Supplemental Methods

Mutagenesis and Cloning

The following modifications were made to a 6.5-kb fragment of genomic Cos2, extending between two KpnI sites in the plasmid vector pCaSpeR4. First, an SphI restriction site was introduced in place of the KpnI site upstream of the Cos2 transcription unit. Next, an AvrII site was introduced near the beginning of intron 1 sequence. Mutations of interest [ΔFu (removes codons for amino acids 540-560 (Ruel et al., 2007)), S182N (Ho et al., 2005), S572A, S572D, S931A, S572A S931A (Ranieri et al., 2012)] were then introduced into the modified Cos2 fragment using the following oligonucleotides:

KpnI to SphI AGA CTC GAG GAA TTC *GCA TG*C GTT GTG AGT ACG GCG

AvrII in intron1 CCA GGT GAG TTT ACT *CCT AGG* ATC TTA TAA GCA TAG

ΔFu GAT CTG GAC GAC AAG ATA [deletion] TAT CTA TCC AAG

S182N CGC GGC CAA GGC AAA *AAC* TAC ACA CTC TAC

S572A AAG GCG GTT ATG CAA *GCC* CAA GAC CGC GAG ATT

S572D AAG GCG GTT ATG CAA *GAC* CAA GAC CGC GAG ATT

S931A ATC ACG GGC CAC CGC *GCC* ATC GAC ACG AGC GAC

S931D ATC ACG GGC CAC CGC *GAC* ATC GAC ACG AGC GAC

The restriction sites or amino acid modifications are in italics, and the underlined bases represent changes from the original sequence of the Cos2 fragment. An *FRT*-flanked transcription terminator cassette FC22 contained on a 3.5-kb NheI fragment (Struhl and Basler, 1993) was cloned into the AvrII site of each construct. The modified genomic Cos2 fragments were then sub-cloned into the pUASattB vector (Bischof et al., 2007) between SphI and KpnI restriction sites and inserted at *att* sites at cytological locations 86F and 53B2 (Rainbow Transgenic Services, Inc.).

Gateway technology was used to make a *UAS-CosΔFu* construct for germline transformation and expression vectors for tissue culture transfection (Invitrogen). The ΔFu mutation was introduced using site-directed mutagenesis into the coding sequence of Cos2 that had previously been cloned

into a pENTR/ D-TOPO vector (Marks and Kalderon, 2011) using the oligonucleotide shown above. The resulting entry vector was transferred to the pTW destination vector from the Drosophila Gateway Vector collection by LR recombination (Invitrogen) to produce pUASt-CosΔFu, which has N-terminal triple Flag tags and was introduced into the Drosophila germline by P-element transformation. Drosophila Gateway vectors pAHW, pAFW, pAMW and pAGW were used to make expression vectors for tissue culture transfection from the Entry clones containing sequences for Cos2, CosΔFu and Fu. The Cos2 proteins were tagged with Flag and Fu was tagged with HA at N-terminal ends.

Germline Excision of Transcriptional Terminator and Genotyping

Animals of genotype *yw hs-flp; smo^{2} FRT42D cos2^{2} / P[y^{+}]CyO; (g>term>Cos2-WT, S572A, S572D, S931A, S572AS931A, S182N* and ΔFu) / TM6B were given a heat shock (37C, 1 h) during the late third instar stage. Adult female progeny were collected 7 days later, and crossed to males with second and third chromosome balancers. Individual male progeny were collected to establish stocks, which were tested for an FRT excision event using two sets of primers [1) 231 and 864_r and 2) 231 and dsRed_bk (231: ACA ATT CCA GAT GCC ATT GG, 864_r: CTG CAA CAG CCC CTC CTT GG, dsRed_bk: TGG AAC TGG GGG GAC AGG ATG). Once stocks were established with excised transcriptional terminators and transferred to stocks of the form *yw hs-flp; smo^{2} FRT42D cos2^{2} / P[y^{+}] CyO; gCos-X / TM6B* the identity of each transgene was verified by PCR and sequencing.

Fly Crosses

Females of the genotype *fu^{M1} / FM7* or *fu^{RX2} / FM7* or *fu^{W3} / FM7* were crossed to *yw hs-flp; C765-GAL4 ptc-lacZ / TM6B* males to generate *fu^{M1} or fu^{RX2} or fu^{W3} / Y; C765-GAL4 ptc-lacZ /* + wing discs.

