Supplementary Information

Cabut/dTIEG associates with the transcription factor Yorkie for growth control

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Supplementary Methods

Fly strains

All *Drosophila* strains and crosses were kept on standard media at 25ºC, except in the case of *cbt* overexpression experiments carrying the GAL80^{ts} allele, which were kept at 17ºC and switched to 29ºC 16h before dissection. The strains used were *Canton S*, *W^{1118}iso; iso2; iso3*, *dTIEG^{S14}FRT40A/CyO-twi:GFP* [1], *UAS-cbt:flag* [2], *ap-GAL4:GFP, ptc-GAL4:GFP, act5C-GAL4, spalt$^{E/Pv}$-GAL4, GAL80^{ts}, nub-GAL4, UAS-GFP, yw {TRIPGL01474}attP2 (RNAicbt*, Bloomington stock center*), w^{1118}; P{GD5044}v15555* (*RNAicbt^{GD5044}*, VDRC*), fj^{p1}*[3], *yki^{B5}FRT42D* [4], *UAS-yki^{S168A}* [5], JB20 *bantam sensor-GFP* (kindly provided by M. Milán), *DIAP1-GFP4.3* [6] and *ex^{e1}FRT40A* [7] (kindly provided by N.Tapon). For twin clones, y*whsflp$^{1.22}$; arm-lacZ FRT40A*/CyO stock was used and heat shock was performed at 48h AEL (after egg laying) 30' at 37ºC. MARCM clones were generated with *ywhsflp$^{1.22}$: UAS-mCD8::GFP; tub-GAL80 FRT40A; tub-GAL4/TM6B* and *ywhsflp$^{1.22}$:tub-GAL4: UAS_GFP/FM6; tub-GAL80FRT42D* stocks and heat shocks were performed at 48h AEL 10' (*ex^{e1}*) and 15' (*yki^{B5}* and *dTIEG^{S14}*) at 37ºC. For flip-out clones y*whsflp$^{1.22}$; act5C-FRT-y^{+}-FRT-GAL4:UAS-GFP* was used and heat shock was performed at 48h AEL 10' at 37ºC.

Antibody staining

The antibodies used were anti-Wg (mouse, 1:10) (Developmental Studies Hybridoma Bank), anti-Vg (rabbit, 1:50) (kindly provided by S. Carroll), anti –En (mouse, 1:10) (Developmental Studies Hybridoma Bank) anti-GAF (rabbit,1:1000) (kindly provided by J. Bernués), , anti-flag (mouse, 1:1000) (Sigma-Aldrich) and anti-βgalactosidase (rabbit, 1:1000) (ICN Biomedicals) .

Bioinformatic analysis

ChIP-Seq analysis was based on single reads of 36 nucleotides aligned against BDGP R5/dm3 assembly. After sequencing, 38257052 reads were obtained and 176262361 could be uniquely mapped to the genome. We ran PeakSeq [8] (parameters READLENGTH=325, MAXGAP=40, MINFDR=0.05 and PVALTHRESH<10^{-40}) to identify the regions significantly enriched in Cbt ChIP-Seq reads in comparison to the normalized input control (Perez-Lluch et al., 2011). The resulting read maps were visualized as custom tracks in the University of California Santa Cruz (UCSC) Genome Bowser [9]. ChIP-Seq raw data, the profile of read counts and the set of Cbt enriched regions are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) under the accession number GSE40958. Cbt putative target genes were filtered considering those genes for which a peak was located in the proximal promoter (at most 1000 upstream of the TSS) or within introns. Gene Ontology (GO) analysis was used employing the DAVID algorithm [10], considering only enrichments with a p-value<0.05. The RNA-Seq data from wing imaginal disc was obtained from a previously published study (Perez-Lluch et al., submitted). ChIP-Seq data for H3k4me3 and H3K27me3 from wing imaginal disc were obtained from a previous study [11]. To find sequence motif enrichment within Cbt bound chromatin, we used the MEME-ChIP algorithm [12], TRANSFAC [13] and JASPAR (http://www.ncbi.nlm.nih.gov/pubmed/24194598). ChIP-Seq data for the transcription factors GAF and Yki [14] were correlated with Cbt ChIP-Seq data. To produce the reads' graphical distribution for each sample around the TSS, we calculated the average number of reads on each position from 1000 bp upstream to 100 bp downstream of the TSS of all genes (according to RefSeq), normalizing by the total number of reads for each ChIP-Seq experiment. To show the location of GAF, Yki and Cbt binding sites along the promoters (-1000 to +100 bp from TSS) of their common target genes, we focused on the region ± 25 bp around the summit of each ChIP peak.

