Supporting Information

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SI Text

Co-IP Experiments. Co-IP experiments were performed essentially as described in ref. 1. Anti-FLAG beads (Sigma) were mixed with cell lysate and incubate for 4 h at 4 °C. Beads were then spun down and washed with RIPA buffer 6 times. SDS-PAGE loading buffer was added and samples heated at 100 °C for 10 min before loading.

To precipitate the extracellular domain of cell surface Fat, pUAST-V5:fat or pUAST-fat:FVH (control) were transfected into S2 cells. Thirty-six hours after transfection, cells were collected by centrifugation and washed twice with cold PBS. Cells were then resuspended with PBS plus 2% BSA and incubated at 4 °C with anti-V5 antibody (Invitrogen) for 30 min. Cells were then collected by centrifugation, washed twice with cold PBS, and then lysed with complete RIPA buffer. The lysate was clarified by centrifugation and the supernatant was incubated with protein G agarose (Amersham Pharmacia) at 4 °C for 4 h, and the standard IP protocol was then followed.

Phosphatase Treatment. For CIP treatment of phosphorylated Fat-ICD from cell cultures, Fat-ICD, and Dco were coexpressed in S2 cells and pulled down with anti-FLAG beads. CIP treatment was performed on beads by incubating at 37 °C for 1 h in NEB Buffer3 (New England Biolabs). SDS-loading buffer was added and samples heated at 100 °C for 10 min before electrophoresis. For CIP treatment of wing disc extracts, the lysate was mixed with SDS loading buffer and incubated at 100 °C for 5 min. After cooling, mixtures were desalted and the buffer was changed to weak detergent buffer (10 mM Hepes pH7.5, 100 mM KCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, and 5 mM DTT) by ultrafiltrating through Microcon YM30 (Millipore). CIP treatment was performed in NEB buffer3 by incubating at 37 °C for 1 h. SDS-loading buffer was added and samples heated before electrophoresis.

***Drosophila* Stocks and Crosses.** Wing discs collected for Western blot analysis of mutants were isolated from *y w; ft^{Grv} FRT40A/CyO,GFP* (an unlinked lethal was removed by recombination to allow recovery of homozygous larvae)

y w; FRT82B dco^3 /TM6b

y w; FRT42D fj^{d1}/L14

y w; ds^{36D} FRT40A/L14 x y w; Df(2L)ED94/CyO,GFP, (referred to as *Df ds* in figures)

y w; ds^{36D} fj^{d1}/CyO,GFP x y w; Df(2L)ED94 fj^{d1}/CyO,GFP

Wing discs collected for Western blot analysis in overexpression experiments were isolated from *y w; tub-Gal4[LL7]/TM6b* crossed to

y w; UAS fj[6a.2]/CyO,GFP

y w; UAS-fj[146.3]/TM6b

y w; GS-ds/CyO,GFP [an insertion of the UAS containing GS element, functionally equivalent to UAS-ds (2)]

y w; UAS-ds[III]/TM6b

y w; UAS-fj[146.3] UAS-ds[III]/TM6b

Wing discs collected for Western blot analysis of Ds overexpression in dco^3 mutants were isolated from *y w; tub-Gal4[LL7] dco^3/TM6b x y w; GS-ds/CyO,GFP; FRT82B dco^3 /TM6b*

Quantitative Western blot analysis of Ds and Ft in animals expressing *UAS-fj* and *UAS-ds* under *tub-Gal4* control revealed that Ds overexpression averages 10-times endogenous Ds across the disc, whereas Fj overexpression averages 100-times endogenous Fj. Because these genes are not uniformly expressed, this overestimates the increased expression in some regions and underestimates it others.

For rescue and overexpression of *dco* we created a *UAS-dco^3* transgene, and assayed *UAS-CKIα*, *UAS-dco-KD* (kinase domain construct, a truncated isoform of wild-type Dco) and *UAS-dco* stocks from J. Jiang. The *UAS-dco* line was lethal in combination with *tub-Gal4*, so we focused on a *UAS-dco-KD* line, previously used in characterization of Wg and Hh signaling (3), and which rescued the lethality and overgrowth phenotypes of dco^3 (Fig. S6). Stocks used included:

y w; UAS-dco-KD/CyO; MKRS/TM2 (3)

y w; UAS-dco; MKRS/TM2 (3)

y w; UAS-CKIα/CyO; MKRS/TM2 (3)

y w; UAS-dco-KD/CyOGFP; FRT82B dco^3 /TM6b

y w; UAS-CKIα/CyOGFP; FRT82B dco^3 /TM6b

y w; en-Gal4/CyO;th-LacZ UAS-dcr2/TM6b

UAS-dco^3 [16R] (3^{rd} chromosome) and *UAS-dco^3 [32R]* (2^{nd} chromosome)

