Supplemental Information

Deubiquitination of Ci/Gli by Usp7/HAUSP regulates Hedgehog Signaling

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Inventory of Supplemental Information

Supplemental Figures and Figure Legends

Figure S1, related to Figure 1. Usp7 selectively regulates Hh pathway through stabilizing Ci protein.

Figure S2, related to Figure 2. P/AxxS motifs on Ci may contribute to its interaction with Usp7.

Figure S3, related to Figure 2. Ci-Usp7 association is regulated by Cos2/Sufu and Ci phosphorylation, but not by ubiquitin modification of Ci.

Figure S4, related to Figure 3. Usp7 blocks Slimb-Cul1- and Hib-Cul3-mediated Ci degradation in S2 cells.

Figure S5, related to Figure 6. Shh promotes HAUSP binding to Gli proteins and HAUSP prevents Gli3 processing.

Figure S6, related to Figure 7. zUsp7 binds zGli proteins.

Table S1, related to Figure 1. The RNAi lines were screened by *MS1096* under Smo^{PKA} background in wings.

Table S2, related to Figure 2. Ci harbors eighteen P/AxxS sites.

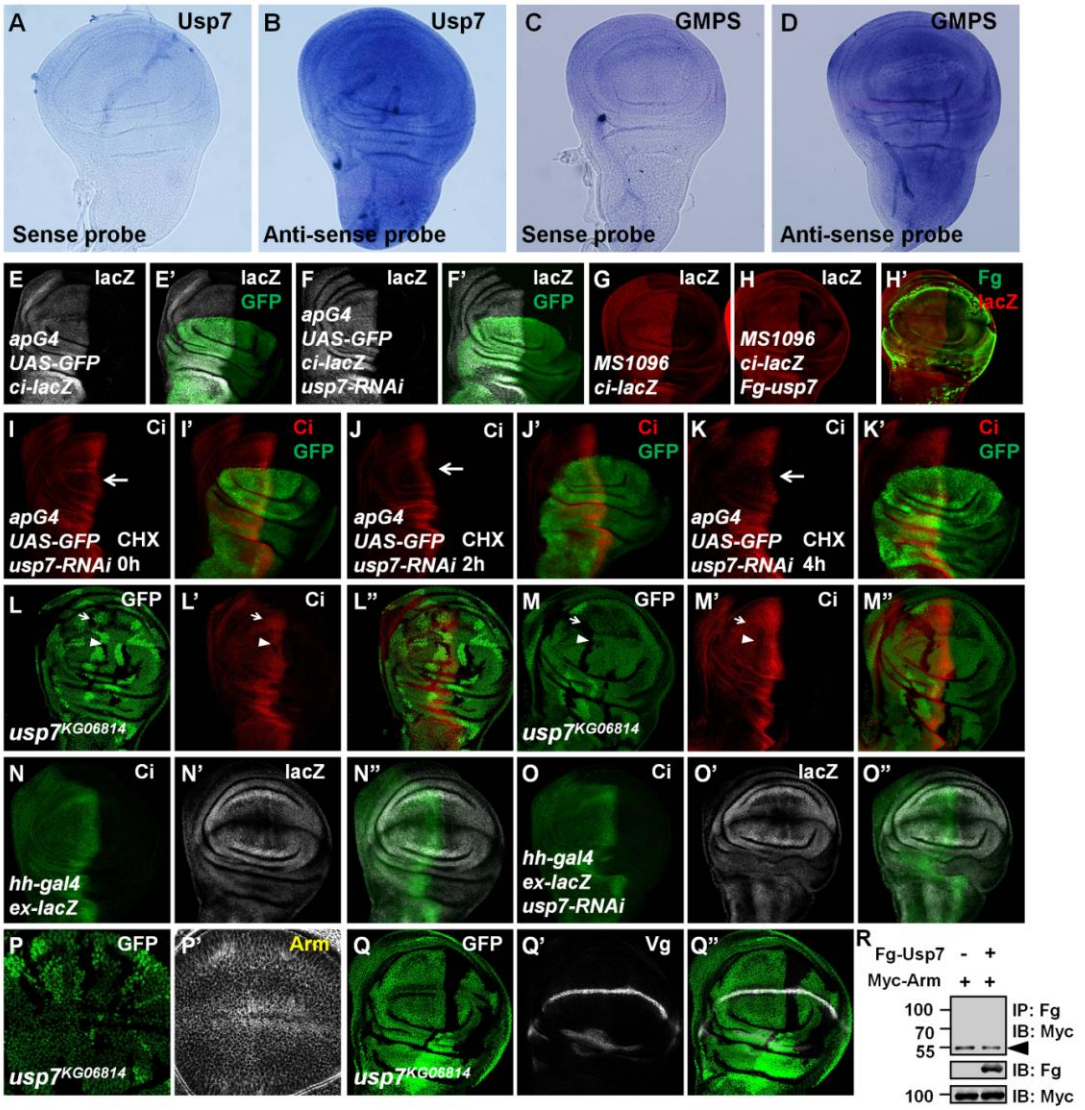

Figure S1, related to Figure 1. Usp7 selectively regulates Hh pathway through stabilizing Ci protein.