RNA extraction and Real-Time PCR

RNA was isolated from wing discs with ZR Mini RNA isolation Kit (Zymo-Research) following the manufacturer's instructions. For retrotranscription, 700 ng of RNA for each condition and retrotranscriptase M-MLV (Promega) were used. For Real-Time PCR, we used Syber green reagent (Roche). At least four independent samples were performed and analyzed following the ΔΔCt method.The following primers were used for RNA quantification:

Cbt (5'CACTAAGGGAAAACAAGTTGG3', 5'TTCTGACTCTTTTGGGCCAC3')
cycD (5'AGCAAGTGATATGGCAAGG3', 5'ATGGCTCTAGATTCCGAGTG3')
cut (5'ATGCTTTCTCTTCTCACCCTC3', 5'CACGACGACCAGGACAAG3')
ap (5'AGGACTGTGCACGGAGATG3', 5'AGGACTGTGCACGGAGATG3')
vg (5'GAAGCTGAAGCAGCGGTG3', 5'GACCGGAGGAGCAGGATG3')
wg (5'TCGAGCGCACGTCCAAGC3', 5'TCGCATCCAGCAGGTCTTC3')
fj (5'GTGCGGGATGTTTTACTTC3', 5'TTCATTAGCTGCCCTGGCAC3')
DIAP1 (5'GGAGAGCTCTTCGATTGGA3', 5'TGATGTCTGCTGCTCTTCC3')
dm (5'ACCCCCTCAGATTCCGATG3', 5'AAGTGGCACGAGGGATTTG3
sply (5'ACTTGAGCTCCACTAAAACG3', 5'AGAACTGACGCTTGCCACG3')

Selected primers were used for ChIP and ChIP-reChIP experiments:

rn (5'GCCAGCTCGCAAGAATTCC3', 5'CTGCGATTTGGCGGCGAG3'),
nub (5'GTCTCTTTTTCCCCAACGG3', 5'CACTCACCACTACTACTGC3'),
cycA (5'TTTCCCGGTCAACACGTCG3', 5'CATATATGGCCACACTGTCG3')
wg (5'GAGGTTGCGCAAATAATCGG3', 5'TGTGCGGTATATAGCCAG3')
vg (5'CGCTCCTTTTGCCAGTTTG3',5'ACGCTGAAATCGAAAATCCG3')
ex (5'GACTGATCCGGTGGGTAAC3', 5'AAAAAAAGTACATGCCACTCC3')
fj (5'GAGCATTAAAATCAACAGAGG3', 5'AGAGGGAGAGCGAAGAGAG3')
dm (5'ACCAGTGTGCGGGAGAGG3', 5'ATCACATTTGGATTCATCACG3')
CG34333 (5'CTTGCAACTCAGCTTCCAC3', 5'CAATGCGATGGTGGAGTGC3')

Co-IP and Western blot assays

For co-immunoprecipitation experiments from S2 cells, two million cells were transfected with pAC-*yki*-HA (kindly provided by N. Tapon), pAct5CPPA-*GAF*-Flag (kindly provided by J. Bernués) or pAC5.1-*cbt*-V5 using Cellfectin reagent (Invitrogen) following the manufacturer's instructions. After 48h, protein extracts were recovered using lysis buffer (50 mM Tris-HCl pH8, 140 mM NaCl, 1.5 mM $MgCl_2$ and 1% NP-40). IP buffer and 2 µl of anti-HA (Roche), anti-flag (Sigma-Aldrich) or anti-V5 (Sigma-Aldrich) antibodies were used for immunoprecipitation. Complexes were precipitated with 35 µl of protein-Sepharose A or G affinity matrix. Protein extraction samples were used as input (INP) and no transfected immunoprecipitated samples as a negative control.

Samples were run on 8% SDS-PAGE gels. Anti-Cbt antibody (1:1000), anti-Yki (kindly provided by KD. Irvine, 1:2000), anti-GAF (kindly provided by J. Bernués, 1:2000), anti-αTub (Molecular probes, 1:2000) and anti-Act5C (Hybridoma Bank 1:1000)were used for Western blot.

