-Ci mAb 2A1. (A) Stimulation of PKA activity leads to hyperphosphorylation of Ci and targets Ci to the proteasome degradation pathway. *lane 1*: extract from untreated *cl-8* cells. Six major charge isoforms are consistently detected. The band representing the unphosphorylated isoform is indicated by an arrow. *lane 2*: extract from *cl-8* cells treated with 10 μM forskolin for an hour. The IEF pattern is similar to that in *lane 1*. *lane 3*: extract from *cl-8* cells treated with 10 μM forskolin and 10 μM MG132 for an hour. The highly phosphorylated isoforms (bracket) accumulate. Most of the highly phosphorylated Ci peptide are degraded when the proteasome is active (compare *lane 3* to *lane 2*). One-hour treatment with MG132 alone did not lead to marked accumulation of these highly phosphorylated isoforms (data not shown). (B) Overall IEF pattern of OreR imaginal discs extract is similar to that of *cl-8* cells extract. In fact, no additional bands were observed when the two extracts are mixed and run in one lane (data not shown). (C) λ-PPase treatment of *cl-8* cell extract. *lane 1*: mock-treated extract. Arrow indicates the unphosphorylated isoform. The slight dephosphorylation was caused by release of endogenous phosphatase activity into the extract during the sonication step required for preparation of these samples (see Material and Methods). *lane 2*: extract treated with λ-PPase for 30 minutes. (D) SDS-PAGE showing effects of forskolin and MG132 on Ci cleavage/degradation. The blot was probed with a monoclonal IgM, 1C2 (C. Motzny, personal communication), which recognizes an epitope in the zinc finger domain of Ci. *lane 1*: extract from untreated cl-8 cells. *lane 2*: one-hour treatment with forskolin leads to a decrease of Ci-155. *lane 3*: one-hour treatment with MG132 leads to a decrease of Ci-75. A marked increase of Ci-155 can be observed after longer treatment with MG132 (3hrs) (data not shown).

PAGE a slight increase in the amount of full-length Ci, accompanied by a slight decrease of Ci-75 (data not shown). This suggests that the basal activity of PKA is low in *cl-8* cells. When *cl-8* cells were treated with the PKA stimulator forskolin, within an hour we observe a decrease of Ci-155 (Fig. 3D, *lane 2*), but there is no marked change in the IEF pattern of Ci (Fig. 3A, *lane 2*). However, there was a dramatic shift in the western pattern when cells were simultaneously treated with forskolin and MG132 (Fig. 3A, *lane 3*). The amount of the unphosphorylated isoform remained constant, while the highly phosphorylated isoforms (marked by a bracket) accumulated. The difference in intensity between *lane 2* and *lane 3*, therefore, represents the amount of PKA-phosphorylated Ci peptides that undergoes proteolysis when the proteasome is active.

Ci proteolysis is inhibited in fu mutants and cos2 mutants

In wild-type wing imaginal discs, full-length Ci is found at high levels in a stripe along the AP compartment boundary and at low levels throughout the rest of the anterior compartment (Fig. 4A). High level Ptc is expressed in a thin stripe along the boundary (Fig. 4A′). In discs mutant for *fu*, the high level Ci stripe is expanded (Fig. 4B), and the Ptc stripe is more diffuse with a modest protein level (Fig. 4B′). In loss-of-function *cos2* clones, Ci protein level is elevated (Fig. 4C) and these clones cell-autonomously express high level Ptc (Fig. 4C′). To examine whether Ci protein level in *fu* and *cos2* mutants is up-regulated through inhibition of proteolysis, extracts from *fu* and *cos2* hypomorphic discs were analyzed by SDS-PAGE and western blot. Proteolysis of Ci is not detectable in *cos2* hypomorphic wings extracts and is significantly inhibited in extracts from both class I and class II *fu* mutant discs (Fig. 4D). Despite the inhibition of Ci proteolysis in *fu* mutants, such animals display evidence of compromised Ci activity, both in reduced *ptc* expression and fusion between LV3 and 4 in adult wings.

