RESEARCH ARTICLE

Contributions of Costal 2-Fused interactions to Hedgehog signaling in *Drosophila*

Eva V. Zadorozny*, Jamie C. Little* and Daniel Kalderon‡

ABSTRACT

The *Drosophila* kinesin-family protein Costal 2 (Cos2) and its mammalian ortholog Kif7 play dual roles in Hedgehog (Hh) signaling. In the absence of Hh, Cos2 and Kif7 contribute to proteolytic processing and silencing of the Hh-regulated transcription factors, *Drosophila* Cubitus interruptus (Ci) and mammalian Gli proteins. Cos2 and Kif7 are also necessary for full activation of full-length Ci-155 and Gli transcription factors in response to Hh proteins. Here, we use classical *fused* alleles and transgenic Cos2 products deficient for Fused (Fu) association to show that Cos2 must bind to Fu to support efficient Ci-155 processing. Residual Ci-155 processing in the absence of Cos2-Fu interaction did not require Suppressor of Fused, which has been implicated in processing mammalian Gli proteins. We also provide evidence that Cos2 binding to the CORD domain of Ci-155 contributes to both Ci-155 processing and Ci-155 silencing in the absence of Hh. In the presence of Hh, Ci-155 processing is blocked and Cos2 now promotes activation of Ci-155, which requires Fu kinase activity. Here, we show that normal Ci-155 activation by Hh requires Cos2 binding to Fu, supporting the hypothesis that Cos2 mediates the apposition of Fu molecules suitable for cross-phosphorylation and consequent full activation of Fu kinase. We also find that phosphorylation of Cos2 by Fu at two previously mapped sites, S572 and S931, which is thought to mediate Ci-155 activation, is not required for normal activation of Ci-155 by Hh or by activated Fu.

KEY WORDS: ***Drosophila*, Hedgehog, Signal Transduction, Costal 2 (Cos), Fused**

INTRODUCTION

Secreted Hedgehog proteins constitute a major family of signaling molecules that guide development, cell proliferation and stem cell behavior in *Drosophila* and in mammals (Hui and Angers, 2011; Briscoe and Therond, 2013; Zhang and Kalderon, 2001; Peng et al., 2013; Petrova et al., 2013; Li et al., 2014). Accordingly, genetic alterations affecting Hedgehog (Hh) signaling are responsible for a variety of developmental defects and cancers, prompting the development of promising therapeutic drugs (Ng and Curran, 2011; Metcalfe and de Sauvage, 2011; Amakye et al., 2013).

The majority of responses to Hh signals are transcriptional changes mediated by the zinc-finger DNA-binding protein Ci in *Drosophila* and a family of three orthologs, Gli1, Gli2 and Gli3, in mammals (Hui and Angers, 2011; Briscoe and Therond, 2013). Full-length Ci-155, like Gli2 and Gli3, is processed by the proteasome to a C-terminally truncated repressor (Ci-75) in the absence of Hh. Proteolytic

Department of Biological Sciences, Columbia University, New York, NY 10027, USA.
*These authors contributed equally to this work

‡Author for correspondence (ddk1@columbia.edu)

processing depends on prior phosphorylation of Ci-155 at a cluster of PKA, CK1 and GSK3 sites, which are conserved in Gli2 and Gli3, and on recognition of those phosphorylated residues by a conserved Cul1-containing E3 ubiquitin ligase. Processing also involves a kinesin-family molecule, Costal 2 (Cos2; Cos – FlyBase), or Kif7 in mammals, which binds to Ci-155 or Gli2/3. In *Drosophila*, Cos2 also binds to PKA, CK1 and GSK3, thereby acting as a scaffold to enhance Ci-155 phosphorylation (Zhang et al., 2005). When Hh binds to Patched (Ptc) and to its co-receptor, Smoothened (Smo) is activated (Hui and Angers, 2011; Briscoe and Therond, 2013). Activated Smo leads to inhibition of Ci/Gli protein processing and activation of full-length Ci/Gli proteins by mechanisms that include gaining increased nuclear access and, likely, dissociation from the inhibitory proteins Cos2/Kif7 and Suppressor of Fused (Hui and Angers, 2011; Briscoe and Therond, 2013). Activation of Ci-155 depends substantially on Fu protein kinase activity in *Drosophila*, while the protein most similar to Fu in mice has no role in Hh signaling (Alves et al., 1998; Ohlmeyer and Kalderon, 1998; Wilson et al., 2009; Zhou and Kalderon, 2011).

Hh signaling has been studied extensively in developing *Drosophila* wing imaginal discs (Ingham and McMahon, 2001). Here, Hh expression is confined to posterior compartment cells, whereas Ci is expressed only in anterior compartment cells. Hh therefore signals in a graded fashion to anterior cells in a central domain of 12-15 cells' width, known as the AP (anterior/posterior) border. Ci-155 processing is substantially inhibited throughout the AP border and the target gene *decapentaplegic* (*dpp*) is transcriptionally induced through most of this region (Methot and Basler, 1999). Induction of *ptc* transcription, which is commonly visualized with a *ptc-lacZ* reporter gene, is restricted to the posterior half of this signaling domain, whereas Engrailed (En) is induced only very close to posterior Hh-secreting cells (Vervoort, 2000). Hh signaling has also been studied biochemically *in vitro* and in tissue culture to define and assess the role of specific protein interactions and modifications, but these inferences are limited by the appreciation that normal Hh signaling depends on maintaining the normal stoichiometry of key signaling components, including Cos2. Here, we investigated the roles of Cos2 binding to Fu and to nucleotides, and the role of Fu phosphorylation sites on Cos2 under physiological conditions.

RESULTS

Fused C-terminal Cos2-binding domain is required for efficient Ci-155 processing

Prior studies have shown that C-terminal truncations of the Fu protein affect Ci-155 processing but there are conflicting claims regarding whether Fu is essential for Ci-155 processing and whether some *fu* alleles simply make Ci-155 processing more sensitive to Hh inhibition (Alves et al., 1998; Wang and Holmgren, 1999; Methot and Basler, 2000; Lefers et al., 2001). Wing discs from male third instar larvae hemizygous for fu^{M1} (encoding only residues 1-80 of the normal Fu protein), fu^{W3} (encoding residues 1-612) and fu^{RX2}

(encoding residues 1-748) (Therond et al., 1996) (Fig. 1A) all exhibited increased Ci-155 levels throughout the anterior compartment that were highest in the broadened AP domain of Hh signaling, suggesting ubiquitously impaired Ci-155 processing that is inhibited further by Hh (Fig. 1B-E). A strong cell-autonomous increase in Ci-155 staining was seen in homozygous *fu* mutant clones for all three alleles in regions beyond the range of Hh and also in anterior *smo fu*^{M1} clones (Fig. 1J; supplementary material Fig. S1A-C), showing a strong Ci-155 processing defect in the absence of any response to Hh.

We then explored the properties of the *fu*^{M1} allele, which is closest to a null, in more detail. The levels of C-terminally tagged Ci from GAL4-driven *UAS-Ci-Myc* transgenes can report Ci-155 stability because transgene transcription is expected to be constant. Thus, Ci-Myc expressed evenly throughout the wing disc is present at lower levels in anterior cells than in AP border or posterior cells because processing in anterior cells eliminates the Myc epitope (Zhou and Kalderon, 2010). This difference is still greater for the Ci variant CiΔCDNΔCORD (henceforth abbreviated as Ci-ΔΔ) (Fig. 1F,H), which appears to be processed more efficiently than wild-type Ci (Zhou and Kalderon, 2010). In *fu*^{M1} mutant discs, Myc staining of both wild-type Ci-Myc and CiΔΔ-Myc was increased in anterior cells to almost the same level as the AP border, confirming impaired processing in the absence of Hh (Fig. 1F-I).