(A-D) The expression patterns of *usp7* and *gmps* in wing discs were determined by *in situ* hybridization with DIG-labeled mRNA probe against *usp7* (B) *or gmps* (D). The sense probes act as negative control (A and C). Notably, *usp7* and *gmps* ubiquitously express in wing discs.

(E-E') A wing disc of *apG4* was stained to show GFP (green) and *ci*-lacZ (white).

(F-F') A wing disc expressing *usp7* RNAi by *apG4* was stained to show

GFP (green) and *ci*-lacZ (white). Knockdown of *usp7* did not affect *ci*-lacZ expression.

(G) A *MS1096* wing disc was stained to show *ci*-lacZ expression (red).

(H-H') A wing disc expressing Fg-*usp7* by *MS1096* was stained to show Fg (green) and *ci*-lacZ (red). Overexpression of *usp7* does not affect *ci*-lacZ expression.

(I-I') Without CHX treatment, knockdown of *usp7* with *apG4* downregulated Ci protein level (red).

(J-K') After treated with CHX for 2hrs (J-J') or 4hrs (K-K'), wing discs expressing *usp7* RNAi with *apG4* was stained to show GFP (green) and Ci (red). CHX treatment fastened Ci degradation caused by *usp7* knockdown.

(L-M") Wing discs carrying $usp7^{KG06814}$ clones marked by lack of GFP (green) were immunostained to show the expression of Ci (red). Compared with clones away from A/P boundary (arrows), the decrease of Ci is more apparent in clones near A/P boundary (arrowheads).

(N-N") A wing disc of *hh*-gal4 was stained to show Ci (green) and *ex*-lacZ (white). Hh promoter drives Gal4 expression in the posterior region in wing disc.

(O-O") Knockdown of *usp7* with *hh*-gal4 did not affect *ex*-lacZ expression in wing discs.

(P-P') A wing disc carrying $usp7^{KG06814}$ clones marked by lack of GFP

(green) was stained to show Arm (white). Arm had no changes in *usp7* mutant cells.

(Q-Q”) A wing disc bearing $usp7^{KG06814}$ clones marked by lack of GFP (green) was stained to show Vg (white). Vg had no changes in *usp7* mutant cells.

(R) Western blots of immunoprecipitates (top) or lysates (bottom two panels) from S2 cells expressing indicated proteins. The arrowhead indicates IgG. Usp7 did not show interaction with Arm.