For rescue of *fat* and dco^3 by Wts overexpression we used

y w; tub-Gal4/CyO-GFP; FRT82B dco^3 /TM6b x y w; UAS-Myc:Wts.2/CyO,GFP; FRT82B dco^3 /TM6b

y w; fat^8 UAS-Myc:Wts.2/CyO,GFP x y w $fat^{G\text{-}rv}$ FRT40A/CyO,GFP; tub-Gal4[LL7]/TM6b

Conventional dco^3 mutant clones in discs were made using

y w; FRT82B dco^3 /TM6b x *y w hs-FLP[122]; FRT82B Ubi-GFP/TM3*

MARCM experiments analyzing D:V5 localization or abdominal polarity were conducted using *y w hs-FLP tub-Gal4 UAS-GFP; UAS-y+/CyO; FRT82B tub-Gal80 x*

y w; UAS-d:V5[50.5]/CyO,GFP; FRT82B dco^3 /TM6b or yw; UAS-d:V5[50.5]/CyO,GFP; FRT82B/TM6b

and *y w hs-FLP tub-Gal4 UAS-GFP; FRT40A tub-Gal4/CyO x y w; arm-lacZ FRT40A/CyO,GFP; UAS-d:V5[9F]/TM6b* or *y w;fat^8 FRT40A/CyO,GFP; UAS-d:V5[9F]/TM6b*

Anti-*Drosophila* Fat Antisera. A GST fusion protein construct including the Fat intracellular domain (gift of H. McNeill) (4) was transformed into *E. coli* BL21 DE3 cells and induced with IPTG. Bacteria were then collected and lysed by sonication in 20 mM Tris·HCl pH8.0 supplemented with 1 mM EDTA and protease inhibitor mixture (Roche). Inclusion bodies were separated from soluble protein by centrifugation and resuspended with 20 mM Tris·HCl, pH 8.0, plus 0.5% Triton X-100. After centrifugation, the pellet was washed with PBS and used to immunize rats (Cocalico Biologicals). After preabsorbtion with embryos, a 1:1,600 dilution of sera was used for tissue staining and a 1:25,000 dilution used for Western blot analysis.

Plasmids and Constructs. Previously published plasmids used included:

pAW-Gal4 (5),

pAct-GFP:V5:His (1),

pMT-dco:V5:His (6),

pMT-dco^{AR}:V5:His (6),

pAct-Per (6),

pMK-CKIα:HA (7),

pMK-CKIα:KN:HA (kinase negative) (7),

pUAS-ft (5).

New plasmids constructed include:

pMT-dco^3:V5:His:

Primers dbt-R4C (5′-G*GA ATT C* (EcoR1) AC CAT GGA GCT GTG CGT GGG TAA CAA ATA TCG-3′) and dbt-E74K

(5′-CAT AAT CTG GTG CGG CAG CAA GGG CGA CTA CAA TGT GAT G-3′) were used to change R4C and E74K on pMT-dco:V5:His by site directed mutagenesis with QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The result was confirmed by sequencing the whole insert.

pUAS-dco^3:V5:His:

To clone dco^3 into pUAST, dco^3 was amplified by PCR with primer dbt-R4C (above) and V5-His-*Xho*I-3 (5′-AA*C TCG AG* (*Xho*I) T CAA TGG TGA TGG TGA TGA TGA CCG GTA CG-3′), restricted by *Eco*RI and *Xho*I and ligated to the same digested pUAST. The insert was confirmed by sequencing. Transgenic flies were generated by standard protocols.

pUAS-ft:FVH (FVH stands for FLAG:V5:His triple tag):

The FLAG:V5:His tag was flanked by KpnI sites at both ends by PCR amplification of pAC5.1/V5 His-A (Invitrogen) with primer KpnI FlagV5His 5 (5′-AA*G GTA CC*(KpnI) *G ACT ACA AGG ACG ACG ACG ACA AGG GT* (*FLAG*) A AGC CTA TCC CTA ACC C-3′) and KpnI FlagV5His 3 (5′-AA*G GTA CC*(KpnI) C CTT AGA AGG CAC AGT CGA GGC-3′) and then digested with *Kpn*I and ligated to restricted pUAS-ft (codon removed).

pUAS-ft intracellular domain truncates:

To generate these constructs, each fragment was amplified by PCR with appropriate primers. Two or 3 overlapping fragments were then mixed together as template to amplify the whole fusion protein with primer ft SPL NotI5 and ft FVH XbaI3 (Gene splicing by overlap extension) (8). The PCR fragments were then digested with *Not*I and *Xba*I and ligated to restricted pUAST. All of the constructs were confirmed by sequencing.