Identification of a reporter highly sensitive to the activation state of Ci

4bslacZ, an artificial reporter construct, contains an enhancer element consisting of four tandem Ci binding sites and four weak Scalloped (Sd) binding sites (Hepker et al., 1999). *4bslacZ* is expressed along the AP boundary in only the pouch region of the wing imaginal disc (Hepker et al., 1999; also see Fig. 5B) due to the combined action of Ci and Vestigial (Vg)/Sd complex (Halder et al., 1998). The width of its expression coincides with that of endogenous Ptc stripe within the pouch. Compared to other enhancer traps/*lacZ* reporters that respond to Ci, *4bslacZ* is only activated at the highest level of Hh signaling. Overexpression of Ci in the anterior compartment fails to activate *4bslacZ* (Hepker et al., personal communication). On the other hand, *4bslacZ* is activated in the posterior wing pouch by a modest level of ectopic Ci (Fig. 5D-F). In discs expressing ectopic Ci, Ptc is activated in the posterior compartment (Alexandre et al., 1996; Hepker et al., 1997; Methot and Basler, 1999) and hence the amount of Hh reaching the AP boundary is reduced. Such sequestering of Hh signal by Ptc was described by Chen and Struhl (1996) and also demonstrated in Bellaiche et al. (1998). As an expected consequence, the endogenous *4bslacZ* stripe is diminished, indicating that in the wild-type situation

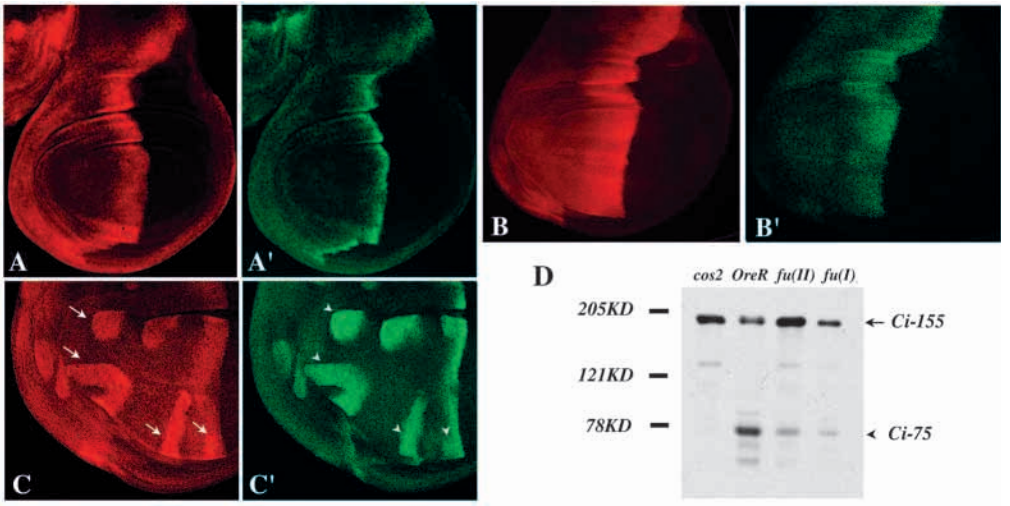

Fig. 4. Mutations in *fu* and *cos2* cause similar inhibition of Ci proteolysis but have opposite effects on transactivation by Ci. A, B and C show Ci staining. A′, B′, and C′ show Ptc staining. (A,A′) A wild-type wing disc. Shown are the expression patterns of Ci (A) and Ptc (A′). (B,B′) A fu^{XR15} wing disc. Compared to wild type, the Ci stripe is broader and Ci-155 level within the stripe is further elevated. The wild-type thin, sharp, high level Ptc stripe along the AP boundary is replaced by a broad, diffuse, modest level expression zone (B′). (C,C′) A wing carrying *cos2* loss-of-function clones. Ci protein level is elevated within the clones (arrows in C). (C′) These clones (arrowheads) cell-autonomously activate high level Ptc (green). (D) Western blot showing the inhibition of Ci proteolysis in *fu* and *cos2* mutant discs. The amount of disc extract loaded in each lane is controlled so that the relative intensity of Ci-155 and Ci-75 bands can be compared between samples with ease. *cos2*: $cos2^{V1}$ $Cos1^{3}$/ $cos2^{W1}$ wing discs. The extract from 5 discs was loaded. *OreR*: *OreR* wing discs. The extracts from 40 discs were loaded. *fu(II)*: fu^{XR15} wing discs. Extract from 20 discs were loaded. *fu(I)*: fu^{1} wing discs. Extract from 20 discs were loaded. The position of the Ci-155 band is marked with an arrow. That of the Ci-75 band is marked with an arrowhead.