We then used an assay that can detect even low levels of Ci-155 processing (Methot and Basler, 2000; Price and Kalderon, 2002; Smelkinson et al., 2007). In this assay, a Ci transgene is expressed throughout the wing disc (using *C765-GAL4+UAS-Ci* transgenes) and conversion to the Ci-75 repressor is assayed in posterior *smo* mutant clones (to block Hh signaling) by examining transcriptional repression of a *hh-lacZ* reporter. No Ci-155 processing is detected by this *hh-lacZ* repression assay in the absence of Cos2 function in *smo cos2* mutant clones (supplementary material Fig. S2A) (Methot and Basler, 2000; Zhou and Kalderon, 2010). However, sufficient Ci-75 was produced in *smo fu*^{M1} clones to give strong repression of the *hh-lacZ* reporter (Fig. 1L), in contrast to previously reported results for *fu*^{4} (Methot and Basler, 2000). We found that *fu*^{4} mutant wing discs have the highest levels of Ci-155 in the AP border region (supplementary material Fig. S1D,E), as seen for the other three *fu* alleles tested, suggesting that some Ci-155 processing persists in the absence of Hh. We therefore suggest that there is residual Ci-155 processing for all *fu* alleles.
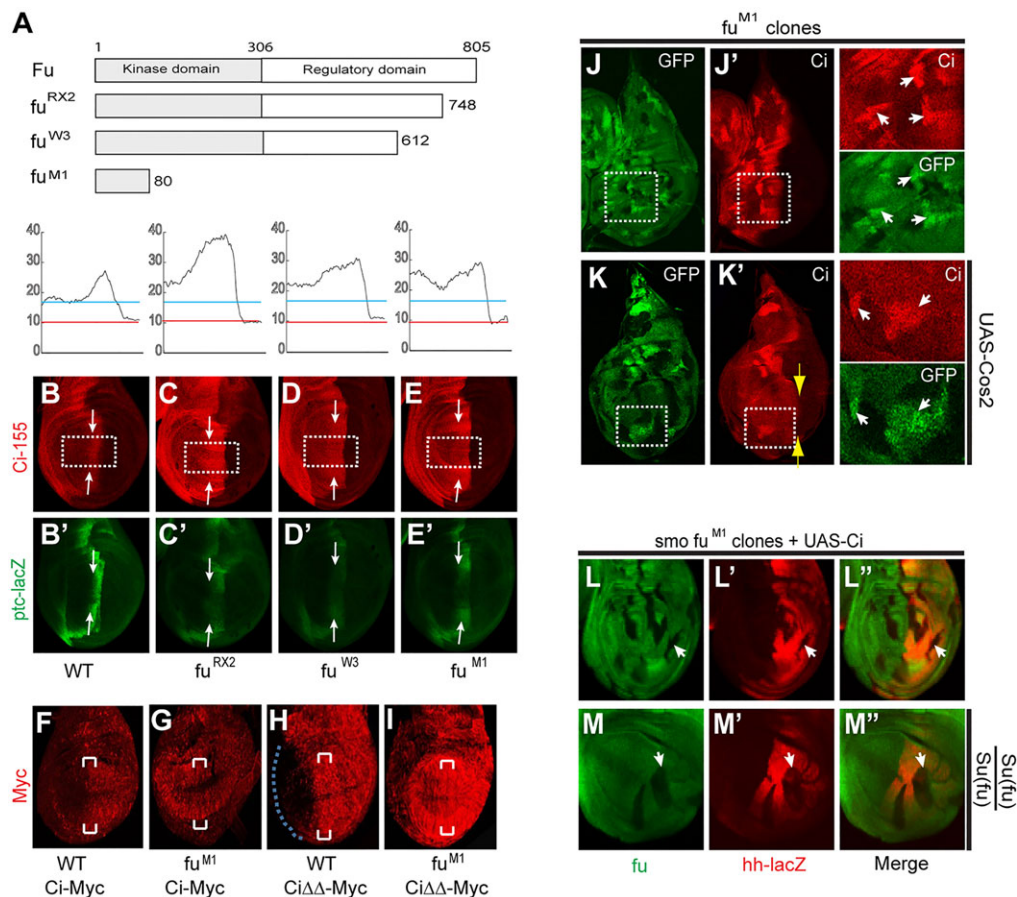
Fig. 1. The C-terminal of Fused is required for efficient Ci-155 processing. (A) Schematic of proteins encoded by *fused* alleles used. (B-E) Full-length Ci-155 (red) and (B′-E′) *ptc-lacZ* reporter of Ci activity (green) in wing discs from male wild-type (WT) or *fu* mutant larvae. Arrows indicate the anterior (left) boundary of *ptc-lacZ* expression. Plots of Ci-155 staining intensity (above B-E) along the AP axis were generated as described in the Materials and methods for the boxed regions. Background posterior Ci-155 levels (red line) and anterior Ci-155 levels (blue line) in wild-type discs are indicated. (F-I) Myc epitope staining (red) of Ci transgenes tagged with Myc at the C terminus and expressed ubiquitously using the *C765-GAL4* driver in wild-type (WT) or *fu*^{M1} mutant wing discs. Brackets indicate the estimated AP border territory from parallel *ptc-lacZ* staining (not shown). (J-K′) Ci-155 levels (red) were increased in anterior *fu*^{M1} clones (white arrows) marked by two copies of a *ubi-GFP* transgene (adjacent to GFP-negative twin-spot clones) in otherwise normal discs (J,J′) and in discs expressing *UAS-cos2* at a high level with the *C765-GAL4* driver (K,K′). Excess Cos2 inhibited Hh signaling and reduced Ci-155 staining at the AP border (yellow arrows). Insets show boxed regions at higher magnification. (L-M″) *hh-lacZ* (red) was repressed by Ci-75 repressor derived from *UAS-Ci* expressed using *C765-GAL4* in posterior *fu*^{M1} *smo*^{2} clones (arrows), marked by loss of Fu staining (green) in otherwise normal discs (L-L″) and in homozygous *Su(fu)*^{LP} (null) discs (M-M″).

We then tested whether excess Cos2 or Su(fu) can compensate for the absence of functional Fu. Excess Cos2 alone does not impair Ci-155 processing and can, in fact, reduce anterior Ci-155 below normal levels (Zhou and Kalderon, 2010). However, Ci-155 levels were still strongly elevated in fu^{MI} clones in discs expressing high levels of a wild-type *cos2* cDNA (Fig. 1K). We also saw strong *hh-lacZ* repression in posterior *smo* fu^{MI} mutant wing disc clones in the complete absence of Su(fu) (Fig. 1M). Thus, residual Ci-155 processing in the absence of functional Fu does not depend on Su(fu) and cannot be restored to normal by excess Cos2. All of the Fu variants that we have shown to have a similar deficit in Ci-155 processing (including Fu 1-748 encoded by fu^{RX2}) lack a C-terminal Cos2-binding domain (Robbins et al., 1997; Monnier et al., 2002), suggesting that Fu must bind to Cos2 to support efficient Ci-155 processing.
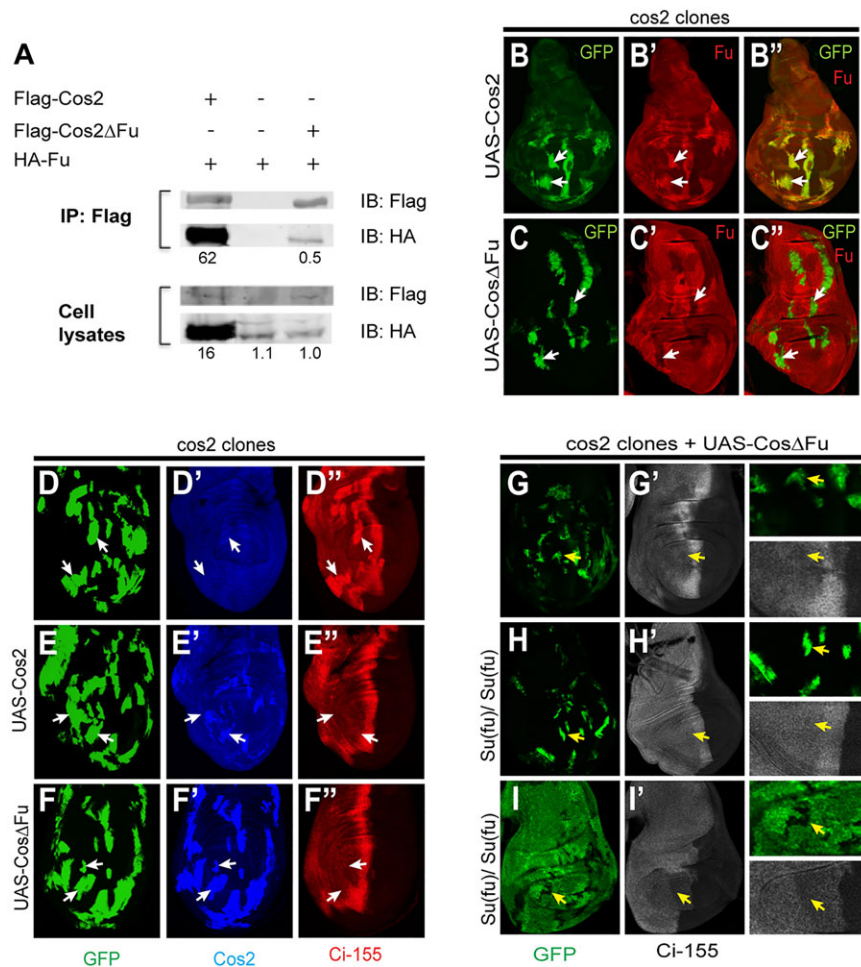
Contribution of the Fu-binding domain of Cos2 to Ci-155 processing

We wished to test further whether Cos2 must recruit Fu to the Ci-155 processing complex by making Cos2 variants deficient for Fu binding. Previous studies identified a very small region of Cos2 (residues 543-605) sufficient for binding the regulatory (non-kinase) domain of Fu (Ruel et al., 2007). We therefore tested whether variants of Cos2 lacking this region could bind Fu by expressing HA-tagged Fu together with Flag-tagged Cos2 proteins in Kc tissue culture cells and measuring co-precipitation of HA-Fu with Flag-Cos2. Flag-Cos2 lacking residues 543-605 was not apparently stable, whereas Flag-Cos2 proteins lacking residues 560-580 or 580-600

co-precipitated HA-Fu efficiently (data not shown). However, Flag-Cos2 lacking residues 540-560 was stable and brought down very little HA-Fu compared with wild-type Flag-Cos2 (less than 1%; Fig. 2A). Although equal amounts of expression constructs were transfected in these experiments, extracts containing wild-type Flag-Cos2 contained far more HA-Fu than extracts from cells with Flag-Cos2Δ540-560 or no transfected Cos2 (Fig. 2A), reflecting previous observations that Cos2 strongly stabilizes Fu protein (Lum et al., 2003; Ruel et al., 2003). Thus, both HA-Fu levels and co-precipitation results indicate that Cos2 lacking residues 540-560 is highly deficient for Fu binding and we henceforth refer to that variant as CosΔFu.