Sequential ChIP (ChIP-reChIP) and Real-Time PCR

ChIP-reChIP was performed as previously described [15]. Drosophila S2 cells overexpressing Yki-HA (10 million for IP) were fixed in Formaldehyde 1%. Samples were sonicated for 30' (Bioruptor , 50% on/off cycle, high). IP buffer and 4 µl anti-HA antibody were used for the first immunoprecipitation. For the second, we pooled 6 IP and used IP buffer and 2 µl of Cbt antibody [16]. Real-time PCRs were normalized against the mock (negative) sample and depicted as fold enrichment. Three independent samples were processed obtaining similar fold enrichment profiles.

Clone analysis

For wing clonal analysis, only clones inside the wing pouch were considered. Big clones possibly resulted from clone fusion and small clones inside the non-proliferative zone of the DV boundary were discarded. Whole stacks were used to measure clonal area using Fiji software. For eye imaginal disc clonal analysis whole stacks were used to measure clonal area using Fiji software. For analysis of clones in the adult eyes threshold of GFP intensity was set and percentage of positive GFP pixels for eye was measured.

Supplementary bibliography

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Supplementary figure legends

Figure S1 - Cbt binding occupancy and correlation with histone marks.

(A) Cbt occupancy profile in a region of chromosome 2L (in black) and the corresponding input control (in grey). Cbt peaks are depicted as boxes in black.
(B) Gene Ontology (GO) term enrichment of Cbt target genes computed by DAVID. The number of genes in each category is shown within the bars, all categories were statistically significant (p-value ≤ 0.05).
(C) Venn diagram showing the intersection between Cbt (blue), H3K4me3 (red) and H3K27me3 (green) target genes.
(D) UCSC Genome Browser overview of Cbt (black), H3K4me3 (red) and H3K27me3 (green) in *H15*, *p38b* and *salm* regions.
(E) FACS analysis showing cells selected for ChIP assay according to size, DAPi staining (death cells were discarded) and GFP levels.

Figure S2 - Motif analysis in Cbt bound DNA

For each motif identified by MEME-ChIP in the sequences of Cbt peaks, we displayed the p-value and the number of sites.

Figure S3 - Cbt association with GAF and Yki and Cbt influence in *fj and bantam* expression.

(A-B) Polytene chromosomes of third instar larvae stained with Cbt (green, A') and GAF (purple, A'') antibodies. Co-staining is observed in several regions. Scale bar = 10 µm. (B) Magnification of a polytene chromosome arm in A showing co-localization in the same regions. Green staining corresponds to Cbt and purple corresponds to GAF. Scale bar = 5 µm.
(C) Western blots showing Yki-HA, GAF-Flag and Cbt-V5 immunoprecipitations from S2 cells. Input (INP), immunoprecipitated samples (+) and negative control (-).

(D-E) Expression of *fj* reporter (green) in wt (D) and *en>cbt* (E-E') wing disc. Scale bar = 50µm.
(F-G) Expression of bantam-sensor (green) in wt (F) and *ptc>cbt* (red) (G-G'') wing discs.

Figure S4 - *cbt* levels influence proliferation and growth in imaginal tissues

(A-D) Wing discs containing clones of *cbt* RNAi (B) and *cbt* overexpression (C). Scale bar = 50µm. (D) Quantification of clone area of control (black), *cbt* RNAi (grey) and *cbt* overexpression (green). Error bars represent SEM. T-test (*) p-value≤ 0.04, n=10.
(E-H) Eye discs containing clones of *cbt* RNAi (F) and *cbt* overexpression (G) Scale bar = 50µm. (H) Quantification of clone area of control (black), *cbt* RNAi (grey) and *cbt* overexpression (green). Error bars represent SEM. T-test (*) p-value≤ 0.03, n=10.
(I-L) Wings expressing *cbt* RNAi (J) and overexpressing *cbt* (K) under $spalt^{E/Pv}$ promoter for 24h. Scale bar = 0.5 mm. (L) Quantification of wing area of control (black) *cbt* RNAi (grey) and *cbt* (green).Error bars represent SD. T-test (*) p-value≤ 0.000001, n=50

Figure S5 - Cbt is required for Yki activity in imaginal tissues

(A-E) Wing discs containing GFP-marked MARCM clones of the ex^{e1} mutant allele (B), *cbt* RNAi ($RNAicbt^{GD5044}$)(C) and ex^{e1}mutant clones with *cbt* RNAi (D). Scale bar = 50µm. (E) Quantification of clone area of control (black), *ex* mutant (grey), *cbt* RNAi (white) and ex^{e1} clones with *cbt* RNAi (green) Error bars represent SEM. T-test (*) p-value≤ 0.03, n=10..
(F-J) Eye discs containing GFP-marked MARCM clones of the ex^{e1} mutant allele (G), *cbt* RNAi ($RNAicbt^{GD5044}$)(H) and ex^{e1}mutant clones with *cbt* RNAi (I). Scale bar = 50µm. (J) Quantification of clone area of control (black), *ex* mutant (grey), *cbt* RNAi (white) and ex^{e1} clones with *cbt* RNAi (green) Error bars represent SEM. T-test (*) p-value≤ 0.02, n=10.