Females of the genotype *w fu^{M1}; P[Fu^{+}] FRT40A Sp / CyO; C765 hh-lacZ / TM6B* were crossed to *yw; Sp / CyO; (UAS-Ci or UAS-CiΔCDNΔCORD) / TM6B* males to generate *w fu^{M1} / Y; (Sp or P[Fu^{+}] FRT40A Sp) / CyO* discs that express *Ci* or *CiΔCDNΔCORD* ubiquitously.

Females of the genotype *w fu^{M1}; P[Fu^{+}] FRT40A Sp / CyO; C765 hh-lacZ / TM6B* were crossed to *yw;*

ubi-GFP Flp38 FRT40A / CyO; (TM2 or UAS-Cos2) / TM6B males to generate *fu* mutant clones marked by two copies of *ubi-GFP* in male wing discs with or without expression of *UAS-Cos2*.

Females of the genotype *w fu^{M1}; P[Fu^{+}] FRT40A Sp / CyO; (FRT82B Su(fu)^{LP}) C765 hh-lacZ / TM6B* were crossed to *yw; smo^{2} Flp38 FRT40A / CyO; UAS-Ci (Su(fu)^{LP}) / TM6B* males to generate *fu smo* mutant clones, detected by the absence of Fu staining, in male wing discs with or without Su(fu).

Females of the genotype *yw hs-flp, UAS-GFP; smo^{2} FRT42D tub-Gal80 hsCD2 P[Smo^{+}] / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; FRT42D cos2^{2} / CyO; (UAS-Cos2 or UAS-CosΔFu) / TM6B* males to generate positively marked *cos2* mutant clones expressing Cos2 or CosΔFu.

Females of the genotype *yw hs-flp, UAS-GFP; smo^{2} FRT42D tub-Gal80 hsCD2 P[Smo^{+}] / CyO; (FRT82B Su(fu)^{LP}) C765 hh-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2} / CyO; (UAS-Cos2 or UAS-CosΔFu) (FRT82B Su(fu)^{LP})(UAS-Ci or UAS- CiΔCDNΔCORD)/ TM6B* males to generate positively marked *smo cos2* mutant clones expressing *UAS-Cos2* or *UAS-CosΔFu* and *UAS-Ci* or *UAS-CiΔCDNΔCORD* with or without Su(fu) to examine Ci processing to a repressor of *hh-lacZ.*

Females of the genotype *yw; FRT 42D cos2^{2}/ P[y^{+}] CyO; ptc-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2}/ P[y^{+}] CyO; gCos2* (*WT, S572A, S572D, S931A, S572AS931A, ΔFu, S182N*) to examine rescue of *cos2* mutant wing discs and whole flies. Females of the genotype *yw hs-flp; FRT42D P[y^{+}] ubi-GFP / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2} (UAS-GAP-Fu) / CyO; TM2 or gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N) / TM6B* males to generate negatively marked *cos2* mutant clones in a wing disc expressing *gCos2 (WT, S572A, S572D, S931A, S572AS931A, ΔFu, S182N)* ubiquitously.

Females of the genotype *yw hs-flp, UAS-GFP; smo^{2} FRT42D tub-Gal80 hsCD2 P[Smo^{+}] / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2} (UAS-GAP-Fu or UAS-SmoD1-3) / CyO; TM2* or *gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N) / TM6B* males to generate positively marked *smo cos2* mutant clones expressing *gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N)* ubiquitously with or without *UAS-GAP-Fu* or *UAS-SmoD1-3*.

Females of the genotype *yw hs-flp, UAS-GFP; smo^{2} FRT42D tub-Gal80 hsCD2 P[Smo^{+}] / P[y^{+}] CyO; C765 hh-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2} UAS-CiΔΔ/ CyO; TM2* or *gCos2 (WT, ΔFu, S182N) / TM6B* males to generate positively marked *smo cos2* mutant clones expressing *gCos2 (WT, ΔFu, S182N)* ubiquitously with *UAS-CiΔΔ* to examine Ci processing to a repressor of *hh-lacZ.*

Females of the genotype *yw flp; FRT42D P[y^{+}] ubi-GFP / CyO; FRT82B Su(fu)^{LP} C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; smo^{2} FRT42D cos2^{2} gCos2 (WT or AA) / P [y^{+}] CyO; Su(fu)^{LP} UAS-Su(fu)-5A / TM6B* males to generate negatively marked cos2 mutant clones in a wing disc expressing *gCos2 (WT or AA)* ubiquitously in discs expressing Su(fu)-5A in place of endogenous Su(fu).