We then examined the properties of CosΔFu in wing discs by expressing GAL4-responsive *UAS-CosΔFu* using the *C765-GAL4* driver but restricting expression to clones homozygous for the null *cos2²* allele by using the MARCM technique (Lee and Luo, 2001). Fu levels are greatly reduced in *cos2* mutant clones (Lum et al., 2003). However, expression of *UAS-Cos2* in *cos2* mutant clones increased the levels of Fu significantly beyond those in surrounding tissue (Fig. 2B). The level of Cos2 in those clones greatly exceeded endogenous levels (Fig. 2E) and presumably accounts for the observed increase in stable Fu protein. Although CosΔFu from *UAS-CosΔFu* was also present in great excess over endogenous levels in *cos2* mutant clones (Fig. 2F), Fu protein levels were much lower in these clones than in surrounding tissue (Fig. 2C). We conclude that the CosΔFu variant does not stabilize Fu in wing discs, consistent with a severe loss of Fu binding.

Surprisingly, Ci-155 protein levels, which are greatly increased in *cos2* mutant clones (Fig. 2D), were restored to normal by expression

Fig. 2. Ci-155 processing by overexpressed Cos2 deficient for Fu association. (A) Kc cells were transfected with DNAs encoding Flag-tagged Cos2 proteins and HA-tagged Fu as shown, followed by immunoprecipitation with Flag antibody (IP: Flag) and western blot with Flag and HA antibodies to measure Fu association with Cos2. Numbers indicate the relative intensity of HA-Fu signals in immunoprecipitates and cell extracts. (B-C″) Fu protein levels (red) were (B-B″) greatly increased in *cos2* mutant clones that express *UAS-cos2* (arrows), marked by GFP (green), but (C-C″) severely reduced in *cos2* mutant clones that express *UAS-CosΔFu*. (D-F) Cos2 levels (blue) were greatly increased in *cos2* clones (arrows), marked by GFP (green), that express (E′) *UAS-Cos2* or (F′) *UAS-CosΔFu* compared with (D′) *cos2* clones alone. Increased Ci-155 (red) in (D″) *cos2* mutant clones was prevented by expression of (E″) *UAS-Cos2* or (F″) *UAS-CosΔFu*. (G-I′) Ci-155 (white) was not elevated in *cos2* mutant clones (arrows), marked by GFP (G-H′) or by the absence of GFP (I,I′), when *UAS-CosΔFu* was expressed in the clone in (G,G′) otherwise normal discs and in (H,H′) $Su(fu)^{LP}$ mutant discs, or (I,I′) when *UAS-CosΔFu* was expressed throughout $Su(fu)^{LP}$ mutant discs. Insets (right) show clone regions at higher magnification.

of *UAS-CosΔFu*, just as for wild-type *UAS-Cos2* (Fig. 2E,F). CosΔFu also supported Ci repressor production from both wild-type Ci and Ci-ΔΔ in posterior *smo cos2* mutant clones, yielding strong repression of the *hh-lacZ* reporter (supplementary material Fig. S2A,B,E,F). Thus, when expressed at much higher levels than normal, Cos2 appears to promote efficient Ci-155 processing, despite disruption of its Fu-binding domain.

We then tested whether Fu is recruited to Ci-155 by Su(fu) when CosΔFu replaces wild-type Cos2. First, we found that *hh-lacZ* was still repressed by Ci repressor derived from either wild-type Ci or Ci-ΔΔ in posterior *smo cos2* clones expressing *UAS-CosΔFu* in discs lacking Su(fu) (supplementary material Fig. S2). Second, we saw that Ci-155 levels were still not elevated relative to neighboring tissue in anterior *cos2* clones expressing *UAS-CosΔFu* in discs lacking Su(fu) (Fig. 2G-I). Hence, Su(fu) is not required to support efficient Ci-155 processing even when Cos2, when expressed in excess, lacks an intact Fu-binding site.

Expression of Cos2 variants at physiological levels to measure *in vivo* function

To test whether efficient Ci-155 processing depended on expressing CosΔFu at abnormally high levels, we sought to express Cos2 variants at physiological levels. A 6.5 kb fragment containing 0.8 kb upstream and 1.7 kb downstream of the *cos2* transcription unit has previously been shown to complement *cos2* function fully (Sisson et al., 1997; Ho et al., 2005). As Cos2 forms dimers (Zhou and Kalderon, 2011; Ranieri et al., 2012) and interacts with many other Hh signaling components, we expected that some variants of Cos2 might dominantly disrupt development, precluding isolation of the desired transgenic flies. We therefore inserted an *FRT*-flanked transcriptional terminator into the first *cos2* intron so that Cos2 expression would be conditional on excision of the *FRT*-flanked cassette. After excising the termination cassette in the female germline we found that none of our Cos2 variants had significant dominant effects. Hence, all experiments were performed with genomic *cos2* transgenes (referred to as *gCos2*) that had undergone germline excision of the transcriptional terminator and retained only an additional *FRT* site and short flanking sequences in the first intron.

The wild-type *gCos2* transgene fully rescued homozygous *cos2²* mutant animals to adulthood, producing fertile flies of normal morphology that could be propagated as a stable stock. A single copy of the genomic *CosΔFu* transgene failed to rescue *cos2* homozygotes to adulthood. Wing discs from the latter larvae showed very high levels of Ci-155 throughout the anterior compartment (Fig. 3A,B). Anterior *cos2* mutant clones in *cos2* heterozygotes carrying the genomic *CosΔFu* transgene also had very high Ci-155 levels, in contrast to controls with a wild-type *gCos2* transgene (Fig. 3H-J). The levels of Cos2 protein produced by wild-type *gCos2* and *gCosΔFu* were similar and slightly lower than the levels produced by two copies of the endogenous *cos2* gene (supplementary material Fig. S3D-G). Thus, CosΔFu expressed at physiological levels does not support efficient Ci-155 processing. However, physiological levels of CosΔFu did allow sufficient Ci processing in *smo cos2* clones to repress *hh-lacZ* in animals expressing CiΔΔ in wing discs (supplementary material Fig. S3A,B), mirroring the inefficient Ci-155 processing phenotype of *fu^M1*.

In addition to facilitating Ci-155 processing, Cos2 has the potential to limit the activity of Ci-155 as an activator of Hh target genes. Thus, the *ptc* reporter, *ptc-lacZ* is strongly induced in *cos2* mutant clones (Fig. 3L′) because Ci-75 repressor is eliminated, Ci-155 levels are increased and Ci-155 is partially activated (Sisson et al., 1997; Wang and Holmgren, 1999; Wang et al., 2000;

Smelkinson et al., 2007). In wing discs with the genomic *CosΔFu* transgene, we did not observe significant *ptc-lacZ* expression in anterior *cos2* mutant clones (Fig. 3N′), suggesting that CosΔFu still inhibits Ci-155 activity even though Ci-155 processing is strongly impaired.

We also constructed and tested a genomic *Cos2-S182N* transgene. The S182N alteration was designed to impair nucleotide binding (Ho et al., 2005) and was later found to prevent Cos2 association with the CORD domain of Ci, which is normally stimulated by nucleotides *in vitro* (Zhou and Kalderon, 2010). Cos2-S182N can promote Ci-155 processing to some degree, but much less efficiently than wild-type Cos2 when overexpressed (Ho et al., 2005; Zhou and Kalderon, 2010). At physiological Cos2-S182N levels, we found that Ci-155 staining was clearly elevated in *cos2* mutant clones (Fig. 3K′) and in the entire anterior compartment of *cos2* homozygotes (Fig. 3C). The genomic *Cos2-S182N* transgene did support strong *hh-lacZ* repression by CiΔΔ in posterior *smo cos2* mutant clones, confirming that it can promote some Ci processing (supplementary material Fig. S3C).

In animals with the *Cos2-S182N* transgene, ectopic *ptc-lacZ* was clearly induced in *cos2* mutant clones (Fig. 3O′), albeit to a level lower than in *cos2* mutant clones with no *cos2* transgene (Fig. 3L′). Thus, Cos2-S182N does not effectively restrict Ci-155 activity. Moreover, direct comparison with CosΔFu, which permits even greater Ci-155 accumulation but no ectopic *ptc-lacZ* induction (Fig. 3J′,N′), shows that the failure of Cos2-S182N to silence Ci-155 in the absence of Hh cannot simply be due to excessive Ci-155 levels or the absence of Ci-75 repressor.

One possible mechanism by which Cos2 restricts Ci-155 activity is by retaining Ci-155 in the cytoplasm. We therefore tested Ci-155 localization in *cos2* clones after incubating discs with the nuclear export inhibitor leptomycin B (LMB). Loss of *cos2* caused increased nuclear accumulation of Ci-155 (Fig. 3D; supplementary material Fig. S3H), as previously reported (Wang and Holmgren, 2000). A similar distribution of Ci-155 was seen in the presence of *gCos-S182N* but Ci-155 was still predominantly cytoplasmic in the presence of *gCosΔFu*, even though overall Ci-155 levels were at least as high (Fig. 3F,G; supplementary material Fig. S3J,K). These data provide the best physiological evidence to date that Cos2 binding to the CORD domain restricts Ci-155 nuclear access and activity.