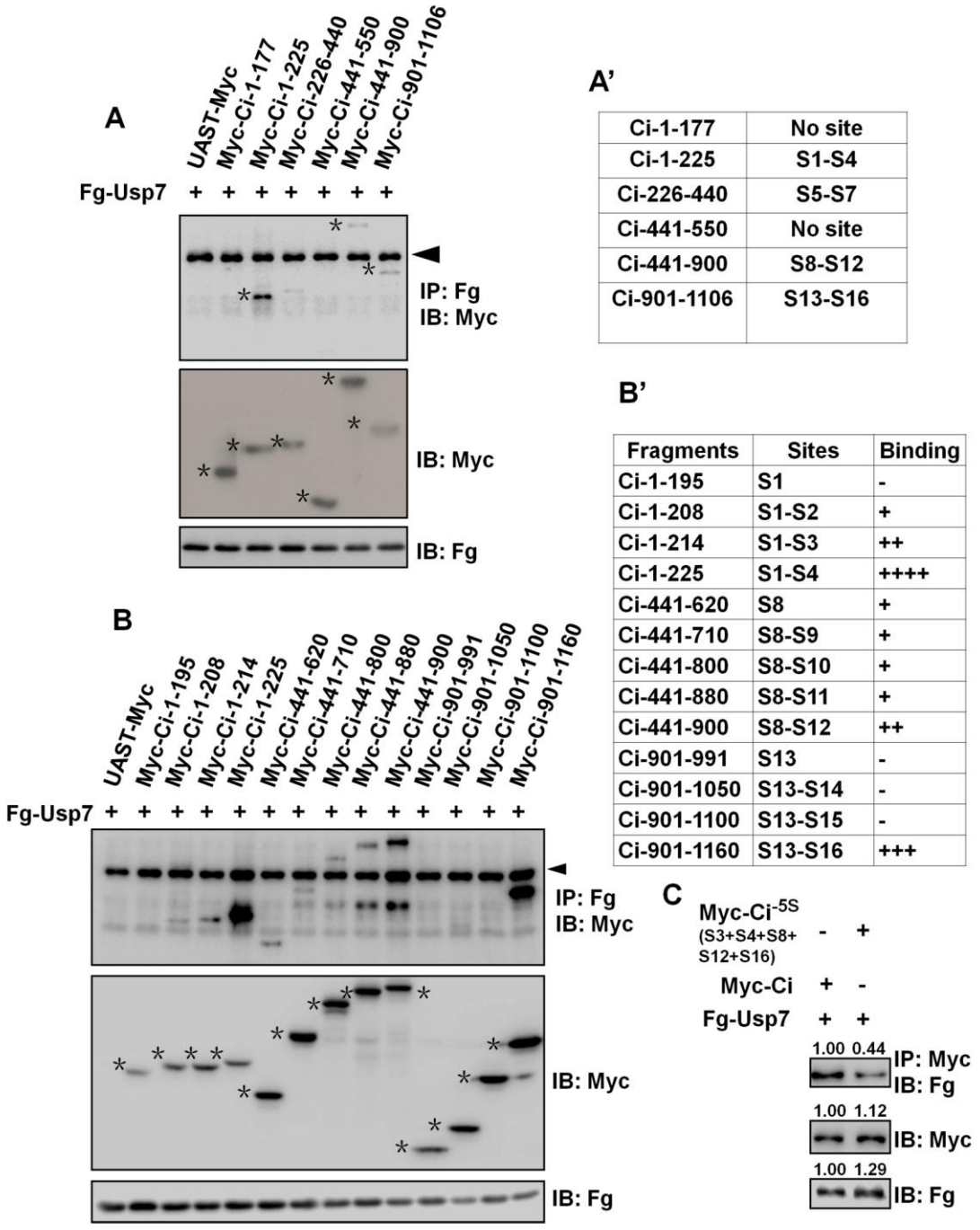

Figure S2, related to Figure 2. P/AxxS motifs on Ci may contribute to its interaction with Usp7.

(A-A') The interactions between Ci truncated fragments and Usp7 in S2 cells. P/AxxS sites sitting on the corresponding fragments were shown (A'). Asterisks marked expressed Ci fragments. The arrowhead indicated

IgG. P/AxxS sites play an important role for Ci binding to Usp7.

(B-B') The interactions between Ci deletion mutants and Usp7 in S2 cells. P/AxxS sites sitting on the corresponding fragments were shown (B'). The arrowhead indicates IgG. Asterisks indicate expressed Ci deletion mutants. S3, S4, S8, S12 and S16 are important for Ci interacting with Usp7.

(C) The interaction between Ci^{-5S} and Usp7. Ci^{-5S} is a mutant form of Ci which Serines on 212, 219, 555, 888 and 1113 replaced by Alanines. Of note, Ci^{-5S} showed a weaker binding to Usp7 than Ci. The relative levels of indicated proteins were measured by Image J.