Primer Sequences ftSPL *Not*I5: 5′-TCT *GCG GCC GC*A TGG AGA GGC TAC TGC TCC-3′

ftSPL SOE3: 5′-TGA AGT TGA GAG TGC TTC TTC TTC GCG GAA AGG CGG CAT-3′

ftL2 SOE5: 5′CCG CGA AGA AGA AGC ACT CTC AAC TTC AAC AAA CAG CCC CTG-3′

ftFVH *Xba*I3: 5′-GG TCT AGA GAT CA GCG GGT TTA AAC TCA ATG GTG-3′

STI-4-SOE: 5′-CTT CTT CGC GGA AAG GCG GCA TAT-3′

TM-SOE: 5′-*GCC GCC TTT CCG CGA AGA AGA* GCC GAT CCT CTC AGC ATT GGC TTC-3′

Construction of attB-P[acman-V5:fat$^+$] and pUAST-V5:ft:

Bacterial artificial chromosome clone BACR11D14 was purchased from BACPAC Resources Center (http://bacpac.chori-.org/). To introduce a V5 tag after the signal peptide, recombineering technology was used (http://recombineering.ncifcrf.gov/). Briefly, a bacterial galK gene was inserted after the *fat* signal peptide coding sequence by amplifying galK with primers galK fatsignal F (5′-cgtggccgaagt-tacgccaccacctatgaacaatatgc-cgcctttccgcgaagaagaagcCCTGTTGACAATTAATCATCGGCA-3′) and galK fatsignal R (5′-gaaatcggcactggtgtccaccgcacgg-gattgcatctcacccgatggcgaggat-gatgaTCAGCACTGTCCTGCTCCTT-3′) (capital letters for amplification of galK gene). After positive selection the galK gene was replaced with the V5 tag donor (5′GGTAAGC-CTATCCCTAACCCTCTCCTCGGTCTC-GATTCTACGCGTACCGGT-3′) via recombination and a negative selection for galK minus. The V5 donor was made by PCR with primers, Ft357up F (5′-cgctttagcgcctcatatttcaccg-3′), FtslV5 R (5′- gcgtagaatcgagaccgaggagagggt-tagggataggcttaccgcttcttcttcgcggaaaggcggca-3′), FtV5cad F (5′-cctctcctcggtctcgattctacgcgtaccggttcatcatcctcgccatcgggtgaga-3′) and Ft718 R (5′-ctagatgcagatacgaggtgtctccac-3′). To make a genomic rescue construct with *V5:fat*, the genomic region between and including Rpl40 and CG3714 was subcloned by recombination into attB-P[acman]-ApR (9). Primers used for making the subcloning donor include Rpl40LA F (5′-aaa ggcgcgcc ctcggagaggccaactaattgcag-3′), Rpl40LA R (5′- cct ggatcc ccttggaggtggagccttctgacacc-3′), CG3714RA F (5′- agg ggatcc aggtgggtgtaatcatttgatttggc-3′) and CG3714RA R (5′-acc ttaattaa caacacttaagtactagaaaaatattaaagacata-3′). Transgenic flies were generated via ϕC31 mediated transgenesis into attP2 (68A4).

To make the pUAST-V5:fat construct, the V5:fat genomic region was subcloned into attB-P (9)-ApR and restriction sites *Not*I and *Kpn*I were introduced into 5′ end and 3′ end respectively. Primers used to make the subcloning donor are ftex1 LA Nt As F (aaaggcgcgccgcggccgcatggagag-gctactgctcctgtttttcc), ftex1 LA Bm R (ctcggatccatttccagc-cacgatctcatactg), ftex8 RA Bm F (aatggatccgaggcgcctc-gagtgtcgagcagcg) and ftex8 RA Kp Pc R (accttaattaaggtacccaacatatattacacgtactcctctgg). V5:fat was then released with NotI and KpnI and ligated into pUAST-attB.