Functions of Cos2 in transducing the Hh signal

In response to Hh, Cos2 has been proposed to facilitate Smo activation (Lum et al., 2003), to promote Fu activation (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011) and to alter its activity in response to phosphorylation by Fu (Ruel et al., 2007; Zhou and Kalderon, 2011; Ranieri et al., 2012). However, these hypotheses have not been tested rigorously because overexpression of Cos2, which has been the inevitable consequence of using *UAS-cos2* cDNA transgenes to date, blocks Hh signaling at the AP border of wing discs (Ho et al., 2005; Ruel et al., 2007; Zhou and Kalderon, 2011), while a similar dose effect has been demonstrated in tissue culture (Lum et al., 2003).

We therefore used genomic *Cos2* transgene variants to examine the roles of Cos2 in responding to Hh. In the presence of *gCosΔFu*, *cos2* mutant clones at the AP border showed greatly reduced *ptc-lacZ* staining compared with neighboring AP cells and complete loss of anterior Engrailed (En) expression (Fig. 3L″-N″). Thus, the response to Hh is drastically reduced when Cos2 cannot bind Fu normally. Ci-155 levels were very high and not distinguishably different in anterior and AP border clones expressing only CosΔFu
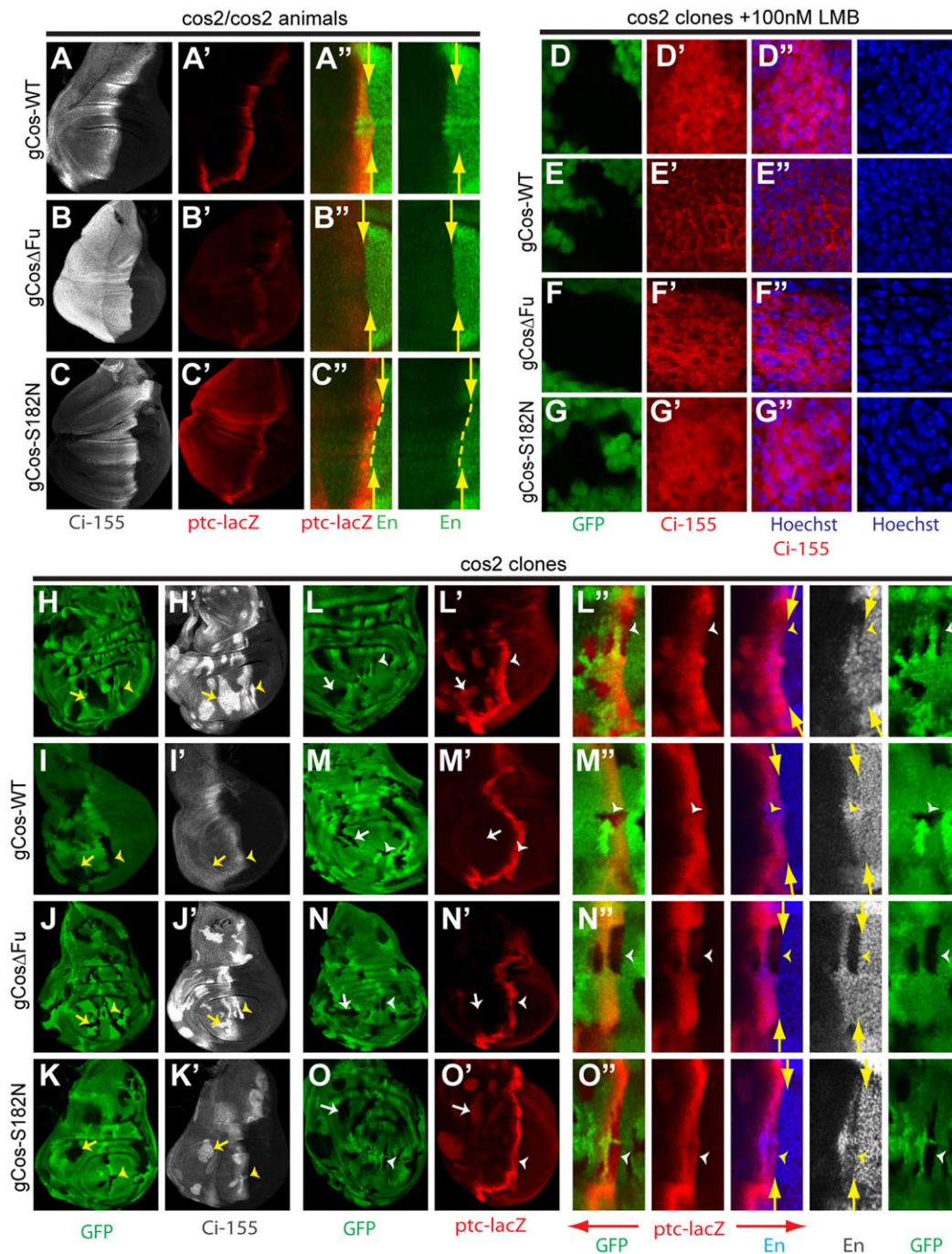
Fig. 3. Properties of Cos2 variants deficient for Fu or Ci-CORD binding when expressed at physiological levels. (A-C″) Wing discs homozygous for cos2² with one copy of genomic transgenes for (A) wild-type Cos2, (B) CosΔFu or (C) Cos2-S182N, showing (A-C) Ci-155 staining (white), (A′-C′) ptc-lacZ staining (red) and (A″-C″) En staining (green) alone (right) or together with ptc-lacZ (red, left) to reveal the exact position of the AP border (arrows and dashed lines) as the posterior (right) edge of ptc-lacZ staining. (D-G″) Wing discs with cos2 mutant clones marked by loss of GFP (green, D-G) and carrying the indicated transgenes, treated with 100 nM LMB for 2 h. Ci-155 (red, D′-G′) is largely absent from nuclei (blue Hoechst staining, D″-G″) in clones expressing Cos-WT (E′) or CosΔFu (F′) but not Cos2-S182N (G′) or no gCos2 transgene (D′). (H-O″) Wing discs with cos2 mutant clones, marked by loss of GFP (green) and (H,L) no Cos2 transgene or one copy of a genomic transgene for (I,M) wild-type Cos2, (J,N) CosΔFu or (K,O) Cos2-S182N, showing (H′-K′) Ci-155 staining (white) and (L′-O′) ptc-lacZ staining (red). (L″-O″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) ptc-lacZ (red) and GFP, ptc-lacZ alone, ptc-lacZ and En (blue), En alone (white), and GFP (green) alone. The AP border is marked with arrows (from ptc-lacZ staining) and GFP-negative clones are marked by arrowheads.

(Fig. 3J′). This finding is consistent with strong impairment of Ci-155 processing whether Hh is present or not, and the absence of Cul3-mediated Ci-155 proteolysis normally seen at the AP border when Hh signaling strongly activates Ci-155 (Jiang, 2006). Likewise, in cos2 homozygous animals with the genomic CosΔFu