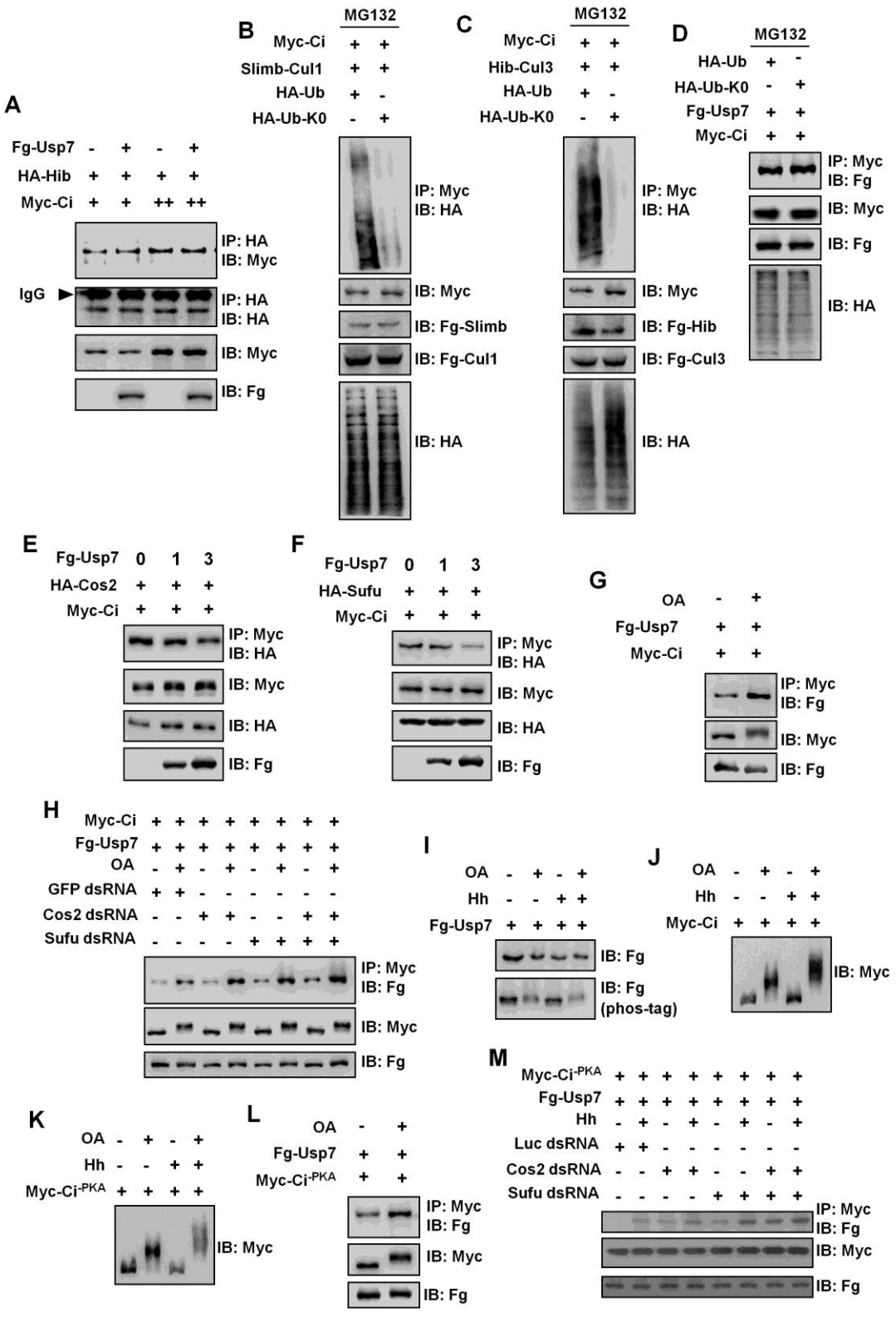

Figure S3, related to Figure 2. Ci-Usp7 association is regulated by Cos2/Sufu and Ci phosphorylation, but not by ubiquitin modification of Ci.

(A) Usp7 did not seem to affect the interaction between Ci and Hib. The transfected S2 cells were treated with MG132 for 4hrs before cell harvestion.

(B-C) Slimb-Cul1 and Hib-Cul3 E3 ligases promoted Ci polyubiquitination. Of note, expressing Ub-K0 inhibited the ubiquitination of Ci.

(D) Ub-K0 expression did not affect the interaction between Ci and Usp7.

(E-F) Usp7 expression inhibited Ci-Cos2 and Ci-Sufu interactions in a dose-dependent manner. S2 cells were treated MG132 for 4hrs before cell harvesting.

(G) OA treatment increased the association between Usp7 and Ci. Transfected S2 cells were treated with 20nM OA for 2hrs prior to harvesting the cells.

(H) OA treatment increased the association between Ci and Usp7 with or without *cos2*/*sufu* knockdown. S2 cells were treated MG132 for 4hrs before cell harvesting.

(I) Hh-conditioned medium treatment did not affect the phosphorylation of Usp7 on regular gel (*Upper*) or on Phos-tag gel (*Lower*).

(J) Hh-conditioned medium treatment increased the phosphorylation of Ci protein.

(K) Hh-conditioned medium treatment promoted Ci^{-PKA} phosphorylation.

(L) OA treatment promoted the interaction between Ci^{-PKA} and Usp7.

(M) Hh-conditioned medium treatment increased the association between Ci^{-PKA} and Usp7 with or without *cos2*/*sufu* knockdown. S2 cells were treated MG132 for 4hrs before cell harvesting.