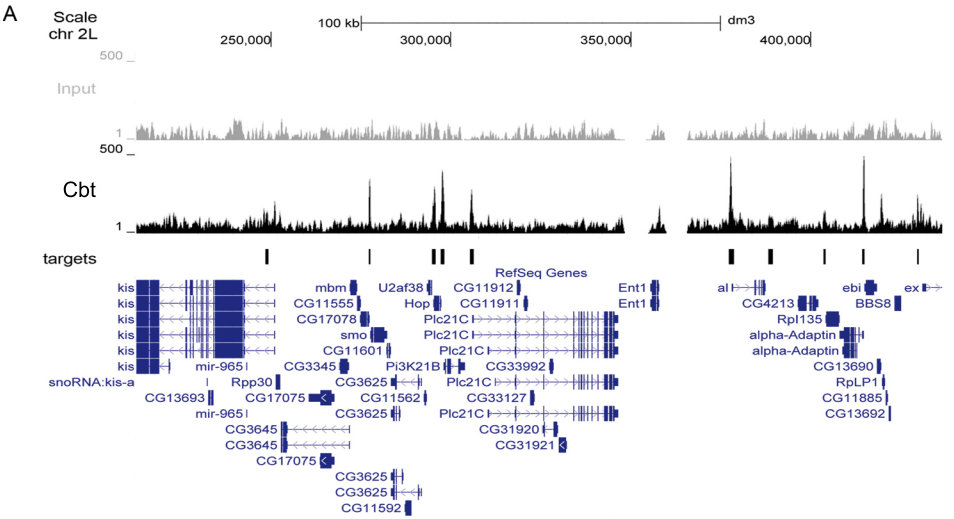
(K-O) Wing discs containing clones expressing *cbt* RNAi (L), *yki*^{S168A} (M) and *yki*^{S168A} with *cbt* RNAi (N). Scale bar = 50µm. (O) Quantification of clone area of control (black) *cbt* RNAi (grey), *yki*^{S168A} (white) and *cbt* RNAi with yki (green). Error bars represent SEM. T-test (*) p-value≤ 0.04, n=10.

(P-T) Eye discs containing clones expressing *cbt* RNAi (Q), *yki*^{S168A} (R) and *yki*^{S168A} with *cbt* RNAi (S). Scale bar = 50µm (T) Quantification of clone area of control (black) *cbt* RNAi (grey), *yki*^{S168A} (white) and *cbt* RNAi with yki (green). Error bars represent SEM. T-test (*) p-value≤ 0.04, n=10.

(U) Wing disc containing clones overexpressing *yki*^{S168A} with *cbt.* Scale bar = 50µm.

(V) Eye disc containing clones overexpressing *yki*^{S168A} with *cbt.* Scale bar = 50µm.