transgene, Ci-155 levels were uniformly high in anterior and AP border cells, there was no anterior En staining and ptc-lacZ expression was confined to a weak AP border stripe, which was wider than normal, presumably because Hh travels further when less Ptc is expressed in AP border cells (Fig. 3A,B).
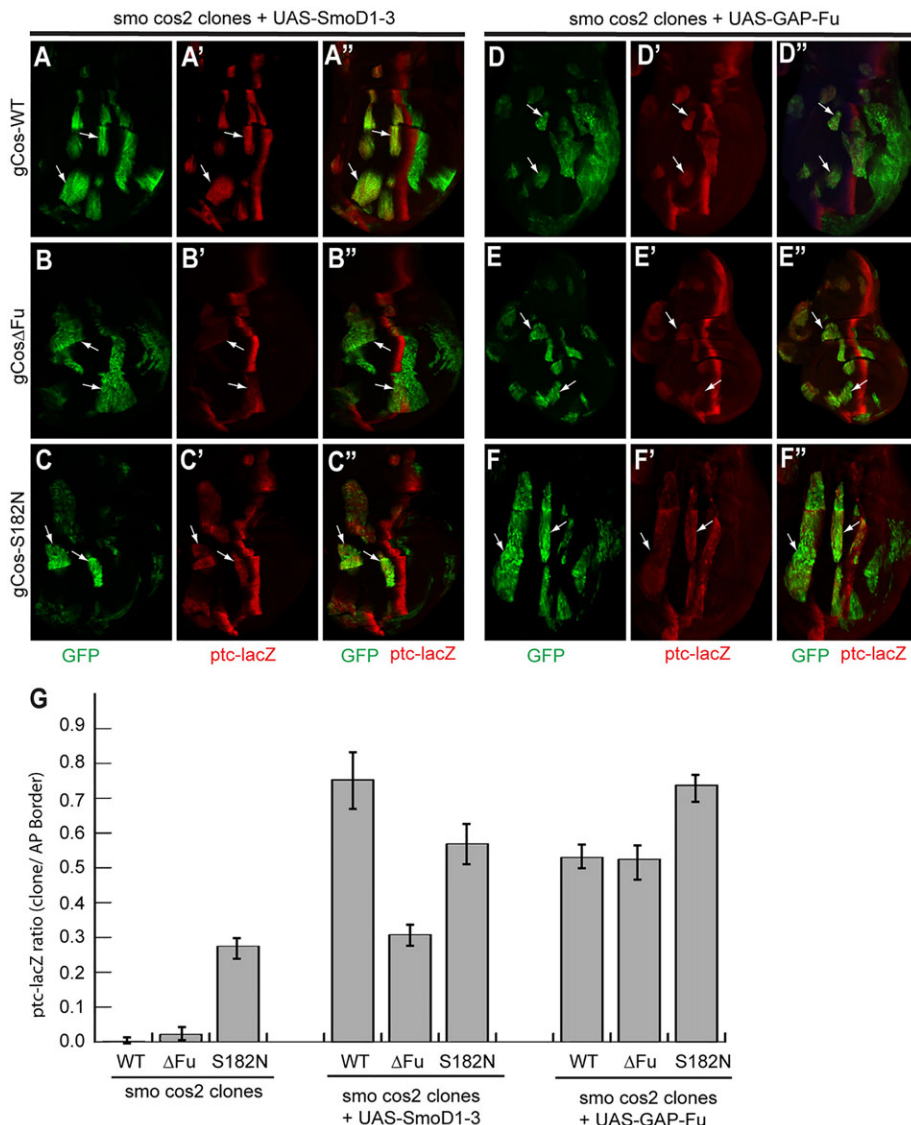
Fig. 4. Cos2 with impaired Fu binding fails to activate Fu in response to activated Smo. (A-F″) Ectopic *ptc-lacZ* (red) was induced in anterior GFP-positive (green) *smo cos2* clones by (A-C″) *UAS-SmoD1-3* or (D-F″) *UAS-GAP-Fu* driven by *C765-GAL4* in discs carrying genomic transgenes for (A-A″,D-D″) wild-type Cos2, (B-B″,E-E″) CosΔFu or (C-C″,F-F″) Cos2-S182N. Induction of *ptc-lacZ* by SmoD1-3 was much weaker for (B-B″) CosΔFu than for (A-A″) Cos2-WT or (C-C″) Cos2-S182N, whereas (D-F″) *ptc-lacZ* induction by GAP-Fu was similar for all three Cos2 transgenes. (G) Measurement of *ptc-lacZ* staining in response to SmoD1-3 and GAP-Fu in the presence of the indicated *gCos2* transgenes. Intensity of *ptc-lacZ* staining relative to the AP border calculated from five anterior clones of each genotype is displayed together with the s.e.m. (see Materials and methods).

In wing discs carrying the genomic *Cos2-S182N* transgene, *cos2* clones at the AP border expressed levels of *ptc-lacZ* close to those in normal AP border cells (Fig. 3O′,O″). These clones also expressed anterior En, although often non-uniformly or at lower levels than normal, indicating only very slightly impaired Hh signaling outcomes (Fig. 3O″). A similar, almost complete rescue of anterior En staining by Cos2-S182N was seen in *cos2* homozygous discs (Fig. 3C).

To examine the response of Cos2 variants to Hh in more detail, we examined Smo, responses to activated Smo and responses to activated Fu. When Smo is activated, it accumulates to higher levels, localizes predominantly at the plasma membrane and is more highly phosphorylated (Briscoe and Therond, 2013). Accumulation of activated Smo is readily observed in wild-type wing discs in posterior cells and in the most posterior AP border cells. We found a similar pattern of elevated Smo staining in *cos2* mutant discs carrying wild-type *gCos2* and *gCos-S182N* (supplementary material Fig. S4A,C). Smo staining was also elevated in posterior and AP border cells of *cos2* mutant discs carrying *gCosΔFu*, although elevated Smo staining differed from that of wild-type wing discs by spreading well beyond the range of *ptc-lacZ* induction at the AP border and at ectopic far anterior sites

(supplementary material Fig. S4B). Thus, both CosΔFu and Cos-S182N can support at least one aspect of Smo activation, Smo accumulation, in response to Hh.

We then expressed constitutively active Smo (SmoD1-3) in *smo cos2* mutant clones in wing discs carrying each of the three *gCos2* transgenes to test their ability to respond to activated Smo. We found high levels of *ptc-lacZ*, similar to those at the AP border in wild-type cells, in anterior clones expressing SmoD1-3 together with wild-type *gCos2*, slightly lower levels for *gCos2-S182N* and much lower *ptc-lacZ* in the presence of *gCosΔFu* (Fig. 4A-C,G). If the limited response of *ptc-lacZ* to both Hh and activated Smo supported by CosΔFu is only because Fu is not activated in these cells, it should be possible to induce strong *ptc-lacZ* expression in cells with CosΔFu by activating Fu synthetically. The *GAP-Fu* transgene encodes a membrane-tethered Fu fusion protein that induces Fu kinase activation even in the absence of Hh, Smo or Cos2 (Claret et al., 2007; Zhou and Kalderon, 2011). Expression of GAP-Fu in *cos2* clones containing the genomic *CosΔFu* transgene induced strong *ptc-lacZ* expression, just as for *cos2* mutant cells with a wild-type genomic *Cos2* transgene or *gCos2-S182N* (Fig. 4D-G), indicating that the defects of CosΔFu in responding to Hh can be attributed to a failure to activate Fu.

DEVELOPMENT
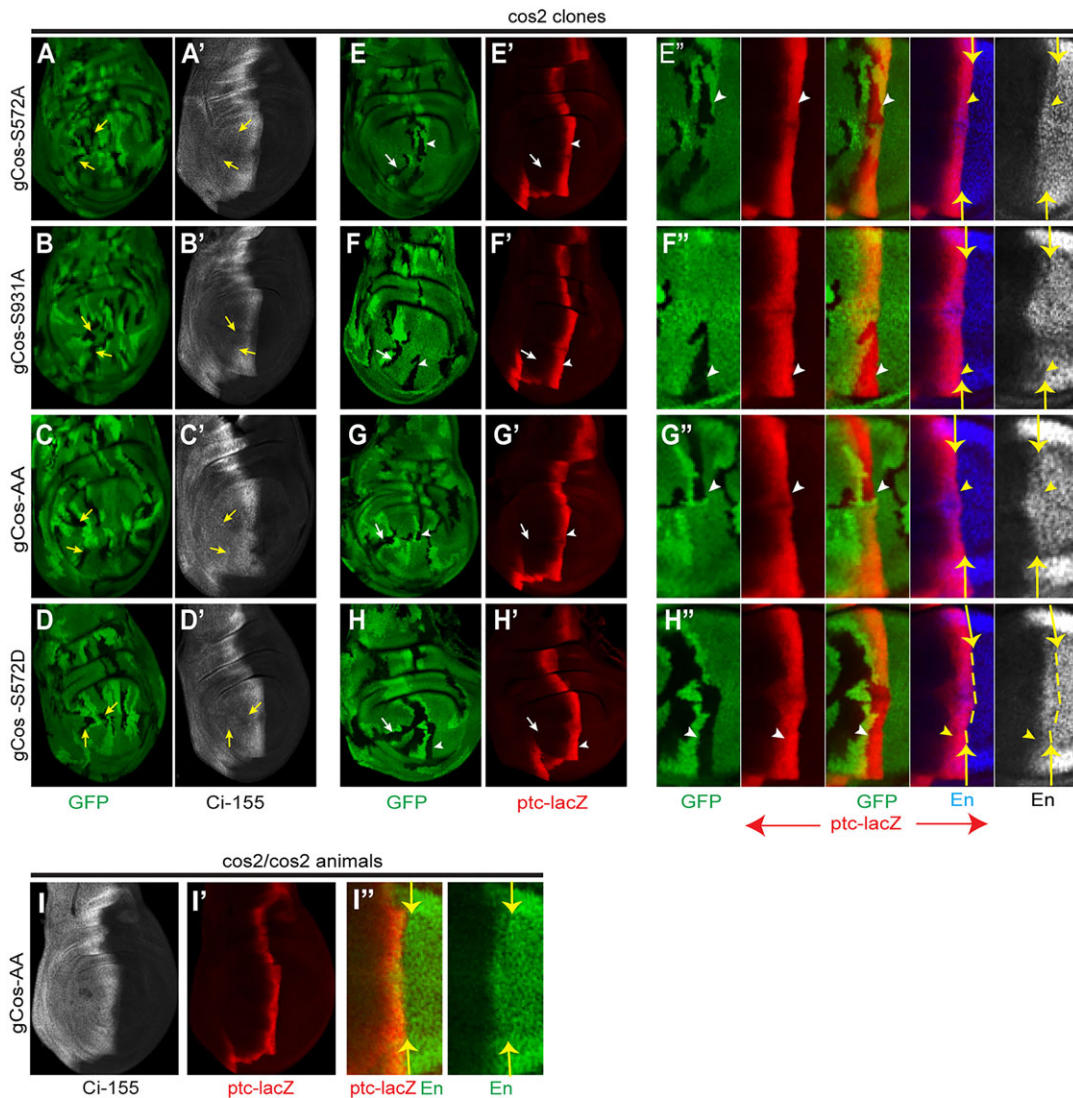
Fig. 5. Cos2 phosphorylation site variants support normal Hh signaling. (A-H) Wing discs with *cos2* mutant clones, marked by loss of GFP (green) and one copy of a genomic transgene for (A,E) Cos2-S572A, (B,F) Cos2-S931A, (C,G) Cos2-S572AS931A (Cos2-AA) or (D,H) Cos2-S572D, showing no changes in (A′-D′) Ci-155 (white) or (E′-H′) *ptc-lacZ* (red) staining in anterior (arrows) or AP border clones (arrowheads). (E″-H″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) GFP (green), *ptc-lacZ* (red), *ptc-lacZ* with GFP, *ptc-lacZ* with En (blue) and En alone (white). The AP border is marked with arrows and dashed lines (from *ptc-lacZ* staining) and GFP-negative clones are marked by arrowheads. (I-I″) Wing disc homozygous for *cos2²* with one copy of the g*Cos2-AA* transgene, showing (I) Ci-155 staining (white), (I′) *ptc-lacZ* staining (red) and (I″) En staining (green) alone (right) or (red) together with *ptc-lacZ* (left) to reveal the exact position of the AP border (arrows).