Supplementary Figure Legends

Figure S1. Hh-independent cell-autonomous elevation of Ci-155 in fu mutant clones.

(A, B) Ci-155 (red) was strongly elevated in (A) fu^{RX2} and (B) fu^{W3} anterior clones. (C) Ci-155 (red) was similarly increased in anterior fu^{M1} smo^{2} mutant clones, marked by loss of Fu (green) staining. Boxed areas in (C, C', C") are magnified below with arrows indicating mutant clones. (D, E) Full-length Ci-155 (red) and (D', E') *ptc-lacZ* reporter of Ci activity (green) in wing discs from male wild-type (WT) or fu^{A} mutant larvae. Arrows indicate the anterior (left) boundary of *ptc-lacZ* expression. Plots of Ci-155 staining intensity (above D, E) along the AP axis were generated as described in Materials and Methods for the boxed regions. Background posterior Ci-155 levels (red line) and anterior Ci-155 levels in wild-type discs (blue line) are indicated. Ci-155 levels were increased throughout the anterior of fu^{A} mutant discs but were highest within the Hh signaling domain at the AP border, marked by *ptc-lacZ* staining.

Figure S2. Ci-155 processing by excess Cos2 deficient for Fu binding does not require Su(fu).

(A-H) Repression of *hh-lacZ* (red), indicating Ci processing, was seen in *smo cos2* mutant clones (arrows) expressing *UAS-CosΔFu*, marked by GFP (green) for both (B-D) wild-type Ci and (F-H) Ci-$\Delta\Delta$ in (B, F) otherwise normal discs, (C, G) *Su(fu)/+* discs and (D, H) *Su(fu)/Su(fu)* discs. Control *smo cos2* mutant clones showed (A) no repression using *UAS-Ci-WT* and (E) only very slight reduction of *hh-lacZ* expression using *UAS- Ci-$\Delta\Delta$.*

Figure S3. Ci-155 processing by Cos2 variants deficient for Fu or Ci CORD binding when expressed at physiological levels.

(A-C) Repression of *hh-lacZ* (red), indicating Ci processing, was seen in *smo cos2* mutant clones (arrows), marked by GFP (green) for wing discs expressing *UAS-Ci-$\Delta\Delta$* and carrying genomic

transgenes for (B) CosΔFu or (C) Cos2-S182N but (A) not in the absence of a Cos2 transgene. (D-F) Cos2 protein (red, D'-F') was not detectable in *cos2* mutant clones (D), marked by loss of GFP (green, D-F) but was partially restored by (E) *gCos-WT* and (F) *gCosΔFu*. Levels of Cos2 were measured (see Materials and Methods) in twin-spots of *cos2* clones ("WT": 2 copies of endogenous wild-type gene), set at 2.0, and compared to Cos2 levels from a single copy of wild-type ("gWT") or CosΔFu ("gΔFu") transgenes in *cos2* mutant clones. Standard error of the mean is shown (n=5). (H-K) Wing discs with *cos2* mutant clones marked by loss of GFP (green, H-K) and carrying the indicated transgenes, treated with no LMB for 2h (as a control for results shown in Fig. 3D-G). Ci-155 (red, H'-K') is largely absent from nuclei (blue Hoechst staining, H"-K") in all clones, even though Ci-155 levels are greatly increased in the absence of a wild-type Cos2 transgene (H', J', K').

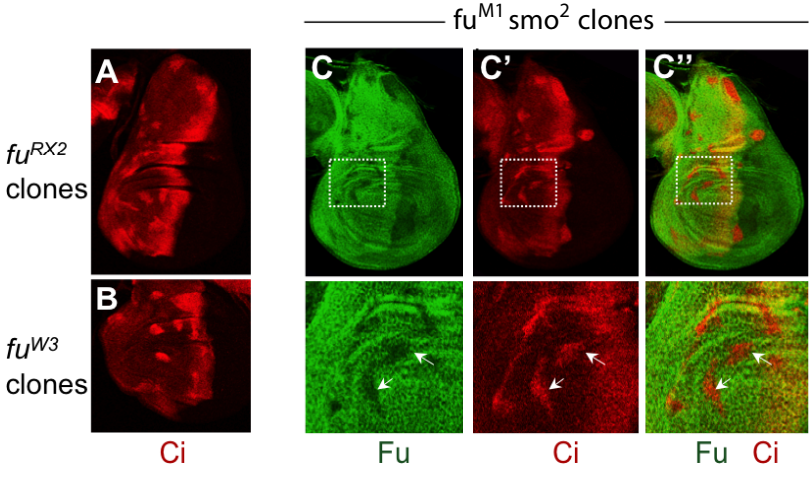
Figure S4. Smo stabilization by Cos2 variants.