Hh signaling is responsible for the expression of *4bslacZ* along the AP boundary. Some Hh signal must have reached the AP boundary, since an elevated Ci stripe is still visible. Yet this elevated Ci is not sufficient to activate *4bslacZ*, suggesting that the induction of *4bslacZ* relies more on the level of Hh signaling than on that of full-length Ci protein. To confirm that *4bslacZ* responds to Ci only when the Hh signaling pathway is fully activated, and that the lack of anterior *4bslacZ* expression in Fig. 5E is not due to some Hh-independent difference between the A and P compartments, we generated discs carrying ptc^{IIW} clones (Fig. 5G-I). As expected, *4bslacZ* is ectopically activated in anterior cells homozygous for ptc^{IIW}. Thus, *4bslacZ* is activated only when the cells receive the highest level of Hh signal (such as those within or immediately adjacent to the posterior compartment) or when such a situation is mimicked (as in strong *ptc* mutants), and the expression of *4bslacZ* can be used to monitor the existence of Ci in the fully activated state.

Loss-of-function PKA or cos2 clones do not fully mimic Hh signaling

Using *4bslacZ* as a sensitive gauge to assess Hh-induced Ci activation, we examined *PKA* and *cos2* loss-of-function clones to assay whether Ci is

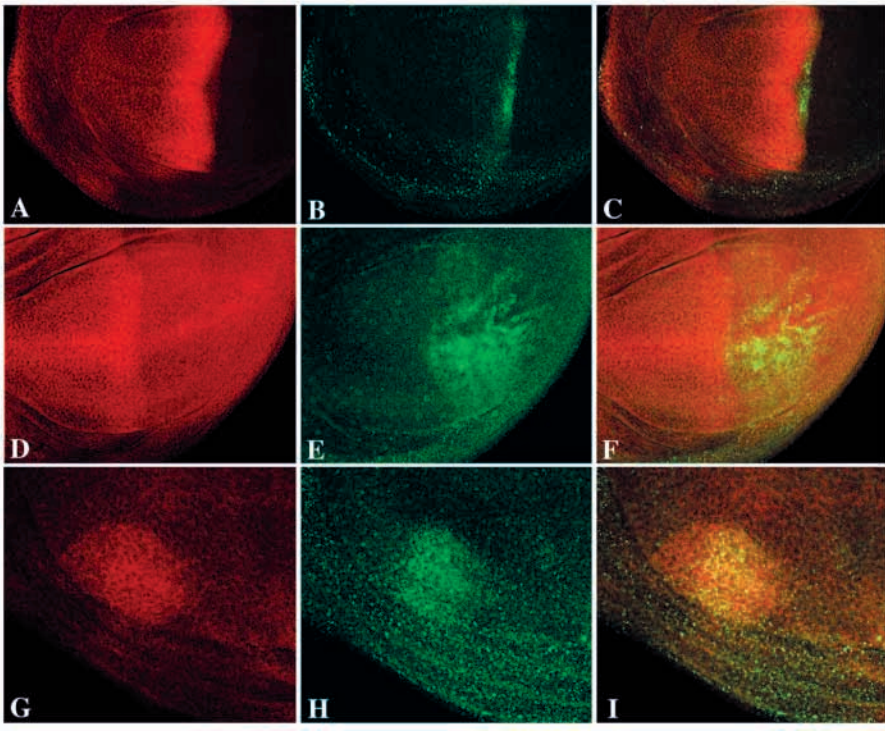

Fig. 5. *4bslacZ* is acitvated by Ci only when high level Hh signaling is present or mimicked. (A-C) Wild-type disc showing (A) Ci expression; (B) endogenous *4bslacZ* expressed in a thin stripe along the AP boundary in the wing pouch; (C) a merged image of A and B. (D-F) A wing disc ubiquitously expressing Ci from an *A5C-FC-ci* transgene. (D) 2A1 staining showing ubiquitous expression of Ci. The endogenous Ci stripe also persists and is visible. (E) *4bslacZ* is activated in the posterior wing pouch, with stronger expression in the ventral quadrant. Note that the endogenous *4bslacZ* stripe is diminished. (F) A merged image of D and E. (G-I) *4bslacZ* is ectopically activated in ptc^{IIW} clones. (G) A ptc^{IIW} clone in the ventral-anterior quadrant. Ci protein level is elevated. (H) β-gal staining showing ectopic activation of *4bslacZ*. (I) A merged image of G and H.