The constructs were verified by restriction fingerprinting and sequencing.

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9. Venken KJ, He Y, Hoskins RA, Bellen HJ (2006) P[acman]: A BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science* 314:1747–1751.

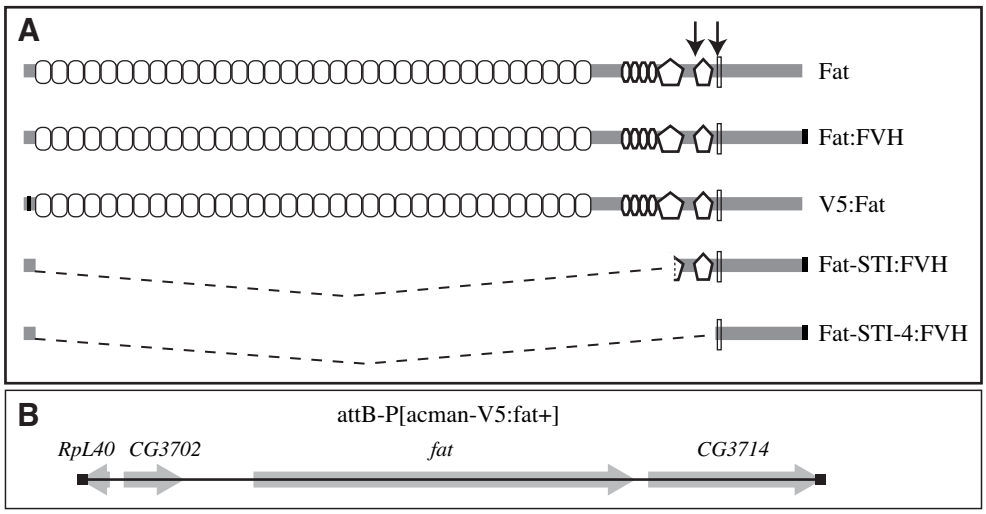

Fig. S1. Schematics of Fat and Fat constructs. (*A*) Schematic of Fat protein in different constructs. Sequence motifs are indicated by shapes: Cadherin domains (ovals), EGF domains (narrow hexagons), Laminin G domains (pentagons), transmembrane domain (rectangle). The approximate location of cleavage (arrows) sites are indicated. In Fat:FVH, a triple FLAG:V5:His_6 tag was fused to the C terminus. In V5:Fat, a V5 tag was incorporated after the signal peptide, at the N terminus. Regions of Fat included in Fat-STI:FVH and Fat-STI-4:FVH are indicated, dashed lines indicated deleted regions. (*B*) Schematic of the genomic DNA included in the attB-P[acman-V5:fat+] genomic construct. The entire region depicted is approximately 39 kb. Gray arrows indicate transcription units of *fat* and nearby genes included in the construct, the *fat* transcription unit is 19.45 kb.