Figure S1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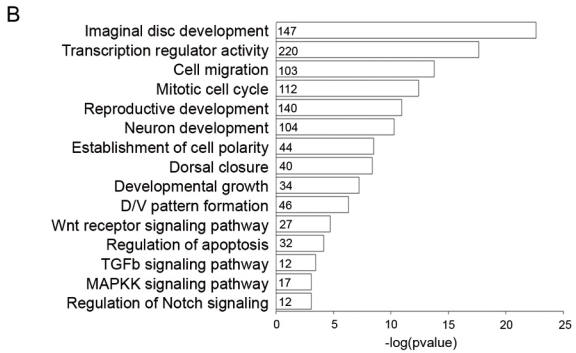
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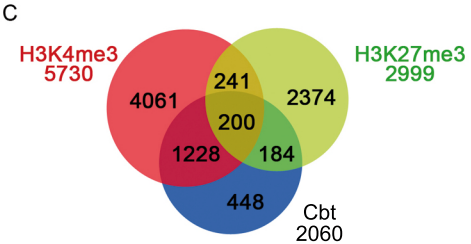
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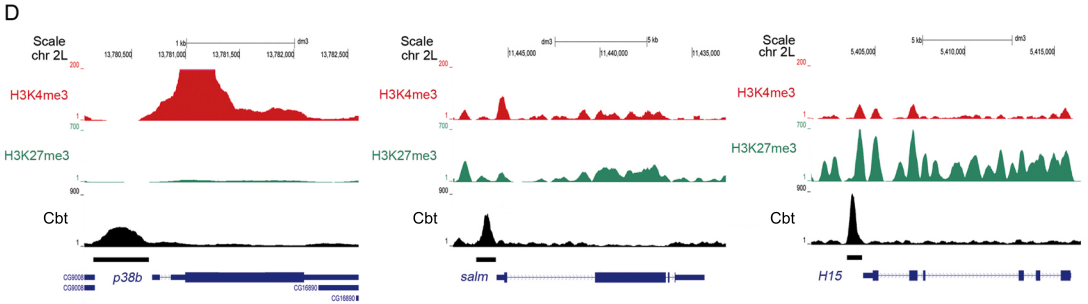
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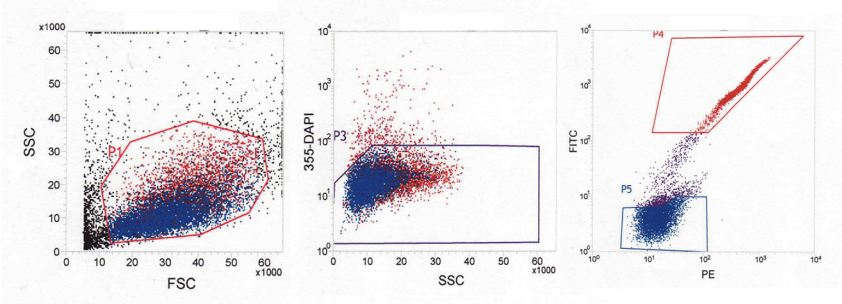
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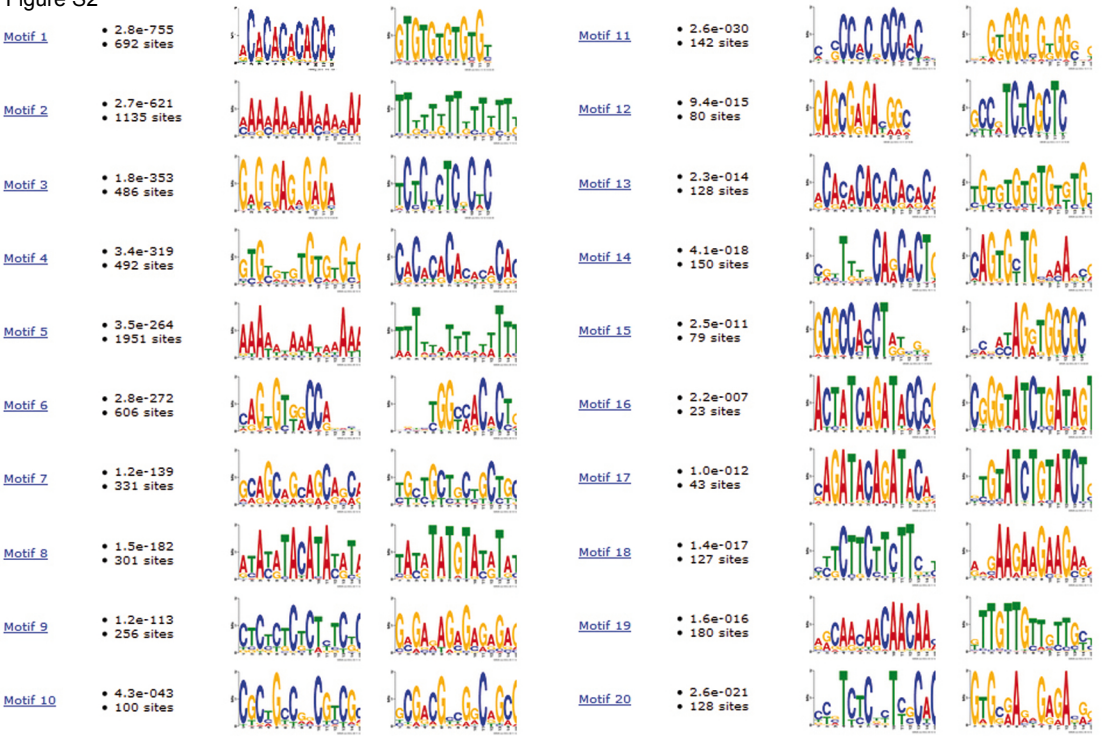