Physiological function of Cos2 sites phosphorylated by Fu

Two residues on Cos2 have been identified as very likely direct Fu kinase targets (Nybakken et al., 2002). Phospho-specific antibodies demonstrated Cos2 S572 phosphorylation in a wide domain at the AP border of wing discs and S931 phosphorylation in a narrower AP domain where Hh signaling is stronger, with both species detected throughout the posterior compartment (Ruel et al., 2007; Ranieri et al., 2012). Biochemically, it was inferred from co-precipitation measurements and by using Ala or acidic residue substituents that phosphorylation at S572 reduced Cos2 binding to Ci and to Smo (Liu et al., 2007; Ruel et al., 2007). Furthermore, Ci transcriptional activity assays in tissue culture showed that Cos2-S572A did not support a positive response to Fu kinase, whereas wild-type Cos2 did, leading the authors to conclude that S572 phosphorylation was essential for normal activation of Ci-155 by Fu (Ruel et al., 2007). However, these

assays used non-physiological levels of Cos2, Fu and Smo. It has also been suggested, based on the properties of overexpressed Cos2 S572 variants in wing discs that phosphorylation of S572 inhibits Ci-155 processing (Ruel et al., 2007; Zhou and Kalderon, 2011).

To test these conclusions in a physiological setting, we used genomic transgenes encoding Cos2-S572A and Cos2-S572D. Surprisingly, we found that both Cos2-S572A and S572D fully suppressed Ci-155 elevation in *cos2* mutant clones, indicating normal Ci-155 processing (Fig. 5A,D). Even more surprising, *cos2* mutant clones at the AP border of discs with genomic *Cos2-S572A* or *S572D* transgenes showed normal strong *ptc-lacZ* expression and anterior En expression (Fig. 5E,H). In fact, *cos2* homozygous animals were fully rescued by each transgene to produce fertile adults of normal morphology. Thus, Cos2 S572 phosphorylation is clearly not essential for Hh to activate target genes appropriately.

smo cos2 clones + UAS-GAP-Fu

GFP   ptc-lacZ   GFP   Ci-155
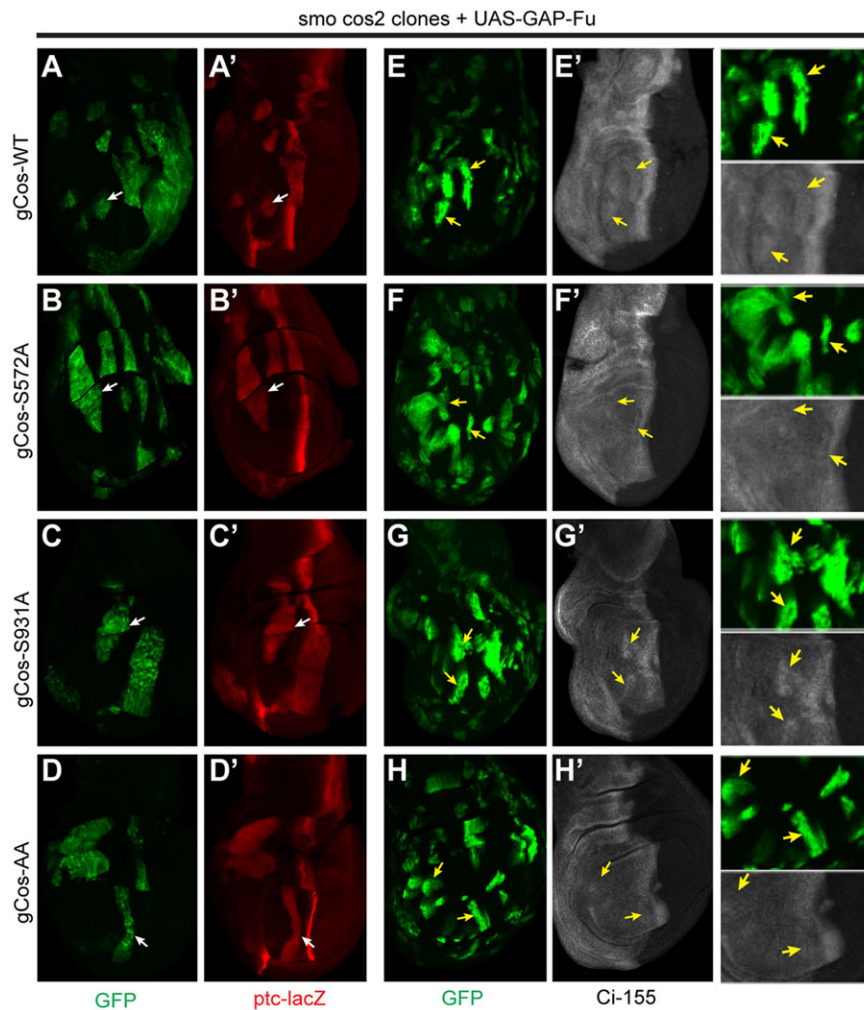
Fig. 6. Cos2 phosphorylation sites are not required to respond to Fu. (A-H′) Anterior *smo cos2* clones (arrows) expressing *UAS-GAP-Fu*, marked by GFP (green), in discs carrying genomic transgenes for (A,E) wild-type Cos2, (B,F) Cos2-S572A, (C,G) Cos2-S931A or (D,H) Cos2-AA (S572A S931A) induced (A′-D′) ectopic *ptc-lacZ* (red) to a similar degree. (E′-G′) Ci-155 staining (white) was mildly elevated in most clones of discs with (E′) wild-type *gCos2* and (G′) *gCos2-S931A*, but not with (F′) *gCos-S572A* or (H′) *gCos-AA*. Insets (right) show clone regions at higher magnification.

To test whether Cos2 S572 was important for responses specifically to Fu kinase, we created *smo cos2* mutant clones that expressed GAP-Fu in animals carrying the different genomic *Cos2* transgenes. The *smo* mutation was included to ensure that we measure only downstream responses to Fu activation, as it has been found that active Fu can also contribute to Smo activation (Claret et al., 2007; Liu et al., 2007). Equally strong induction of *ptc-lacZ* was seen for *gCos2-WT* and *gCos2-S572A* transgenes, showing that Ci-155 activation by Fu does not require S572 phosphorylation (Fig. 6A,B). Ci-155 levels were slightly elevated by activated Fu in *cos2* mutant clones with the wild-type *gCos2* transgene but no clear elevation was seen for *gCos2-S572A* (Fig. 6E,F). These results are consistent with S572 phosphorylation being important for Fu kinase to increase Ci-155 levels (Zhou and Kalderon, 2011). Unfortunately the sensitivity of this assay is limited because activated Ci-155 is labile (Ohlmeyer and Kalderon, 1998; Jiang, 2006).

Prior studies of Cos2-S931A and S931D variants in combination with S572A and S572D variants suggested that S931 phosphorylation enhanced Cos2-Smo binding and that it also increased Ci-155 activation, associated with reduced Ci-155 binding and increased Ci-155 nuclear entry (Ranieri et al., 2012). The relevant assays were, however, conducted with non-physiological levels of Cos2, Fu and Smo proteins, principally in tissue culture cells. We found that genomic *Cos2-S931A* and *S931D* transgenes fully rescued *cos2* homozygous mutant animals, producing normal patterns of Hh target gene expression in wing discs (data not shown). Full rescue was also

seen for Cos2-S931A when examining *cos2* mutant clones (Fig. 5B,F) and in response to activated Fu in *smo cos2* mutant clones (Fig. 6C,G), as described previously for Cos2-S572A. To determine whether S572 and S931 phosphorylation might act redundantly, we also tested Cos2-AA (S572A S931A). This genomic transgene was constructed directly without a transcriptional terminator cassette, along with an analogous wild-type *cos2* transgene. Both transgenes rescued *cos2* mutant clones (Fig. 5C,G) and *cos2* homozygous mutant animals fully (Fig. 5I), producing normal adult wing morphologies and normal patterns of Hh target gene expression in wing discs. Furthermore, Cos2-AA supported strong *ptc-lacZ* induction by activated GAP-Fu in *smo cos2* mutant clones with no significant increase in Ci-155 levels, just like Cos2-S572A (Fig. 6D,H). We also tested the response to a different activated Fu kinase transgene, *Fu-EE*, with similar results. Fu-EE, like GAP-Fu, produced a small increase in Ci-155 levels in the presence of the wild-type Cos2 transgene but no clear increase in the presence of Cos2-AA (supplementary material Fig. S5A,B), providing further evidence that Cos2 phosphorylation is required for Fu kinase to increase Ci-155 levels.