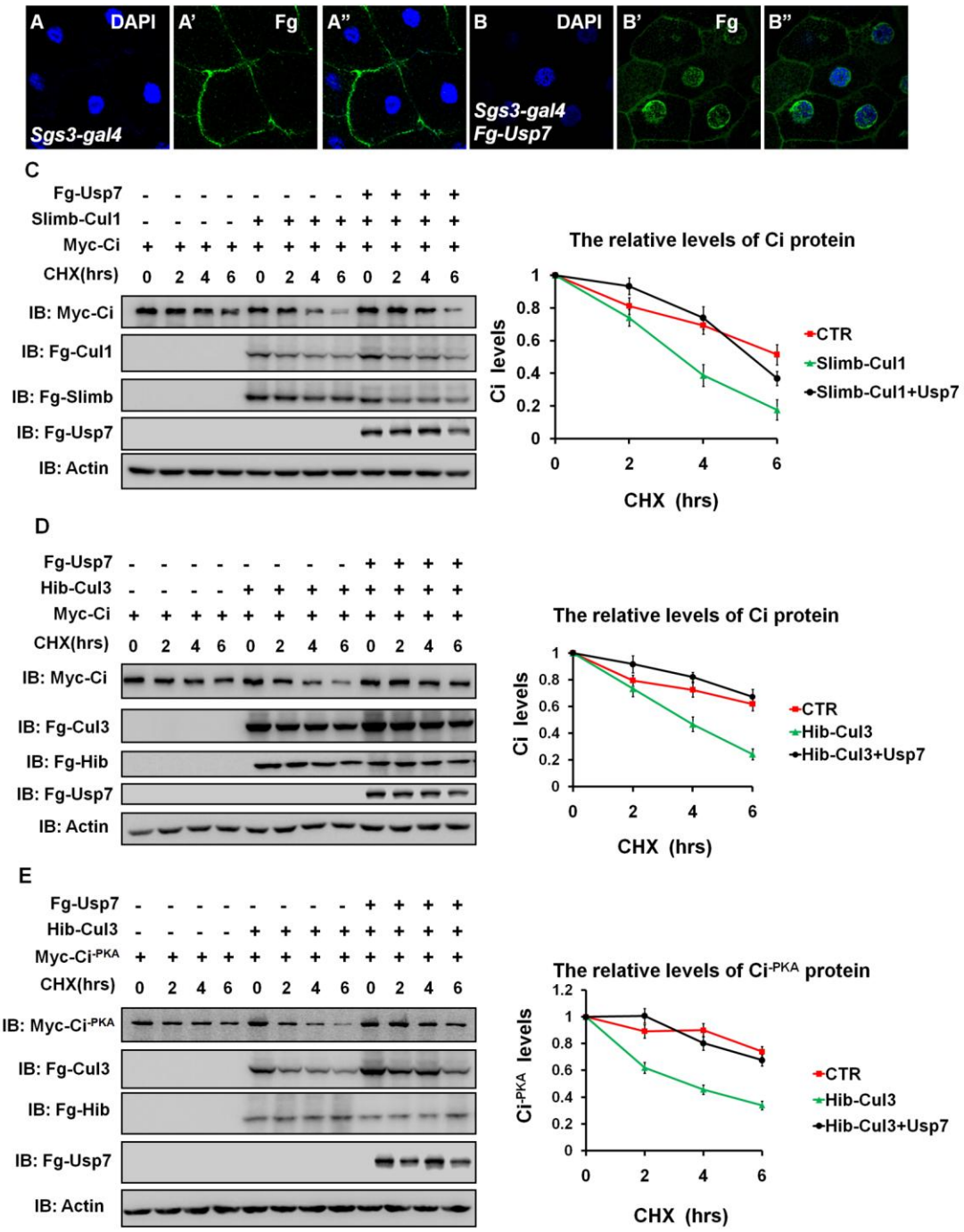

Figure S4, related to Figure 3. Usp7 blocks Slimb-Cul1 and Hib-Cul3-mediated Ci degradation in S2 cells.

(A-B") Control salivary gland cells (S-S") and salivary gland cells expressing Fg-*usp7* by *Sgs3*-gal4 (T-T") were stained to show DAPI (blue) and Fg (green). Of note, the cell membrane had some unspecific signals.

(C-E) Western blots of lysates from S2 cells expressing indicated proteins and treated with CHX for the indicated time intervals. Quantification analyses were shown on right of autoradiogram. The results were presented as means±SD of values from three independent experiments. Notably, Usp7 could hamper Slimb-Cul1-mediated Ci degradation (C). Usp7 also counteracted Ci and Ci^{-PKA} destabilization by Hib-Cul3 E3 ligase (D-E).