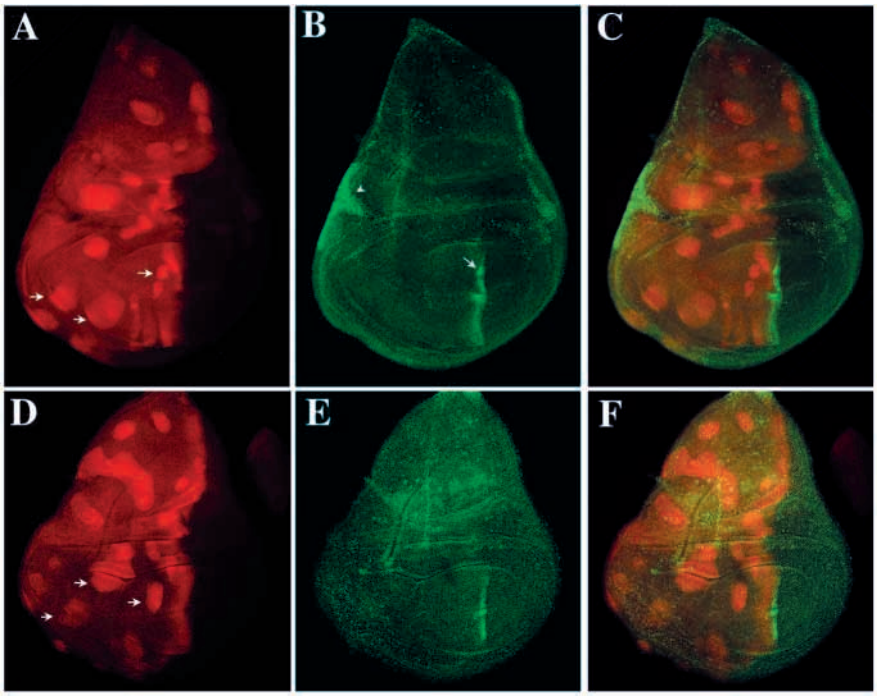

Fig. 6. Ci is not fully activated in anterior *PKA* and *cos2* loss-of-function clones. (A-C) *4bslacZ* is not activated in anterior *PKA* loss-of-function clones. (A) *PKA* loss-of-function clones are marked by autonomous elevation of Ci protein level (arrows). (B-C) No ectopic expression of *lacZ* (green) is observed in these clones. The endogenous *4bslacZ* stripe is marked in B with an arrow. The hinge staining in B (arrowhead) is parapodial membrane staining. (D-F) High level Ci in *cos2* loss-of-function clones fails to activate *4bslacZ*. (D) *cos2* loss-of-function clones are marked by up-regulation of Ci protein level (arrows). (E-F) These clones do not express *lacZ* (green).

automatically activated by these mutations. When *PKA* loss-of-function clones were generated in discs carrying the *4bslacZ* reporter transgene, *4bslacZ* was not activated in the clones whether they were away from or close to the AP boundary (Fig. 6A-C). Similarly, *cos2* loss-of-function clones failed to activate *4bslacZ* (Fig. 6D-F). Therefore, ectopic, modest level Ci in the posterior compartment (Fig. 5F) exhibits higher activity than does overexpressed Ci in *PKA* or *cos2* mutant clones. It shows that although *PKA* or *cos2* clones mimic Hh-induced Ci stabilization and leads to ectopic expression of *ci* target genes, they do not automatically mimic Hh-induced Ci activation.