Su(fu) and Cos2 do not appear to be redundant targets for responses to Fu kinase activity

As Su(fu), as well as Cos2, becomes hyper-phosphorylated during Hh signaling in a Fu-dependent manner (Lum et al., 2003; Ho et al., 2005), it was thought that Su(fu) phosphorylation probably contributes to Hh signal transduction, specifically in response to

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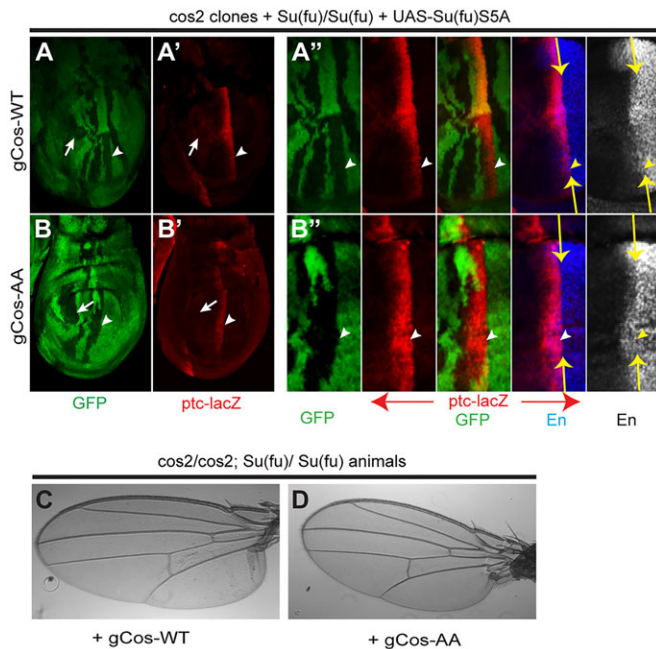
Fig. 7. Cos2 phosphorylation sites are not required to respond to Hh even in the absence of normal Su(fu) phosphorylation. (A,B) *Su(fu)* mutant wing discs with *UAS-Su(fu)-5A* (which lacks known Fu phosphorylation sites) expressed ubiquitously using *C765-GAL4*, and one copy of a genomic transgene for (A) wild-type Cos2 or (B) Cos2-AA. In *cos2* mutant clones, marked by loss of GFP (green) (A′,B′), *ptc-lacZ* staining (red) was unchanged. (A″,B″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) GFP (green), *ptc-lacZ* (red), *ptc-lacZ* with GFP, *ptc-lacZ* with En (blue) and En alone (white). The AP border is marked with arrows (from *ptc-lacZ* staining) and GFP-negative clones are marked by arrowheads. (C,D) Wings from *cos2²/cos2²; Su(fu)^LP/Su(fu)^LP* animals carrying two copies of the (C) gCos-WT or (D) gCos-AA transgene on the second chromosome.

activated Fu. However, after identifying the major Fu-dependent phosphorylation sites on Su(fu), it was found that altering those sites had no effect on Hh signaling or responses to Fu (Zhou and Kalderon, 2011). We therefore removed the known Fu phosphorylation sites on Su(fu) and on Cos2 simultaneously in case these two targets of Fu act redundantly. We found that *cos2* clones in the anterior and at the AP border were still fully rescued by the genomic *Cos2-AA* transgene in wing discs homozygous for the null *Su(fu)^LP* mutation but expressing a Su(fu) variant (Su-5A) with five serine residues (constituting Fu phosphorylation sites and neighboring residues) altered to alanine (Zhou and Kalderon, 2011) (Fig. 7A,B). Furthermore, animals containing Cos2-AA and Su-5A as the only functional versions of Cos2 and Su(fu) developed into adults of normal morphology. Similar results were seen in Su(fu)-null animals expressing only Cos2-AA (Fig. 7C,D). Thus, even though Fu kinase activity is essential for normal responses to Hh, neither the known Fu phosphorylation sites on Cos2 nor those on Su(fu) are required to respond to Hh.

DISCUSSION

We have investigated the role of Cos2 in Hh signaling for the first time using variants expressed at normal levels in *Drosophila* tissues. This approach is crucial because Cos2 collaborates with several direct binding partners and its actions have been found to be extremely dose sensitive. Our results provide definitive evidence for prior proposals that Cos2 association with Fu is essential for Ci-155 processing (in the absence of Hh) and for Fu kinase activation (in response to Hh), and suggest that Cos2 binding to the CORD

domain of Ci-155 promotes both Ci-155 processing and Ci-155 silencing. Our results also contradict prior assertions that phosphorylation of specific Cos2 sites (S572 and S931) by Fu is essential for Ci-155 activation in response to Hh.

Functions of Cos2, Fu and Su(fu) in Ci-155 processing and silencing in the absence of Hh

Cos2 is generally regarded as the major mediator of Ci-155 processing (Zhang et al., 2005), whereas the role of Fu is less clear. Here, we find that three class II *fu* alleles (encoding truncated proteins) strongly impair Ci-155 processing in a cell-autonomous manner, whereas even *fu^M1*, which encodes only 80 amino acids of the N-terminal kinase domain, still permits a low level of Ci-155 processing. All of these *fu* alleles encode proteins lacking a C-terminal Cos2-binding domain (Robbins et al., 1997; Monnier et al., 2002), suggesting that a low level of Ci-155 processing can occur in the absence of Fu but efficient processing requires Fu to bind to Cos2. Our evidence specifically argues against previous assertions that Fu is absolutely required for Ci-155 processing (Methot and Basler, 2000) and that some Fu proteins that cannot bind Cos2 are nevertheless able to promote efficient Ci-155 processing in the absence of Hh (Wang and Holmgren, 1999). The requirement for Fu in Ci-155 processing may be explained by the recent finding that Fu, rather than Cos2, is principally responsible for recruiting PKA to Ci-155 (Ranieri et al., 2014).

Consistent with our deductions from *fu* alleles, we found that a Cos2 variant with strongly impaired Fu binding (CosΔFu) supported low-level Ci-155 processing (detected by a sensitive *hh-lacZ* repression assay) but not efficient Ci-155 processing (measured by Ci-155 staining) when CosΔFu was expressed at physiological levels. Surprisingly, Ci-155 processing appeared normal when CosΔFu was strongly overexpressed. Given the failure of excess Cos2 to rescue the processing defects of *fu^M1*, we suggest that some Fu protein is recruited by CosΔFu to Ci-155 processing complexes when CosΔFu is overexpressed, likely due to some residual direct binding between Fu and CosΔFu. Ci-155 processing by excess CosΔFu was not detectably impaired by eliminating Su(fu), suggesting that Su(fu) does not contribute to Fu recruitment even under conditions of greatly reduced Cos2-Fu binding, despite the potential for Su(fu) to bind to both Fu and Ci-155. It appears therefore that Su(fu) has no detectable role in Ci-155 processing, in contrast to evidence of an important role in Gli2/3 processing in mammals (Kise et al., 2009; Humke et al., 2010).

Three domains on Ci-155 that bind Cos2 have been identified biochemically but Ci-155 processing was disrupted in wing discs only when two of these (the zinc fingers and the CORD domain) were deleted simultaneously, with no discernible negative consequence of deleting the third (CDN) region (Wang et al., 2000; Wang and Jiang, 2004; Zhou and Kalderon, 2010). Surprisingly, deletion of the CORD domain, especially in combination with the CDN region, appears to enhance Ci-155 processing (Zhou and Kalderon, 2010). It was therefore suggested that Cos2 binding to CORD might compete with alternative CORD binding interactions, which might otherwise reduce processing, perhaps by masking PKA, CK1 or GSK3 phosphorylation sites. A key test of that idea involved Cos2-S182N, which cannot bind the CORD region but still associates normally with other regions of Ci-155 (Zhou and Kalderon, 2010). Here, we found that Cos2-S182N expressed at physiological levels supported only low levels of Ci-155 processing. Moreover, excess Ci-155 spared from processing induced low levels of *ptc-lacZ*, whereas a similar processing defect due to CosΔFu resulted in no ectopic *ptc-lacZ*. CosΔFu also retained Ci-155

in the cytoplasm more effectively than Cos2-S182N in wing discs treated with the nuclear export inhibitor LMB. Hence, it appears that Cos2 binding to CORD normally promotes Ci-155 processing and also limits the nuclear access and transcriptional activity of Ci-155 when Ci processing is impaired. This suggests that Cos2-CORD interaction may be regulated during Hh signaling in order for Ci-155 to be fully activated.

Functions of Cos2-Fu association

Recently, the mechanism of Fu kinase activation has come into focus. Fu kinase activation depends on phosphorylation of putative Fu and CK1 sites in the activation loop of Fu (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). It is promoted by association of Fu molecules and requires the region of Fu that binds to Cos2, suggesting that activated Smo clusters Cos2-bound Fu molecules to stimulate their cross-phosphorylation and consequent full activation. Here, we provide further evidence for that model by showing that Cos2 deficient for binding to Fu (CosΔFu) fails to support strong Ci-155 activation in response to Hh or activated Smo.