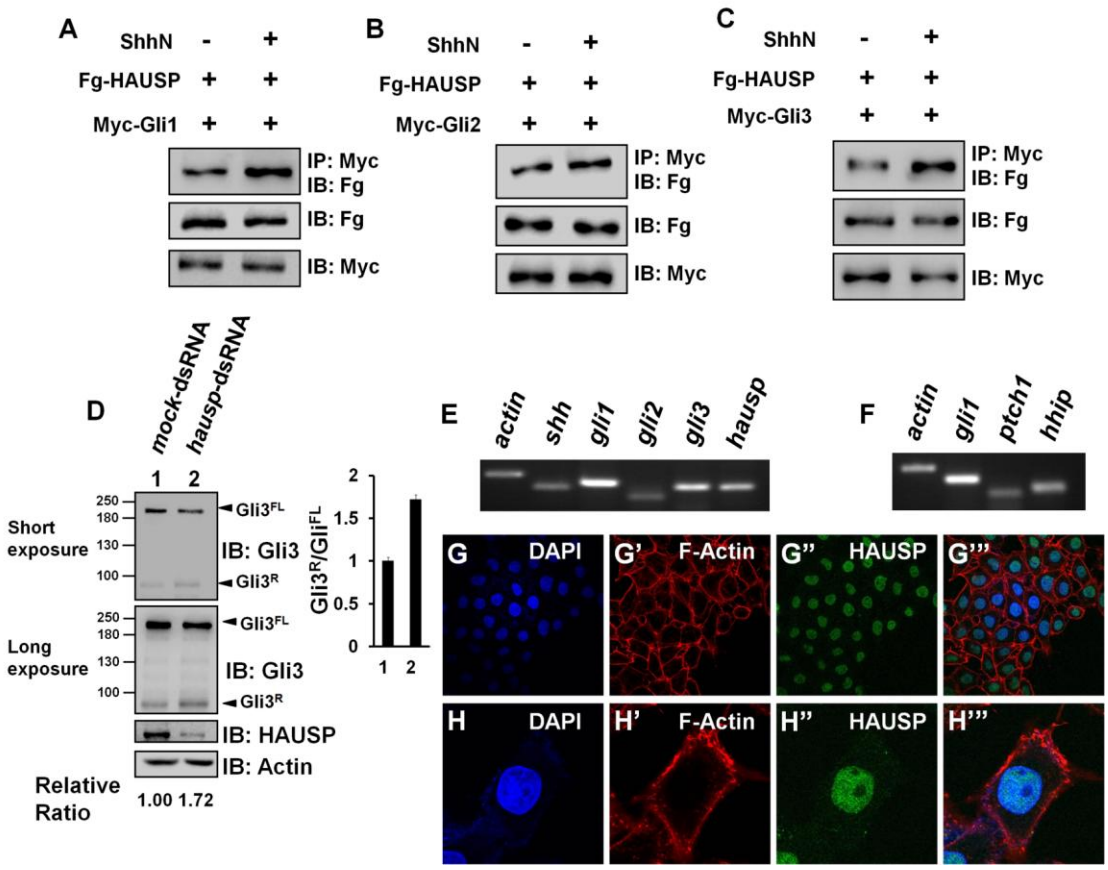

Figure S5, related to Figure 6. Shh promotes HAUSP binding to Gli proteins and HAUSP prevents Gli3 processing.

(A-C) ShhN expression promoted Fg-tagged HAUSP binding to Myc-tagged Gli1 (A), Gli2 (B) and Gli3 (C) in 293T cells.

(D) Western blot analysis of whole cell lysates of 293T transfected with *mock*-dsRNA or *hausp*-dsRNA with indicated antibodies. Knockdown of *hausp* promoted Gli3 processing. The ratio result was presented as means ±SD of values from three independent experiments.

(E) RT-PCR analysis of expression levels of the components of Hh pathway (*shh*, *gli1*, *gli2* and *gli3*) and *hausp* in 293T cells. *actin* acts as a positive control.

(F) RT-PCR analysis of expression of several Hh target genes (*gli1*, *ptch1*

and *hhip*) in 293T cells. *actin* acts as a positive control.

(G-H’”) Wild type 293T cells were stained to show DAPI (blue), F-Actin (red) and HAUSP (green). Of note, endogenous HAUSP mainly localized in nuclei, with a small proportion localized in the cytoplasm.

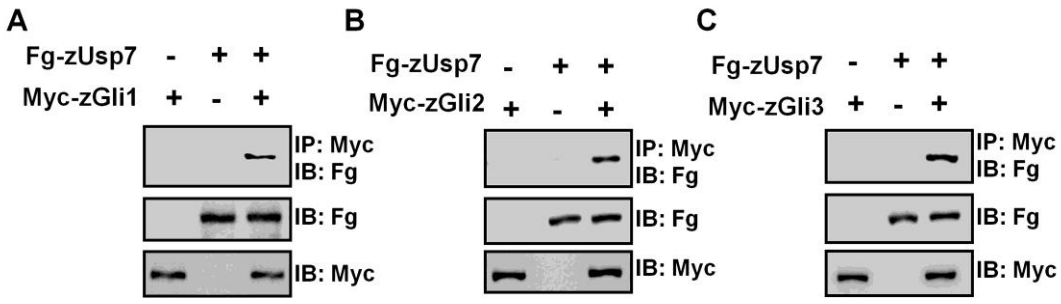

Figure S6, related to Figure 7. zUsp7 binds zGli proteins.

(A-C) zUsp7 interacted zGli1 (A), zGli2 (B) and zGli3(C). The indicated plasmids expressed in 293T cells.

CG number	gene name	VDRC/NIG transformant ID	*ptc*-lacZ staining
CG12082*		12082R-1	No change
CG15817		v100992 v41605	
CG3016		v110616	
CG32479*		v37859 v37858	No change
CG4165	Usp45	v110286 4165R-2	
CG1490*	Usp7	v18231	Decrease
CG12359	Ulp1	12359R-4 v106625	
CG3431*	Uchl3	3431R-1 v103481	No change
CG5486	Usp47	5486R-3 v103743	
CG4166*	Not	v45775 v45776	No change
CG11025	Isopeptidase-T-3	v105416	
CG1945	Faf	v2955 1945R-1	
CG5384	Usp14	v110227	
CG5794	puf	v106192	
CG5798*	Usp8	v107623	No change
CG7023	Usp12/46	v100586	
CG7288		7288R-1 7288R-2	
CG14619		v104382	
CG30421		v33727 v103553	
CG5603*	CYLD	v101414 v15340 5603R-2	No change
CG8494	Usp33	v42609	
CG8334		v18982 v18981	
CG8445	BAP1	v107757 8445R-2	
CG4265	Uchl1	v26468 4265R-2	
CG5505	Usp36	v11152	
CG2904	Ec	2904R-1 v106671	

Table S1, related to Figure 1. The RNAi lines were screened by

***MS1096* under Smo^{PKA} background in wings.** The asterisks indicate the RNAi-mediated reduction of these genes induced adult wing phenotypes. The asterisk-marked genes were selected to knock down via *MS1096* for checking the changes of *ptc*-lacZ expression in wing discs.