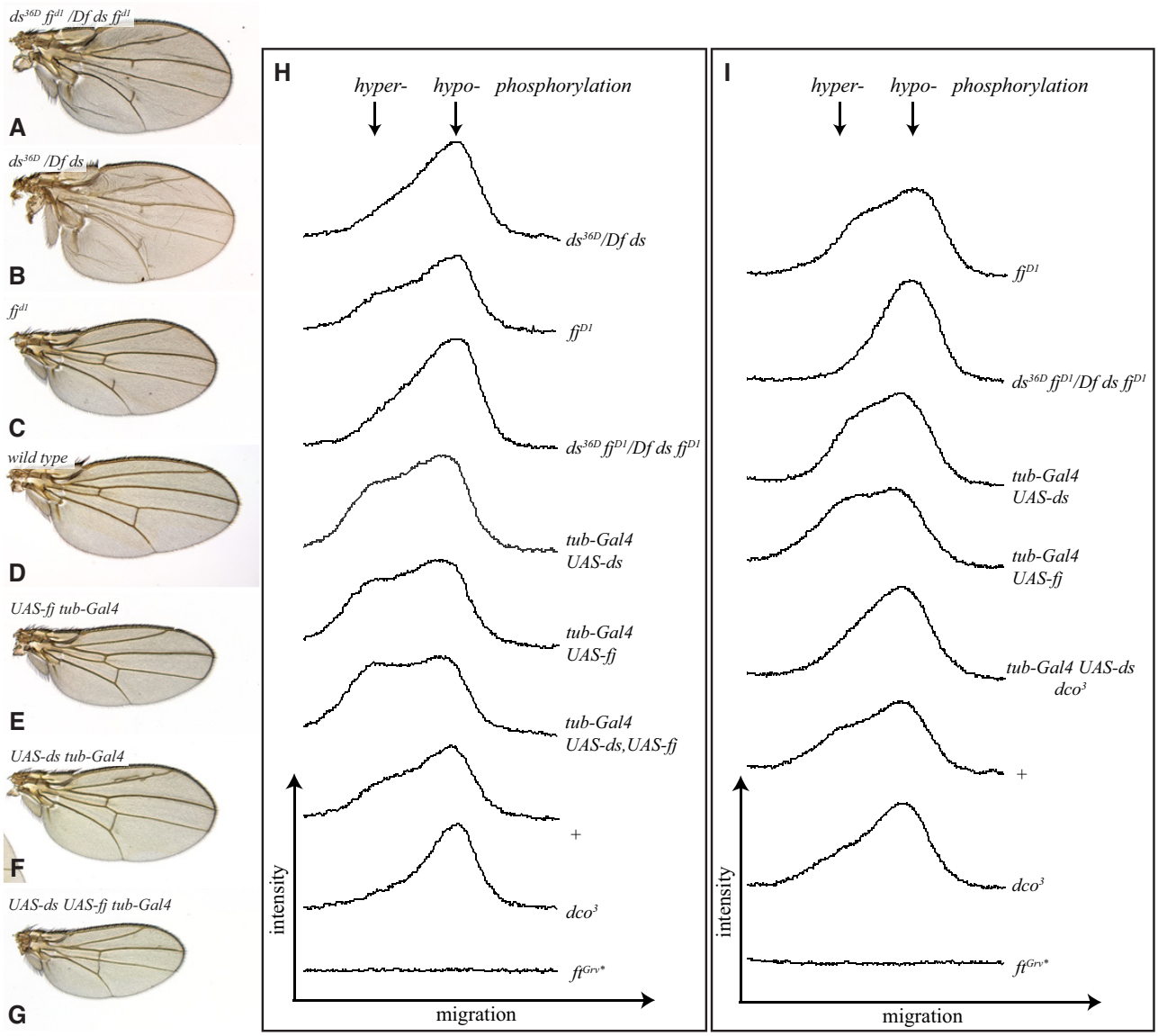

Fig. S2. Wing size and Fat phosphorylation in different genotypes. Adult wings from (*A*) fj^{D1} ds^{36D}/ fj^{D1} *Df ds*, (*B*) ds^{36D}/*Df ds*, (*C*) fj^{D1}, (*D*) wild-type (Oregon-R), (*E*) *tub-Gal4 UAS-fj*, (*F*) *tub-Ga4 UAS-ds*, and (*G*) *tub-Gal4 UAS-ds UAS-fj*. (*H* and *I*) Line scanning traces generated from Western blot analyses (as depicted in Fig. 2) of Fat protein in lysates of different genotypes. Relative intensity traces across bands from 10 traces were averaged.

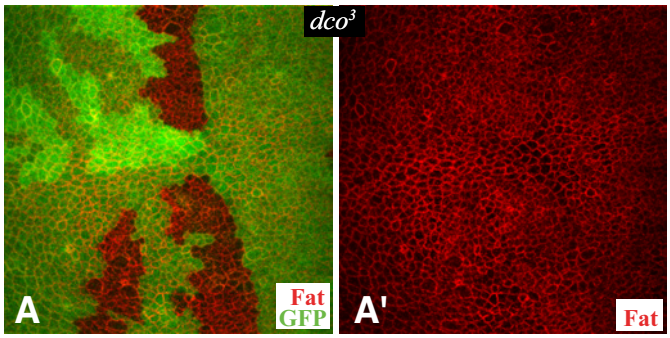

Fig. S3. Fat staining in *dco*^{3} mutant clones. Example of a wing imaginal disc containing clones of cells mutant for *dco*^{3} (marked by absence of GFP, green), and stained for expression of Fat (red). Fat staining is unaffected by the clones.