(A-C) Smo staining (green) in homozygous $cos2^2$ mutant discs was highest in the posterior compartment and the posterior edge of the AP border, as normal, for discs carrying genomic transgenes for (A) wild-type Cos2 and (C) Cos2-S182N but elevated Smo staining extended much further anterior when (B) CosΔFu replaced endogenous *cos2* and was also ectopically elevated in far anterior regions. (A'-C') Staining with *ptc-lacZ* (red) marks the AP border (arrows in A"-C"). The two panels on the right in (A"-C") show close-up views of Smo staining (green) and *ptc-lacZ* (red) around the AP border.

Figure S5. Cos2 phosphorylation sites do play a role in Ci-155 stabilization by Fu.

(A, B) In anterior *smo cos2* clones (arrows) expressing *UAS-FuEE*, marked by GFP (green), Ci-155 (white) was slightly elevated in discs carrying (A') the wild-type Cos2 genomic transgene *gCos-WT* but not (B') *gCos2-AA*.

Figure S1


fu^M1 smo^2 clones
A
B
C
C'
C''
fu^RX2 clones
fu^W3 clones
Ci
Fu
Ci
Fu Ci

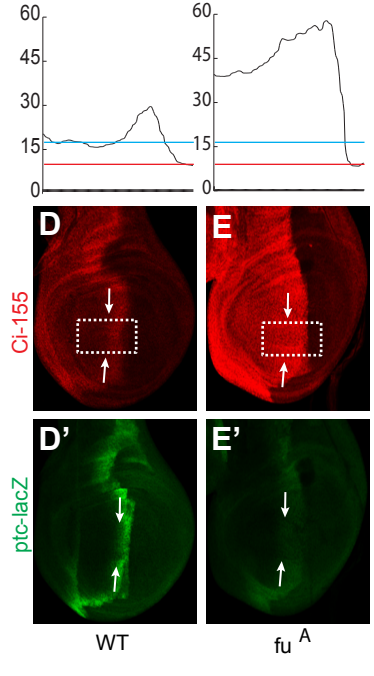

60
45
30
15
0
60
45
30
15
0
D
E
Ci-155
D'
E'
ptc-lacZ
WT
fu A

Figure S2

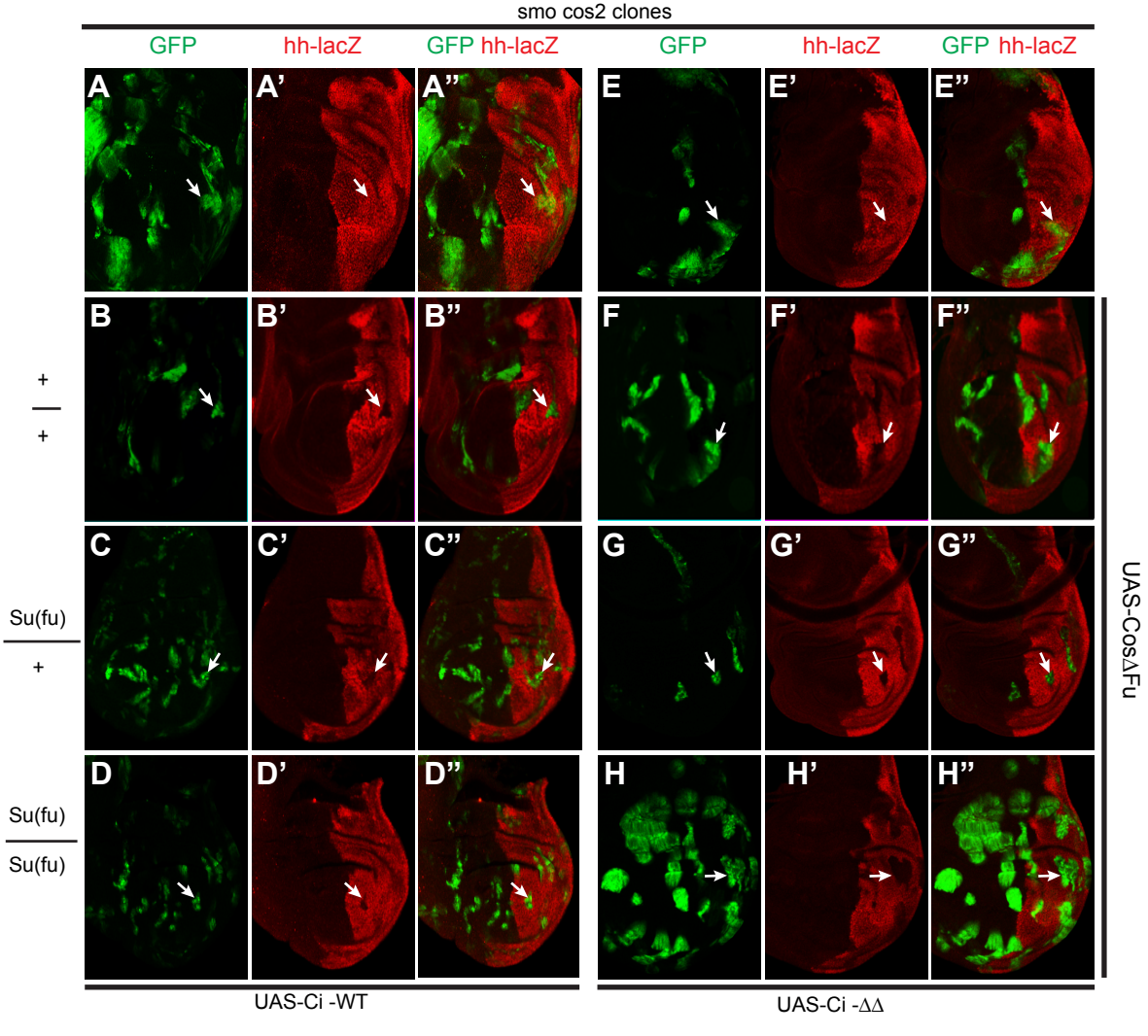

smo cos2 clones
GFP
hh-lacZ
GFP hh-lacZ
GFP
hh-lacZ
GFP hh-lacZ
A
A'
A''
E
E'
E''
B
B'
B''
F
F'
F''
C
C'
C''
G
G'
G''
D
D'
D''
H
H'
H''
+
+
Su(fu)
+
Su(fu)
Su(fu)
UAS-CosΔFu
UAS-Ci -WT
UAS-Ci -ΔΔ