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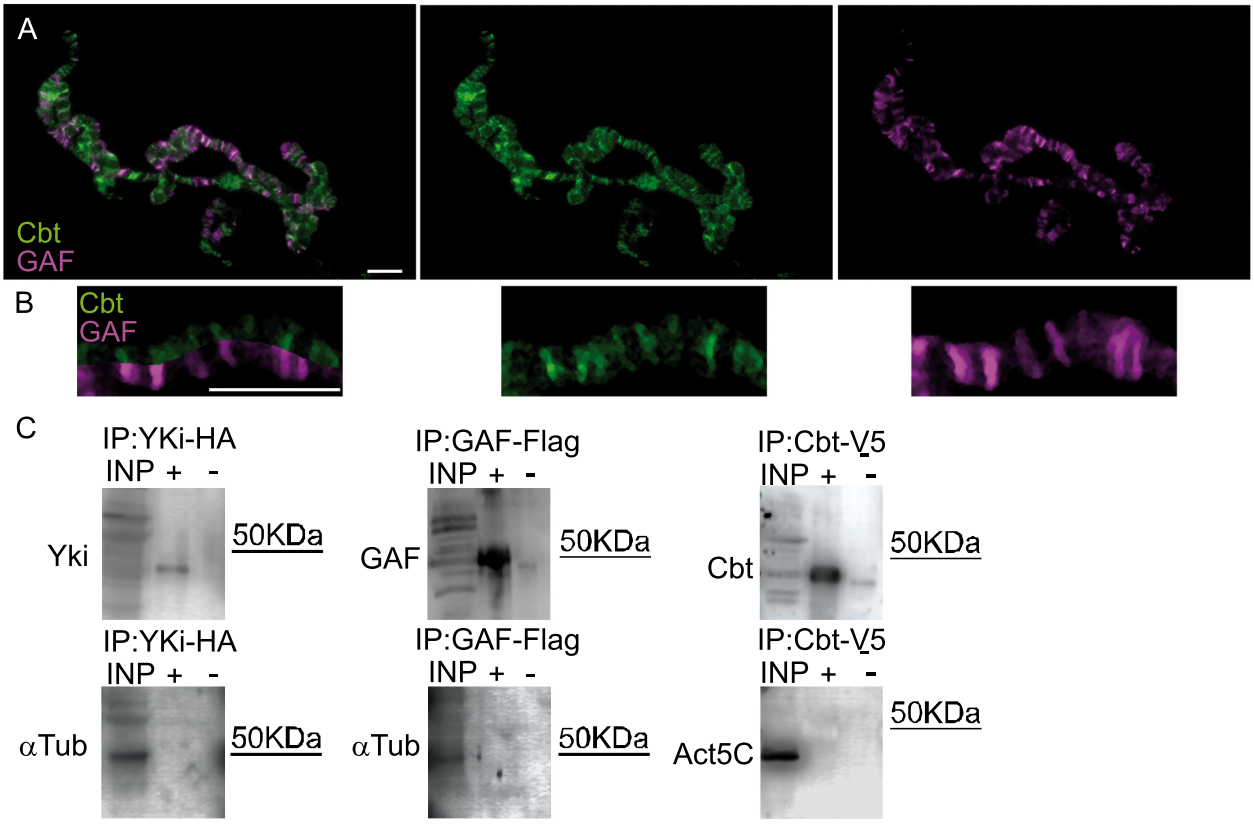
Figure S3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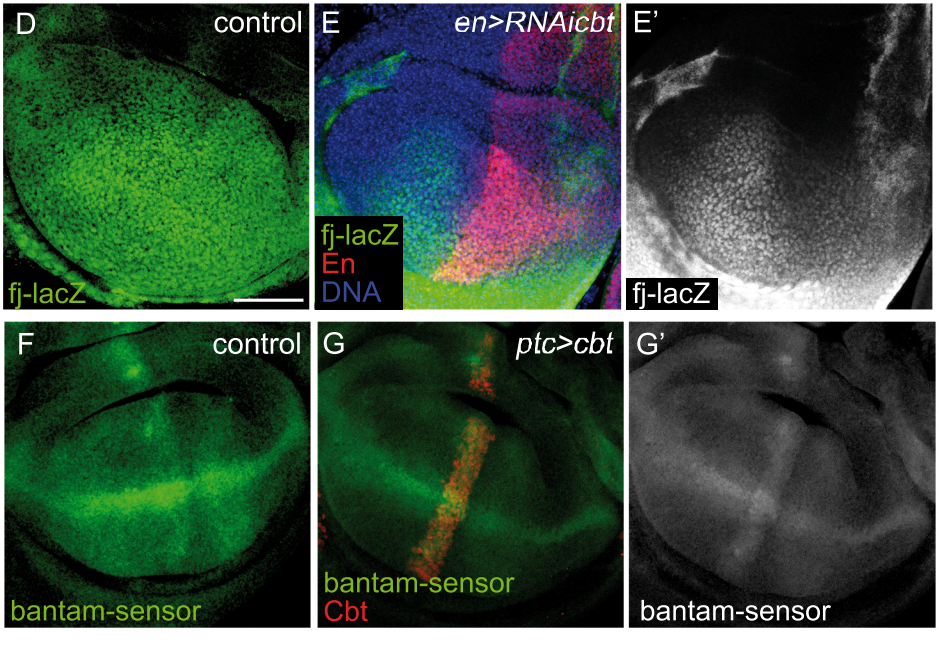