We found that Cos2 must also bind to Fu to stabilize Fu. What might be the purpose of Fu stabilization by Cos2? One possible purpose is to prevent spurious Fu activation. Fu is activated by aggregation and a positive-feedback loop of cross-phosphorylation. By ensuring that excess Fu is unstable and almost all Fu molecules are associated with Cos2, Fu activation can be limited to circumstances where Cos2 molecules are aggregated. Conversely, stabilization of Fu by Cos2 allows the relatively low abundance Cos2 protein to compete better with other potential Fu partners for Fu binding, thereby priming Fu for Cos2-dependent activation. A second potential purpose of Fu stabilization by Cos2 concerns Ci-155 processing. If Cos2 and Fu each recruit kinases to Ci-155 and an effective processing complex must contain multiple components (Ci-155, Cos2, Fu, PKA, CK1 and GSK3) stabilization of Fu by binding to Cos2 would ensure that productive associations of Fu with PKA, CK1 or GSK3 are not distributed throughout the cell but concentrated in Ci-Cos2-Fu complexes. It also seems likely, though currently untested, that the presence of Fu enhances Cos2 association with Ci-155. Otherwise it would be hard to understand why excess Cos2 does not impair Ci-155 processing by forming a high proportion of Ci-155 complexes lacking Fu, or how excess CosΔFu can process Ci-155 efficiently despite extremely low levels of Fu.

Mechanism of Ci-155 activation by Fu kinase

As both Su(fu) and Cos2 bind directly to Ci-155 and both have been shown to limit nuclear accumulation and activity of Ci-155 under some conditions, it has been suggested that Fu kinase might overcome these inhibitory actions by phosphorylating one or more of these components directly. Su(fu) is phosphorylated in response to Hh and activated Fu (Lum et al., 2003; Ho et al., 2005), but alteration of the identified phosphorylation sites had no measurable effect on either the silencing activity of Su(fu) or on the ability of Hh or Fu to oppose that silencing (Zhou and Kalderon, 2011). By contrast, the two identified sites of Hh-stimulated Fu-dependent phosphorylation on Cos2 appeared to play a significant role (Ruel et al., 2007; Ranieri et al., 2012). It was variously argued that Cos2 S572 phosphorylation contributed to Smo activation, promoted Cos2 degradation, reduced association with Ci-155 and blocked Ci-155 processing (Liu et al., 2007; Ruel et al., 2007; Zhou and Kalderon, 2011). Here, we found that an S572A substitution in Cos2 did not impair Ci-155 processing or silencing in the absence of Hh

or any aspect of the response to Hh, allowing the development of morphologically normal and fertile adults.

We did find some support for the assertion that Ci-155 stabilization by activated Fu depends on S572 phosphorylation (Zhou and Kalderon, 2011), seemingly consistent with the finding that Cos2-S572D associates poorly with Ci-155 in co-precipitation experiments (Ruel et al., 2007). However, here we found that physiological levels of Cos2-S572D supported efficient Ci-155 processing. In addition, the impaired Ci association of Cos2-S572D seen by co-precipitation of tissue culture cell extracts (Ruel et al., 2007) was not observed using in vitro binding experiments that employed isolated Cos2-binding domains of Ci (Zhou and Kalderon, 2010). Hence, it remains unclear whether the S572D substitution is a good mimic of phosphorylation or exactly how S572 phosphorylation might affect Cos2 binding to Ci-155 or to other partners in order to impair Ci-155 processing.

Phosphorylation of Cos2 on S931 was suggested to be important for high-level Hh signaling (Ranieri et al., 2012). This idea was supported by transcriptional reporter assays in tissue culture cells that used transfected Cos2 variants together with carefully titrated doses of Smo and Fu. Increased transcriptional response for phosphomimetic alterations and impaired responses for Ala substituents in these experiments were ascribed principally to alterations of S931, although the most clear-cut differences were seen only when both S572 and S931 were altered in analogous fashion (Ranieri et al., 2012). We found that both Cos2-S931A and Cos2-AA (S572A S931A) behaved exactly like wild-type Cos2 in silencing Ci-155 in the absence of Hh, supporting a normal Hh response at the AP border of wing discs and allowing the development of morphologically normal fertile adult flies. The only minor deficit noted was a failure of Cos2-AA to support a small increase in Ci-155 levels in response to activated Fu, exactly as seen for Cos2-S572A.

There is some evidence that the response to Hh at the AP border of wing discs may include some redundancy. For example, it appears that Fu kinase activity can inhibit Ci-155 processing (via Cos2 S572 phosphorylation) but Fu kinase is not necessary for Hh to inhibit Ci-155 processing fully (Zhou and Kalderon, 2011). We therefore tested whether altering Fu phosphorylation sites on both Su(fu) and Cos2 might reveal some redundant actions. However, we saw no abnormalities in Hh responses in cos2 mutant clones at the AP border of wing discs expressing Cos2-AA and Su(fu) with Ala substituents at phosphorylation sites in place of endogenous Su(fu). Conceivably, a third set of Fu-dependent changes may need to be blocked in order to reveal a contribution of the known Fu phosphorylation sites on Cos2 or Su(fu). For the present, it is clear that key targets of Fu kinase remain to be identified.

MATERIALS AND METHODS
Mutagenesis and cloning
A 6.5 kb fragment of genomic Cos2, extending between two KpnI sites in the plasmid vector pCaSpeR4 had been used to rescue Cos2 function in flies (Sisson et al., 1997). We introduced modifications in order to incorporate this fragment into an ATT vector, add an excisable transcription termination cassette in the first intron, and modify Cos2-coding sequence, all by using oligonucleotide-mediated mutagenesis (QuikChange, Stratagene), as detailed in the methods in the supplementary material.

Cell culture, transfection, immunoprecipitation and western blot analysis
Kc cells were kept at 25°C in Schneider's Drosophila media +5% FBS +1% penicillin-streptomycin (Gibco). Three 10 cm plates were seeded with 1×10^7 cells and were given fresh media after 24 h. The cells were transfected

3-4 h later with *Actin-HA-Fu* together with *Actin-Flag-Cos2*, *Actin-HA-Fu* or *Actin-Flag- CosΔFu* (8 μg each) using a calcium phosphate protocol (Invitrogen). Cells were given fresh medium 24 h later and were harvested after 48 more hours. The cells were lysed at 4°C in 1 ml lysis buffer [50 mM Hepes (pH 7.5), 1.25 mM EDTA, 50 mM NaF, 0.5 mM Na_2VO_3 0.5% NP-40, 1 mM DTT, protease inhibitors (mini complete, Roche)]. The lysates were incubated with mouse anti-Flag antibody conjugated to agarose beads (Sigma) for 2 h at 4°C. The immunoprecipitates were washed three times for 10 min in lysis buffer. The western blots were probed with mouse anti-Flag and rabbit anti-HA antibody (ab9110, Abcam). Secondary antibodies Alexa Fluor-680 and Alexa Fluor-800 were visualized with LI-COR Infrared imager, and the bands were quantified using LI-COR Odyssey Software.

Immunohistochemistry

Wing disc clones were generated by heat-shocking late first or early second instar larvae for 1-2 h at 37°C. Late 3rd instar larvae were dissected into 4% paraformaldehyde on ice and rocked at 25°C for 30 min. For measurements of Ci-155 nuclear access, discs were dissected in cold PBS and treated with 100 nM LMB (or no LMB as a control) in Schneider's Drosophila cell media for 2 h prior to fixation. Fixed samples were rinsed with 1× PBST (1× PBS +0.1% Triton X-100+0.05% Tween 20) and placed in the blocking buffer [5% goat serum, 0.5% BSA, 50 mM Tris (pH 7.0), 150 mM NaCl, 0.5% BSA] for 2 h at 4°C. The discs were stained with rabbit (MP Biomedicals) and mouse (Promega) anti-β-galactosidase antibody for *lacZ* products, mouse monoclonal 4A6 antibody to Myc (Millipore), rat monoclonal 2A1 antibody to Ci, mouse monoclonal antibodies 17E11 to Cos2 and 4D9 to En (Developmental Studies Hybridoma Bank, University of Iowa, USA), mouse monoclonal antibody 5D6 to Cos2, and rabbit antibody to Fu (Ascano et al., 2002). Secondary antibodies were Alexa Fluor-488, -546, -594, -647 or -680 (Molecular Probes). A Zeiss LSM700 confocal microscope was used to examine the fluorescent staining.

Measurements from fluorescent images

In Fig. 1, the areas within a rectangle were quantified and intensity plots for Ci-155 staining were constructed using Image J software (NIH, Bethesda, MD). The *y*-axis shows the average fluorescence intensity over the height of the rectangle at each point on the *x*-axis.

In Fig. 4, the average fluorescent intensity (I) of a fixed area within GFP-expressing clones and the normal AP border (no clones) was measured using Image J software. Five separate clones (from at least three wing discs) were measured for each genotype, together with five samples at the AP border and of anterior territory with no clone ('background'). The signal due to each clone was calculated relative to the signal due to Hh at the AP border as $(I_{clone}-I_{background})/(I_{AP\ Border}-I_{Background})$ before deriving the mean signal and standard error for each clone genotype.

Fly crosses

Transgenic flies and genotypes for all experiments are described in the methods in the supplementary material.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.112904/-/DC1

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