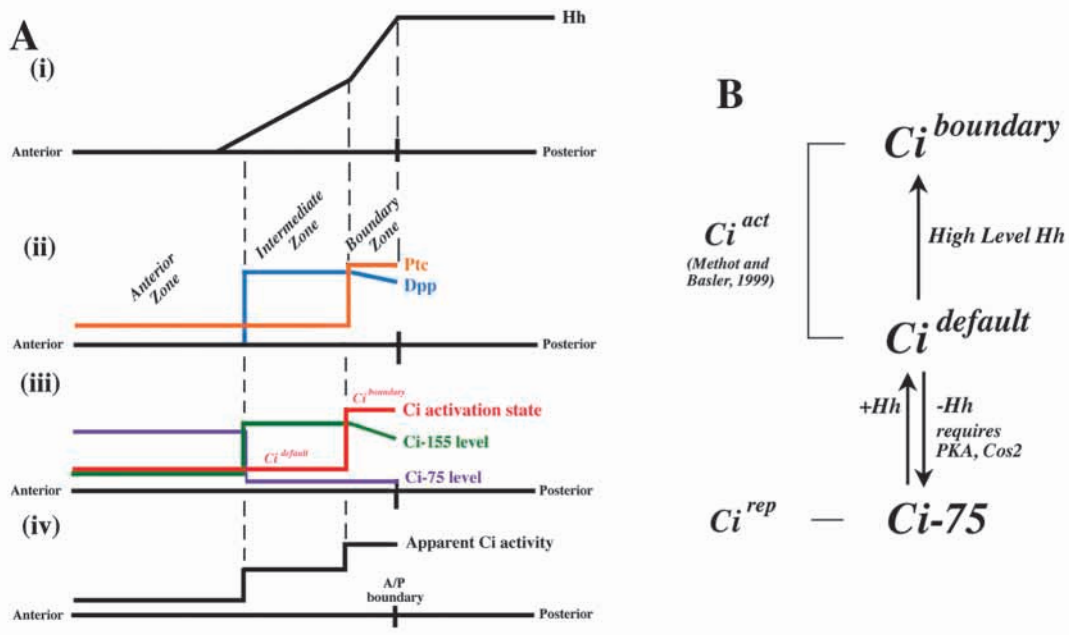

Fig. 7. (A) Three levels of apparent Ci activity corresponding to three zones along the AP axis with different sets of gene expression and different levels of Hh signaling. (i) Hh signal originates from the posterior compartment and diffuses into the anterior. Immediately anterior to the AP boundary, a high level of Ptc sequesters Hh protein, resulting in a steep decline of the Hh signal. (ii) Anterior cells are divided along the AP axis into three zones based on the expression of *ci* target genes. A blue line marks the expression of *dpp*; the orange line marks that of *ptc*. (iii) The protein levels of Ci-155 (green line), Ci-75 (purple line) and the state of Ci activation (red line) also vary along the AP axis. $Ci^{boundary}$ exists only in the Boundary Zone. Away from the boundary Ci exists as $Ci^{default}$. (iv) The three levels of apparent Ci activity. The apparent Ci activity in each zone is a combined readout of Ci activation state and Ci-155 protein level in that zone. (B) The transition between different states of Ci. Hh signaling inhibits the proteolysis of Ci into Ci-75. Proteolysis also requires the functions of PKA and Cos2. Ci-75 mediates the repressor function of Ci (Ci^{rep}), while $Ci^{default}$ and $Ci^{boundary}$ both mediate the activator function (Ci^{act}). The activation from $Ci^{default}$ to $Ci^{boundary}$ happens only when the cells receive a high level of Hh signaling, such as the Boundary Zone cells.

DISCUSSION

Apparent Ci activity is determined by its protein level and its state of activation

Cells in the anterior compartment of wing pouch express different sets of ci target genes depending on their distance from the AP boundary. Based on the expression profile of Ci and its target genes (summarized in Fig. 7A), we can divide cells along the AP axis into three zones. The Anterior Zone consists of cells more than 8-9 cell diameters away from the boundary. Cells in this zone express low level Ci, low level Ptc and no Dpp. The Intermediate Zone, marked by expression of high level Ci, low level Ptc and high level Dpp, corresponds to cells between 8-9 and 2-3 cell diameters away from the boundary. Cells immediately adjacent to the boundary (within 2-3 cell diameters) fall into the Boundary Zone, marked by medium level Ci, high level Ptc, and medium level Dpp. The enhancer element *4bslacZ* is also expressed in this zone.

Expression of *ci* target genes is a consequence of the overall activity of many individual Ci peptides, which is determined by both the potency of each peptide and the number of peptides present. In the following discussion, the overall Ci activity observed for a cell, judged by the expression of target genes, is termed "apparent activity" of Ci. The specific activity, or potency, of each peptide is defined as its "activation state". In a wild-type wing disc, there are three levels of apparent Ci activity corresponding to the three zones (Fig. 7A-iv). In the Boundary Zone, Ci has the highest apparent activity, activating both *ptc* and *4bslacZ* to high levels. *dpp* expression in this region is likely subjected to a partial repression by anterior En (Blair, 1992; Methot and Basler, 1999). In the Intermediate Zone, Ci exhibits an intermediate level of apparent activity, inducing strong expression of *dpp* but not high level *ptc* nor *4bslacZ*. In the Anterior Zone, Ci has the lowest apparent activity.