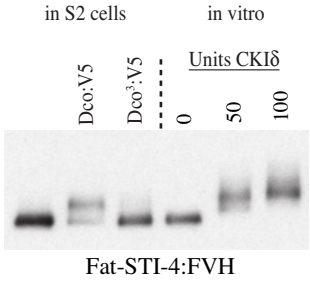

Fig. S4. Phosphorylation of Fat by CKIδ in vitro. Western blot analysis of products of a kinase assay with mammalian CKIδ shows that purified Fat-STI-4:FVH can be directly phosphorylated by CKI in vitro. Under these conditions, the extent of phosphorylation is proportional to the amount of enzyme, for comparison protein phosphorylated in S2 cells was run on the same gel.

RFRGKQEKIG SLSCGVPGFK IKHPGGPVTQ SQVDHVLVRN LHPSEAPSPP VGAGDHMRPP
VGSHHLVGPE LLTKKFKEPT AEMPQPQQQQ QRPQRPDIIE RESPLIREDH HLPIPPLHPL
PLEHASSVDM GSEYPEHYDL ENASSIAPSD IDIVYHYKGY REAAGLRKYK ASVPPVSAY~~T~~
HHKHQNSGSQ QQQQQHRHTA PFVTRNQGGQ PPPPPTSASR THQSTPLARL SPS~~S~~EL~~S~~SQQ
PRILTLHDIS GKPLQSALLA TTS**S**SGGVGK DVHSNSER~~S~~L N~~S~~PVMSQL~~S~~G Q~~S~~SSA~~S~~RQKP
GVPQQQAQQT SMGLTAEEIE RLNGRPRTCS LI~~S~~TLDAVS**S** S~~S~~EAPRVSSS ALHMSLGGDV
DAH**S**ST~~S~~T~~T~~DE **S**GNDSFTCSE IEYDNN**S**LSG DGKYSTSKSL LDGRSPV~~S~~RA LSGGETSRNP
PTTVVKTPPI PPHAYDGFES **S**FRGSLS~~T~~LV ASDDDIANHL SGIYRKANGA ASPSAT~~T~~LGW
EYLLNWGPSY ENLMGVFKDI AELPDTNGPS QQQQQQTQVV STLRMPSSNG PAAPEEYV

Fig. S5. Predicted CKI sites in the Fat ICD. The sequence of the Fat ICD (amino acids 4,610 through 5,147) in single letter code. Three web-based kinase site prediction programs were used to identify potential CKI sites within the Fat ICD: NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) predicts 7 sites, ELM (http://elm.eu.org/) predicts 15 sites, and KinasePhos (http://kinasephos2.mbc.nctu.edu.tw/index.html) predicts 36 sites. The predictions only partially overlap, so the total number of sites predicted by at least 1 program is 46. The sites predicted by the programs are identified by *underlining* (KinasePhos), bold font (NetPhosK), or strikethrough (ELM).