Figure S3

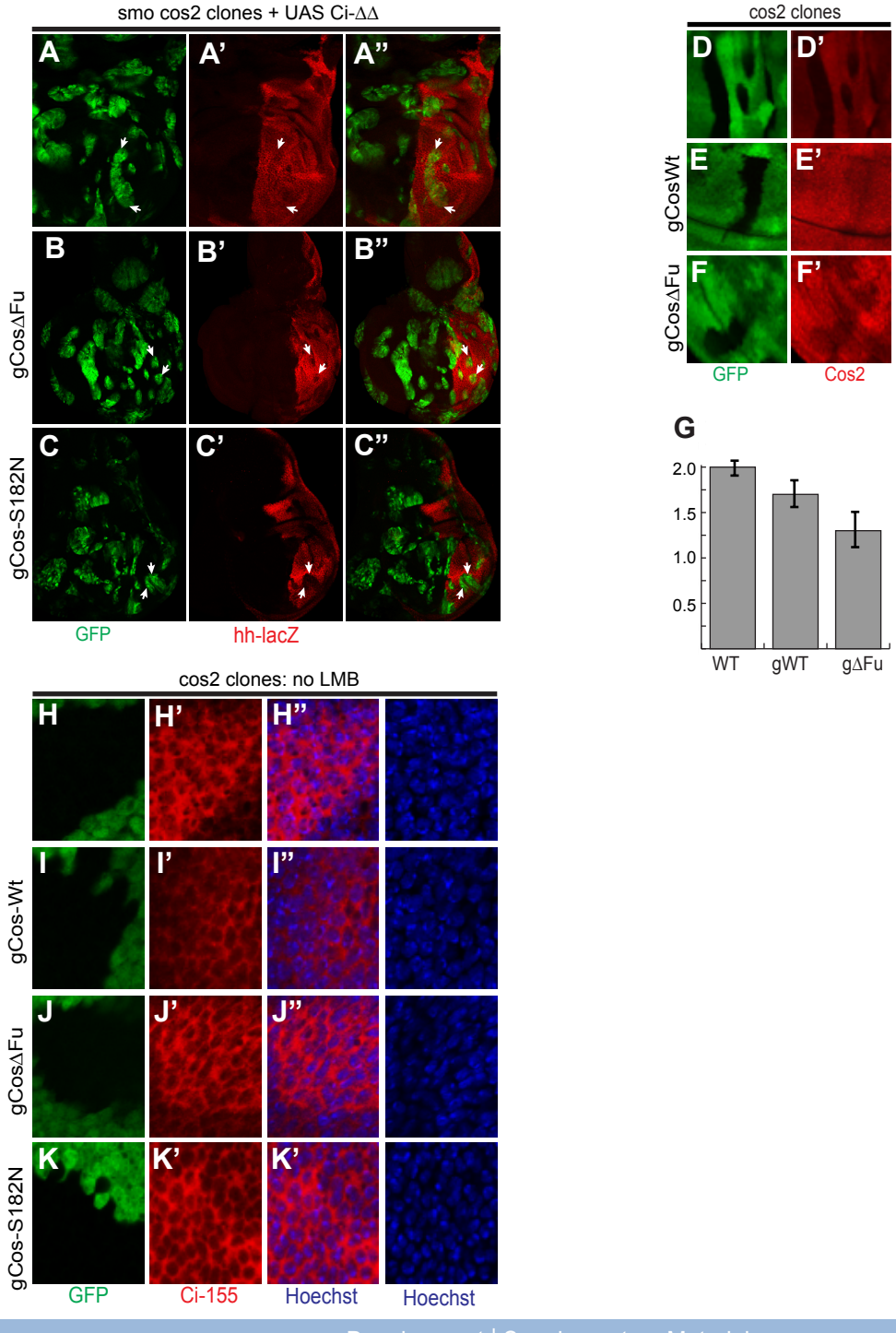

smo cos2 clones + UAS Ci-ΔΔ
A
A'
A''
B
B'
B''
C
C'
C''
gCosΔFu
gCos-S182N
GFP
hh-lacZ
cos2 clones
D
D'
E
E'
F
F'
gCosWt
gCosΔFu
GFP
Cos2
G
2.0
1.5
1.0
0.5
WT
gWT
gΔFu
cos2 clones: no LMB
H
H'
H''
I
I'
I''
J
J'
J''
K
K'
K'
gCos-Wt
gCosΔFu
gCos-S182N
GFP
Ci-155
Hoechst
Hoechst

Figure S4

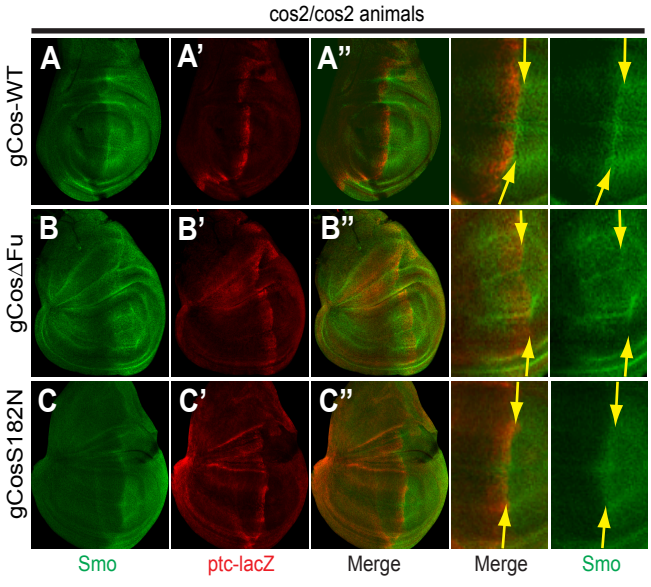

cos2/cos2 animals
gCos-WT
gCosΔFu
gCosS182N
A
A'
A"
B
B'
B"
C
C'
C"
Smo
ptc-lacZ
Merge
Merge
Smo

Figure S5