Although there are three levels of apparent Ci activity, we find evidence for only two activation states. *4bslacZ*, whose sensitivity allows us to monitor Hh-induced Ci activation, is expressed only in the Boundary Zone, suggesting that Ci is activated only in this zone. Ci in the activated state, therefore, is given the name $Ci^{boundary}$. The high level of Ci in the Intermediate Zone indicates that cells in this zone must receive some level of Hh signaling (Fig. 7A-i), which inhibits Ci proteolysis. Despite the high protein level, Ci in the Intermediate Zone is not sufficiently activated to induce *4bslacZ* expression, and is given the name $Ci^{default}$. The lack of proteolysis in the Intermediate Zone allows $Ci^{default}$ to accumulate, resulting in high level expression of *dpp*. In the Anterior Zone, the majority of Ci is proteolytically cleaved into Ci-75. Although the Anterior Zone and the Intermediate Zone differ in Ci protein levels and the expression patterns of target genes, at present there is no evidence that they differ in the state of Ci activation. In fact, when Ci stabilization is mimicked in the Anterior Zone by making *PKA* loss-of-function clones, the expression of high level *dpp* is also mimicked (Pan and Rubin, 1995). This observation is consistent with the idea that the two zones share the same activation state of Ci, $Ci^{default}$, but differ in the levels of full-length Ci. In summary, the three levels of apparent Ci activity correspond to $Ci^{boundary}$, high level $Ci^{default}$, and low level $Ci^{default}$, respectively.

Attenuation of Hh signal by Ptc along the boundary is important for proper regulation of Ci apparent activity

The boundary between the Anterior Zone and the Intermediate Zone corresponds to a division between cells with low Ci levels and those with high Ci levels, and is likely to coincide with the anterior border of Hh signaling. Between this line and the AP boundary, cells in both the Intermediate Zone and the Boundary Zone receive some level of Hh signaling and show stabilization of Ci. (The relatively lower Ci level in the Boundary Zone probably reflects partial transcriptional repression by anterior En. See Eaton and Kornberg, 1990; Blair, 1992.) However, the activation from $Ci^{default}$ to $Ci^{boundary}$ happens in only the Boundary Zone (Fig. 7A-iii), suggesting that it takes place when the level of Hh signaling is above a certain threshold. The change in the level of Hh signaling across the anterior compartment is illustrated in Fig. 7A-i. In the Boundary Zone, cells express high level Ptc, which both transduces and sequesters Hh signaling. The presence of high level Ptc creates a steep decline of the Hh signal. Consequently, cells receiving Hh are divided into those receiving high level Hh signal (the Boundary Zone) and those receiving lower level Hh signal (the Intermediate Zone). Its role in regulating Hh distribution makes Ptc essential for proper regulation of the apparent activity of Ci.

PKA and cos2 loss-of-function clones do not fully mimic Hh signaling

Study of Hh-induced Ci activation has been complicated by the fact that in almost all the assays, a high level of Ci protein seems to suffice for the activation of Ci target genes. This is well illustrated in the case of loss-of-function *PKA* clones, in which elevated Ci protein levels are associated with strong activation of Hh target genes such as *ptc* and *dpp* (Jiang and Struhl, 1995; Jonhson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). It has been difficult to tell whether the activation of downstream genes is due to elevated Ci alone, or if Ci is activated in addition to being stabilized in these clones.

Progress was made in a recently published study through a combination of manipulating the expression of an uncleavable deletion construct of Ci and examining *smo* loss-of-function clones in the posterior compartment (Methot and Basler, 1999). Importantly, it demonstrated that inhibition of Ci proteolysis is not sufficient to activate Ci. However, the assays as described did not address the question of whether in vivo stabilization and activation of Ci are regulated simultaneously as two consequences of the same process, or regulated separately through different mechanisms. The sensitivity of *4bslacZ* allowed us to address this question. While a modest level of Ci in cells receiving high level Hh signal can activate *4bslacZ* and clones lacking ptc activate *4bslacZ*, high level Ci in *PKA* or *cos2* loss-of-function clones cannot. We conclude that Ci is stabilized but not activated in *PKA* or *cos2* mutant clones. In other words, these mutations mimic one aspect of Hh signaling but not the other, and Ci in the clones exists as $Ci^{default}$ instead of $Ci^{boundary}$.