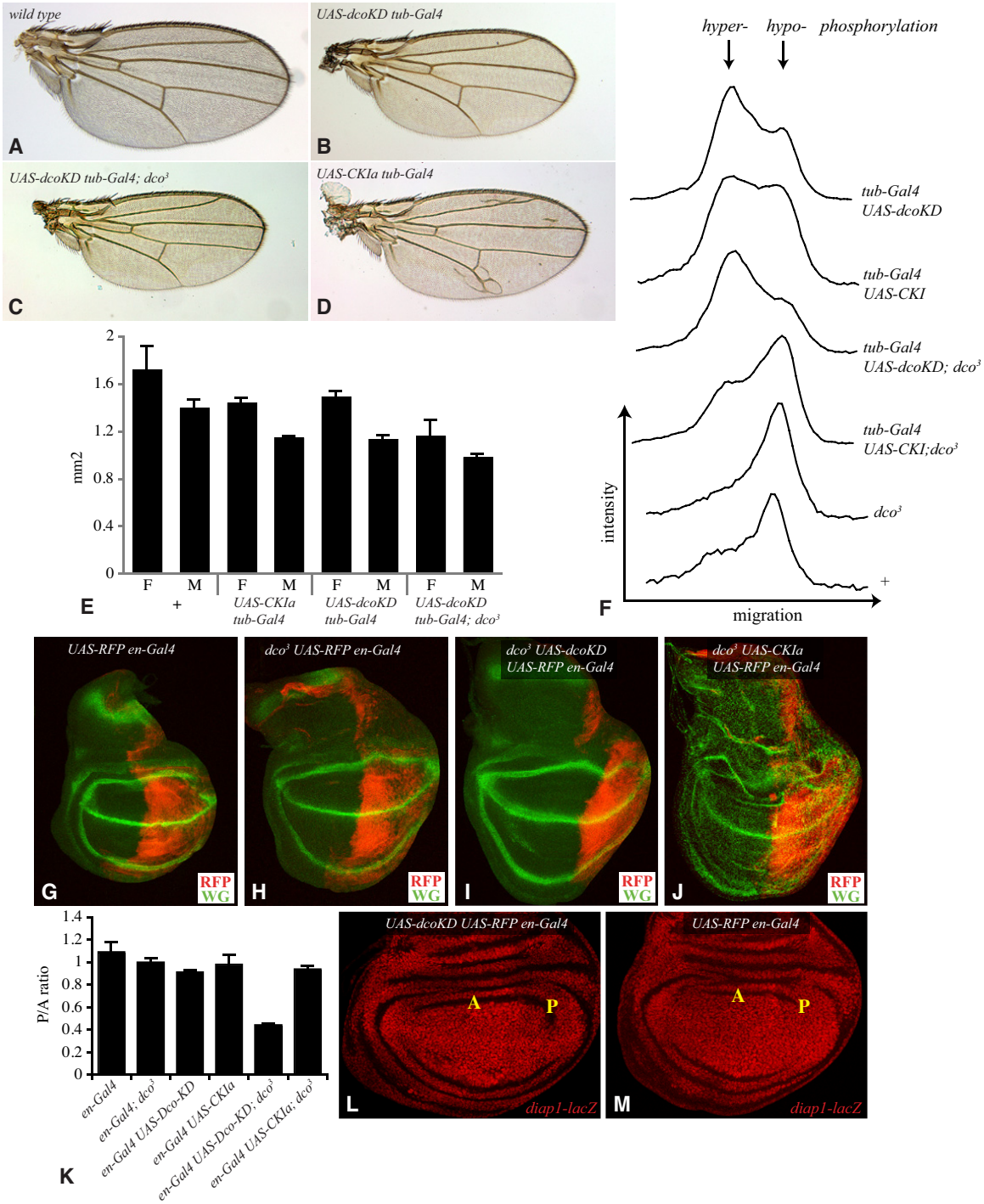

Fig. S6. Analysis of CKI overexpression. (*A–D*) Adult wings from males expressing (*A*) *tub-Gal4* (*wild-type* control), (*B*) *tub-Gal4 UAS-dco-KD*, (*C*) *tub-Gal4 UAS-dco-KD* in dco^3 mutants (rescue experiment), and (*D*) *tub-Gal4 UAS-CKIα*. (*E*) Measures of average wing size (F = female, M = male) for animals of the 4 genotypes depicted in (*A–D*). (*F*) Line scanning traces generated from Western blot analyses (as depicted in Fig. 3*E*) of Fat protein in lysates of the indicated genotypes. (*G–J*) Wing imaginal discs stained for WG expression, and with posterior (P) cells identified by expression of *UAS-RFP* under *en-Gal4* control. (*G*) Wild-type, anterior (A) and P compartments of the wing pouch (region demarcated by ring of WG expression) are similar in size. (*H*) dco^3 mutant, A and P compartments are similar in size. (*I*) dco^3 mutant rescued in posterior cells by expression of UAS-dco-KD, consequently the P compartment is much smaller than the A compartment. (*J*) dco^3 mutant in which expression of UAS-CKIα is expressed in P compartment cells; the A and P compartments are similar in size, indicating that the overgrowth phenotype has not been rescued. (*K*) Measures of the relative sizes of the P to A compartment in the wing pouch for animals of the indicated genotypes. (*L* and *M*) Wing imaginal discs stained for expression of a *diap-lacZ* reporter that is known to be a direct downstream target of Fat-Hippo signaling. (*L*) *en-Gal4 UAS-dco-KD*; a slight decrease in *diap1-lacZ* expression in P cells was detected. (*M*) *en-Gal4* control.

Table S1. Primers for generating Fat truncates

Construct	PCR template	Primers for 5′ fragment		PPrimers for 3′ fragment	
ft-STI	ft:FVH	ft_SPL_NotI5	ft_SPL_SOE3	ft_L2_SOE5	ft_FVH_XbaI3
ft-STI-4	ft:FVH	ft_SPL_NotI5	STI_4_SOE	TM_SOE	ft_FVH_XbaI3