Site (NO.)	Amino acid sequence	Site (NO.)	Amino acid sequence
S1	^{178}AAG^{181}S	S10	^{728}ASA^{731}S
S2	^{203}PGG^{206}S	S11	^{869}PNP^{872}S
S3	^{209}ASI^{212}S	S12	^{885}PGC^{888}S
S4	^{216}ALS^{219}S	S13	^{944}PPS^{947}S
S5	^{252}ASG^{255}S	S14	^{997}AIA^{1000}S
S6	^{321}PNL^{324}S	S15	^{1063}AMT^{1066}S
S7	^{346}AAF^{349}S	S16	^{1110}PVG^{1113}S
S8	^{552}PGC^{555}S	S17	^{1209}ALP^{1212}S
S9	^{680}ASD^{683}S	S18	^{1360}PDV^{1363}S

Table S2, related to Figure 2. Ci harbors eighteen P/AxxS sites. S1 to S7 locate on the N-terminus of Ci (CiN). S8 to S16 sit on the M-region of Ci (CiM). S17 and S18 locate on C-region of Ci (CiC).

Supplemental Experimental Procedures

Constructs, Mutants, and Transgenes

The constructs for expression in S2 cells were used as follows: the HA-Ci, Myc-Ci, Myc-CiN, Myc-CiM, Myc-CiC, Fg-Hib, Fg-Cul3, HA-Cos2, Fu-YFP and Sufu-YFP constructs have been previously described (Shi et al., 2011; Zhang et al., 2006). To construct Fg-Usp7, HA-Usp7, Fg-Slimb, Fg-Cul1, HA-GMPS, Fg-Ub, and HA-Ub plasmids, we amplified the corresponding cDNA fragments, and then cloned them into pUAST-3×Fg or pUAST-3×HA vectors. Fg-Usp7^{CA} was generated using PCR-based site-directed mutagenesis at the background of Fg-Usp7. The Fg-tagged Usp7N (aa1-234), Usp7M (aa210-600), Usp7C (aa587-1129), Usp7-MATH (aa90-220) and Usp7-ΔMATH were constructed by inserting the corresponding coding sequences into the pUAST-3×Fg vector. The constructs for expression in mammal cells were prepared as follows: Fg-Ub, Fg-hSpop, Fg-hCul3, Myc-hGli1, Myc-hGli2, Myc-hGli3, Myc-zGli1, Myc-zGli2, Myc-zGli3 and Fg-zUsp7 were cloned into pcDNA3.1 vector. Fg-tagged HAUSP and HAUSP^{CA} were prepared from pPB-CAG-HAUSP vector (Ma et al., 2012) (a generous gift from Dr. Yang Shi, Harvard University, USA), and also sub-cloned into pcDNA3.1. N-terminally Fg-tagged Itch plasmid used in the study was generated via sub-cloning of Itch coding sequence from pCMV-Itch-GG (Wei et al., 2012) (a kind gift from Dr. Lin Li, Shanghai

Institutes for Biological Sciences, China) into the pcDNA3.1 vector. The RNAi lines that targeted *usp7* (v18231), and *gmps* (v24152 and 9242R-1) were obtained from the Vienna *Drosophila* RNAi Center (VDRC) or National Institute of Genetics (NIG). *MS1096*, UAS-Dicer2, *ptc*-lacZ, *kn*-lacZ, *dpp*-lacZ, *ap*G4, UAS-GFP, *ci*-lacZ, *hib*-RNAi, HA-*hib*, and Fg-*slimb* have been described (Flybase) (Zhang et al., 2006). $usp7^{KG06814}$ was obtained from Bloomington. The Fg-*usp7*, HA-*usp7*, Fg-$usp7^{CA}$, Fg-*usp7-ΔMATH*, HA-*gmps*, and Fg-*hausp* transgenic flies were generated by injection of constructs into *Drosophila* embryos according to the methods described previously (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The parental strain for all germline transformations was w^{1118}. All stocks used in this study were maintained and raised under standard conditions.