Role of Fu, PKA and Cos2 in the Hh-induced Ci stabilization pathway

Although Ci-155 is stabilized and Ci-75 reduced in *fu* mutants,

we do not take this observation as evidence that Fu kinase normally promotes Ci cleavage/degradation. The lack of proteolysis is more likely a reflection of changes in Hh signaling dynamics. Without Fu kinase activity, *ptc* expression is disrupted. In these mutants, the wild-type thin, sharp Ptc stripe is replaced by a broad, diffuse stripe with reduced Ptc protein level. Consequently, Hh signal diffuses further into the anterior compartment and instructs more cells to stabilize Ci. The overall decrease in proteolysis therefore may be due to the increase in the number of cells receiving Hh signal. Consistent with this model, in *fu* mutants Ci levels are not elevated in cells located far away from the boundary which receive no Hh signal (Fig. 4B; also see Fig. 5d in Ohlmeyer and Kalderon, 1998). The elevation of Ci within *fu* clones at the AP boundary (Ohlmeyer and Kalderon, 1998) can also be explained by changes in Hh signaling dynamics. *fu* mutant cells express lower level Ptc than neighboring cells while receiving the same level of Hh signal. Hence the repression of Smo is diminished and stabilization of Ci is more efficient in mutant cells. Significantly, Ci levels are low in *fu smo* double mutant clones (Ohlmeyer and Kalderon, 1998), demonstrating that Hh signaling is required for the up-regulation of Ci in *fu* mutant tissue. These data indicate that in *fu* mutants, Ci proteolysis is regulated by Hh signal as in wild-type animals. An intriguing observation is that in class II *fu* mutants, Ci level is slightly increased away from the boundary (Alves et al., 1998; Fig. 4B). It was postulated that class II Fu mutant protein, which cannot associate with Cos2, may affect the efficiency of Ci proteolysis by disrupting the integrity of the Cos2/Fu/Ci complex. Therefore, in this case it may be the loss of Fu as a structural component, rather than the loss of Fu as a kinase, that is compromising Ci proteolysis away from the boundary. Taken together, Fu kinase activity is required neither for Ci proteolysis in cells lacking Hh signal, nor for Ci stabilization in cells receiving Hh signal (Fig. 7B). On the other hand, Ci protein levels are consistently elevated in *PKA* or *cos2* mutant clones irrespective of position relative to the AP boundary, suggesting that these two molecules function downstream of Hh in the pathway that regulates Ci stability (Fig. 7B).

In *PKA* mutant clones as well as in *fu* mutant cells near the AP boundary, Ci protein accumulates to levels higher than that of the wild-type endogenous Ci stripe (see Fig. 4B for *fu*; see Fig. 6A for *PKA* clones). The high level $Ci^{default}$ in *PKA* clones strongly activates *dpp* and *ptc*. In comparison, the high level Ci in *fu* mutants activates *ptc* only to a modest level (Fig. 4B′). The trace amount of Ci-75 present in *fu* mutant discs (see Fig. 4D) could interfere with transactivation by full-length Ci. Alternatively, Ci in *fu* mutants could exist in an unique state whereby their specific activity is lower than that of $Ci^{default}$.

Opposing forces in determination of Ci subcellular distribution

Ci^{NZn}, Ci-75 and Ci-155 all carry the same bipartite NLS, but the two truncated molecules are nuclear while Ci-155 is cytoplasmic. A region immediately C-terminal to the NLS (P617–R836) was able to inhibit nuclear accumulation of a fusion protein (Fig. 2), demonstrating that this region represents a cytoplasmic targeting domain which overcomes the function of the NLS. This region, however, does not account for all the cytoplasmic targeting elements on Ci, because a deletion construct missing this region still accumulates in the cytoplasm. Therefore the subcellular distribution of Ci is controlled by two opposing forces, a nuclear directing force of the NLS and a cytoplasmic directing force contributed by more than one element.

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