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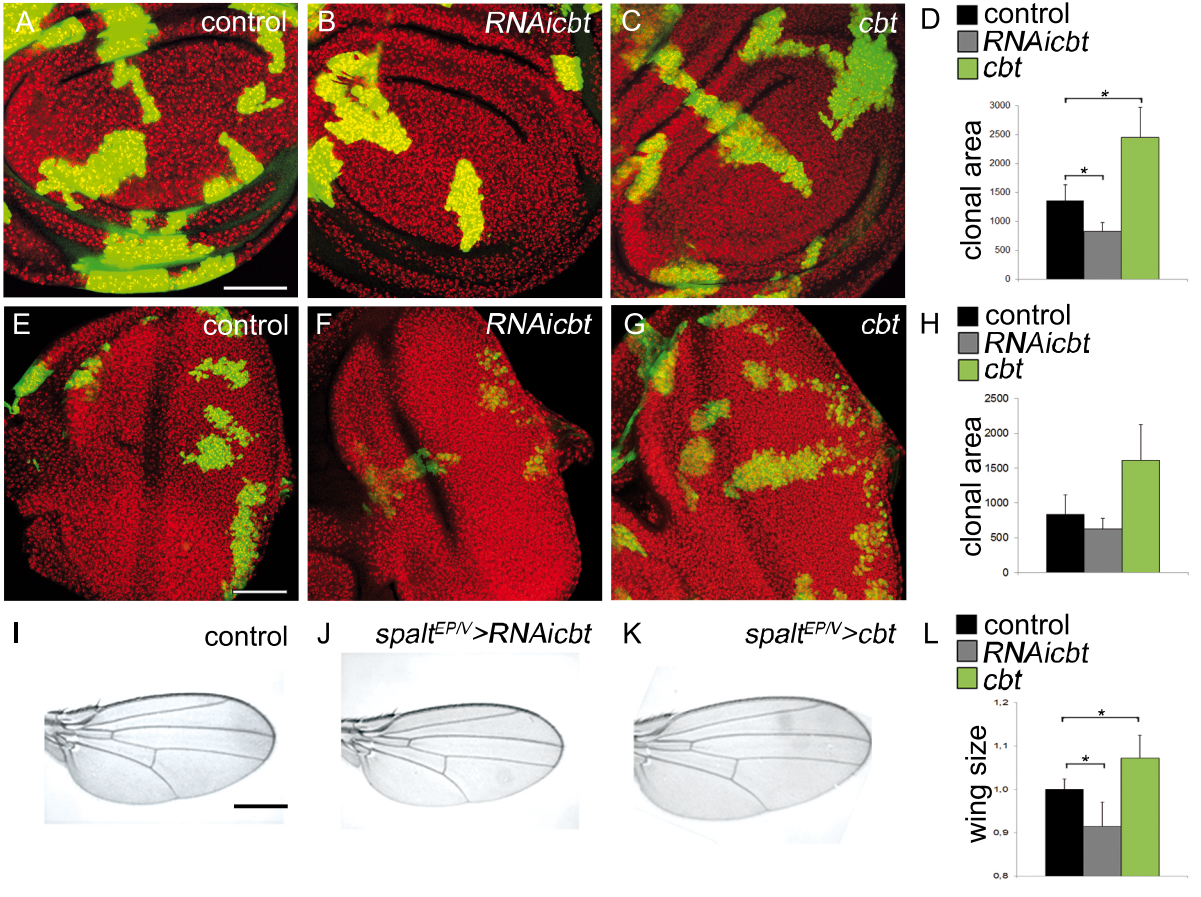
Figure S4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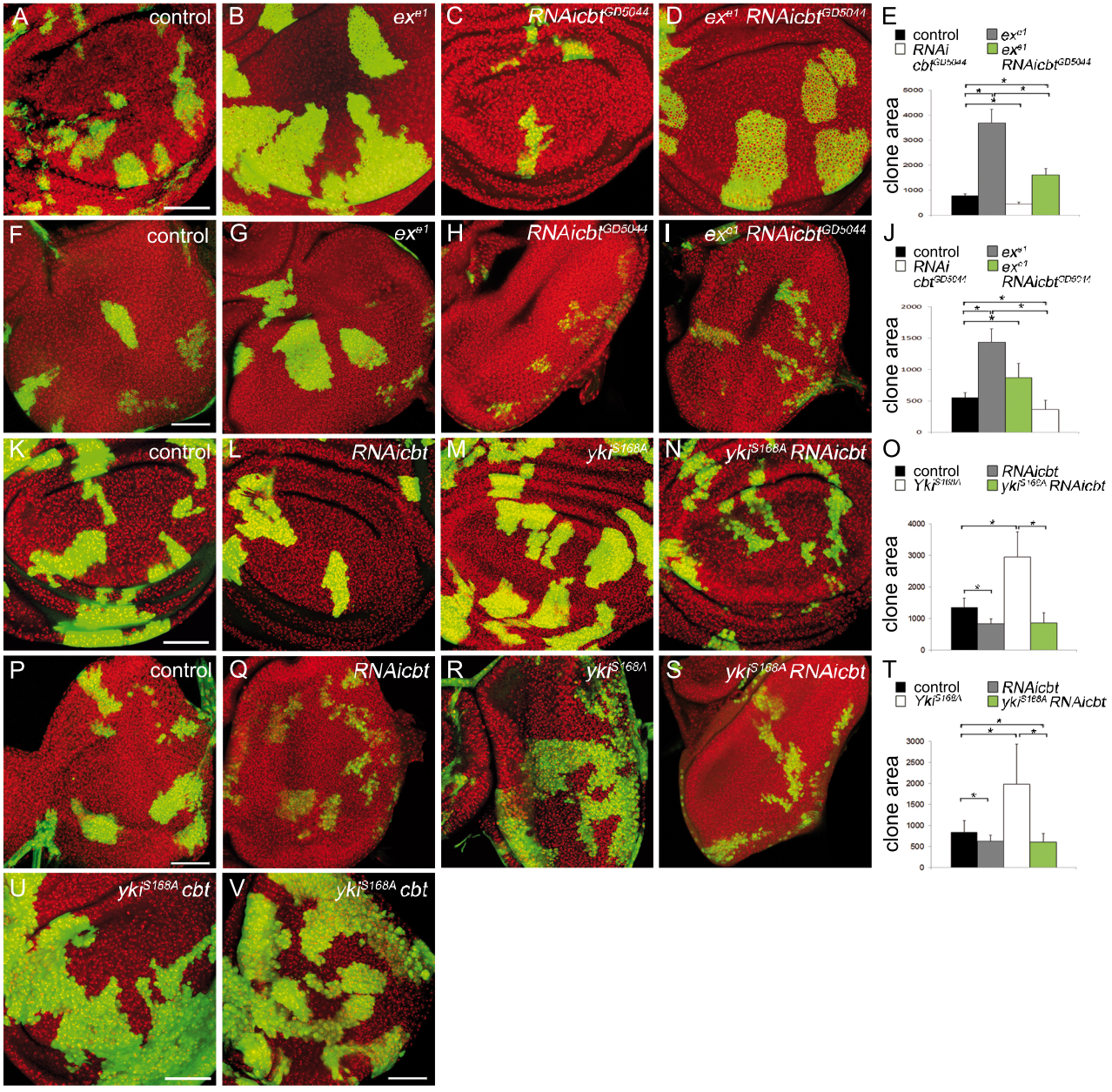
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