Immunostaining and *In Situ* Hybridization of Wing Discs

Immunostaining and *In situ* hybridization of imaginal discs were performed with standard protocols (Wang et al., 1999). For CHX treatment on wing discs in Figure S1, larvae were cut in half and cultured in M3 medium (S8398, Sigma) containing 2.5% FBS (F0718, Gibco), 2.5% fly extract, and 0.5 mg/ml insulin (Sigma) with CHX at a final concentration of 100μg/ml for indicated times at room temperature. Then, larvae were fixed and subject to following regular processing. Images were captured with FV10-ASW 2.0 Olympus confocal microscope.

Antibodies were in this study: rat anti-Ci (2A1) (1:50; DSHB); mouse anti-β Gal (1:500; Sigma); mouse anti-Arm (1:50; DSHB); mouse anti-En (1:50; DSHB); rabbit anti-Fg (1:200; Thermo); mouse anti-HA (F7) (1:200; Santa Cruz); rabbit anti-HA (Y11) (1:200; Santa Cruz) and DAPI (1:1000; Santa Cruz). Secondary antibodies used in this study were bought from Jackson ImmunoResearch, and were diluted at 1:500.

Cell Culture, Transfection, Immunoprecipitation, Western Blot, Cell Immunostaining

S2 cells were maintained in Schneider's *Drosophila* medium (S9895, Sigma) supplemented with 10% FBS (F0718, Gibco) and 1% penicillin/streptomycin (P0781, Sigma). Clone8 cells were maintained in M3 medium. 293T, 3T3 and MEF cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS and 1% penicillin/streptomycin. Transfection of S2 and Clone8 cells was performed using calcium phosphate according to the manufacturer's instructions (Invitrogen). Mammalian cells were transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48hrs after transfection, cells were harvested for immunoprecipitation and western blot analysis with standard protocols as described (Zhang et al., 2005; Zhang et al., 2006). The Phos-tag gel assay was carried out as described previously (Jiang et al., 2014; Shi et al., 2014). Two-step immunoprecipitation in Figure 5D was performed as

previously described (Zhang et al., 2013b). Hh-conditioned medium was obtained from Hh stable cell line of S2 cells after 24hrs induced by 0.7mM CuSO4, and was added to cells at 50% of the medium for 24hrs stimulation before cells were harvested. Cell immunostaining was performed as protocols have been described (Zhang et al., 2013a). The following antibodies were used for immunoprecipitation and western blot: mouse anti-HA (F-7) (1:2500; Santa Cruz); mouse anti-Myc (9E10) (1:2500; Santa Cruz); mouse anti-Fg (M2) (1:5000;Sigma); mouse anti-Ub (P4D1) (1:1000; Santa Cruz); mouse anti-Actin (1:5000; Genscript); rat anti-Ci (2A1) (1:1000; DSHB); rabbit anti-CiN (ABclonal Technology); rabbit anti-Gli1 (1:1000; Abcam); rabbit anti-Gli2 (1:1000; Proteintech); rabbit anti-Gli3 (1:1000; Santa Cruz); rabbit anti-HAUSP (1:1000; Santa Cruz); goat anti-mouse HRP (1:10000; Abmax) and goat anti-rabbit HRP (1:10000; Abmax).

GST Fusion Protein Pull-down Assay

GST fusion proteins were produced in *E.coli BL21*, and purified with glutathione agarose beads (GE, Healthcare). GST fusion protein-loaded beads were incubated with cell lysates derived from S2 cells expressing epitope-tagged proteins at 4 ^{o}C for 2h. The beads were washed three times with PBS, followed by western blot analysis.

RNA Isolation, Reverse Transcription, and Real-time PCR

About 20 zebrafish embryos at 24 hpf were lysed in TRIzol (Invitrogen)

for RNA isolation following standard protocols. 1μg RNA was used for reverse transcription by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO). Real-time PCR was performed on ABI Fast7500 with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). 2-ΔΔCt method was used for relative quantification. The primer pairs used were as follows: *zusp7*, 5'- ATGAACCACCATCACA-CCCAG-3' (forward) and 5'-TGTCATCCTCCATGTCCTCC-3' (reverse); *fkd4*, 5'-GCTTCACTGAACCATTT-CGCA-3' (forward) and 5'-CTGA-GCCATAATACATCTCGCTG-3' (reverse); *hhip*, 5'-CTTACGAGCCA-AGTGTGAACTG-3' (forward) and 5'-TGCTGTCTTTCTCACCGTCC--3' (reverse); *ptch2*, 5'-TCCTCCTTATGAGTCCCAAACAG-3' (forward) and 5'-CATGAACAACCTCAACAAACTTCC-3' (reverse); *nkx2.2b*, 5'-CAAATATCCAGTGCCGTCAGC-3' (forward) and 5'-CGCTCTAA-CTCAAAGGTTTGAGTC-3' (reverse); *gapdh*, 5' –CATCACAGCAA-CACAGAAGACC-3' (forward) and 5'-ACCAGTAAGCTTGCCATT-GAG- 3' (reverse).

Supplemental References

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