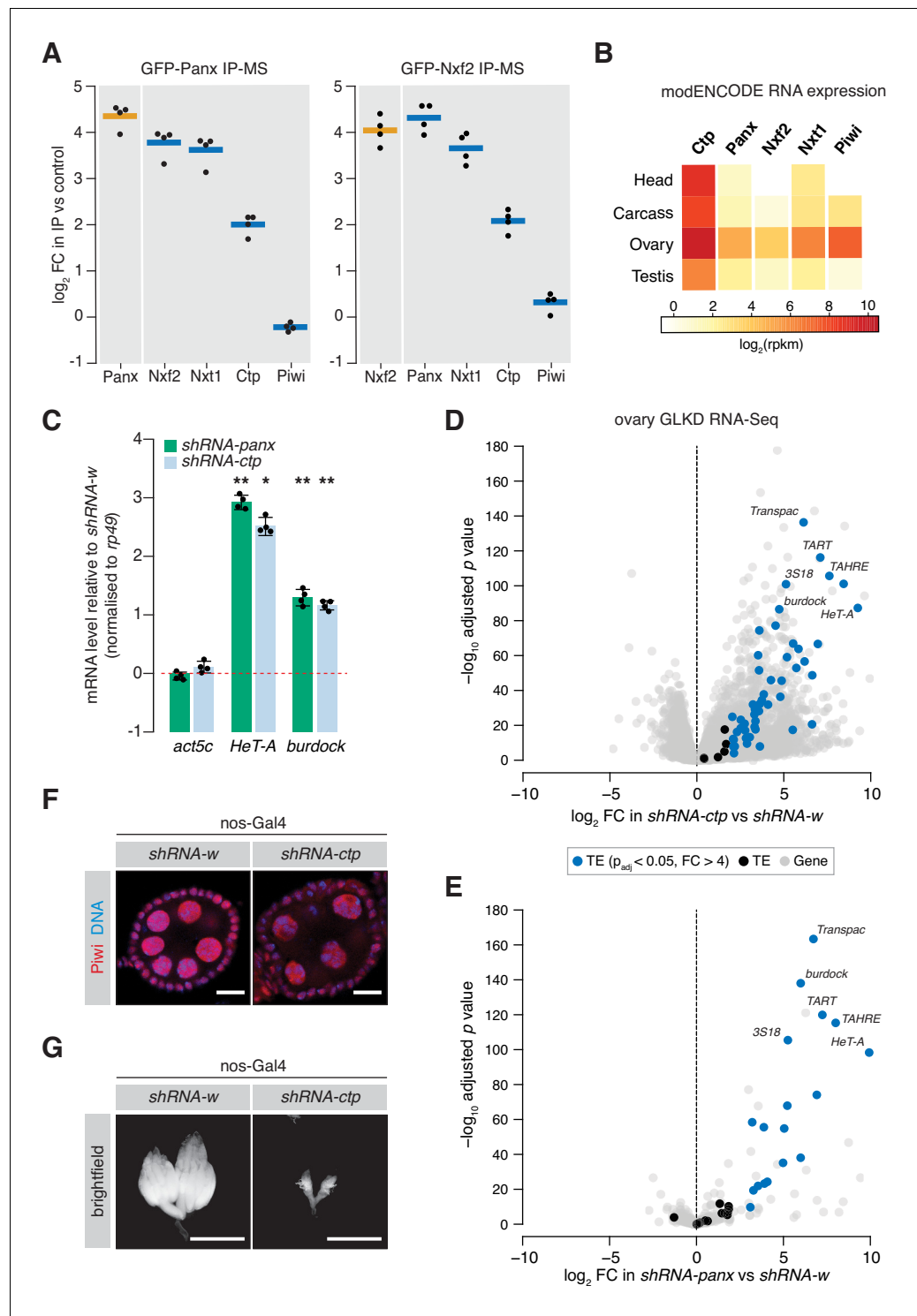# Figures and figure supplements

Dimerisation of the PICTS complex via LC8/Cut-up drives co-transcriptional transposon silencing in *Drosophila*

**Evelyn L Eastwood** *et al*

Figure 1. Ctp associates with the PICTS complex and is required for transposon silencing in the fly germline. (**A**) Enrichment plot showing the fold-change of PICTS complex proteins and Piwi in the GFP-Panx and GFP-Nxf2 IPs vs the control (n = 4). (**B**) Heatmap showing expression levels of *ctp*, *cdlc2*, *panx*, *nxf2*, *nxt1*, and *piwi* in various *Drosophila* tissues. (**C**) Bar graph showing fold-changes in ovary RNA levels of the house-keeping gene *act5c* and germline-specific transposons *HeT-A* and *burdock* upon germline knockdown of the indicated gene as measured by qPCR. (*) p<0.05 (**) p<0.001 (unpaired t-test). Error bars indicate standard deviation (n = 4). (**D**) Volcano plot showing fold-change and significance (adjusted p value) of genes and transposons between *ctp* GLKD and control as measured by RNA-seq. TE, transposable element. (**E**) As in D but comparing *panx* GLKD with control. (**F**)

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Figure 1 continued

Immunofluorescence images showing Piwi localisation in ovaries upon knockdown of the indicated gene. Scale bar = 10 µm. (**G**) Images showing ovary morphology visualised by DAPI staining upon knockdown of the indicated gene. Scale bar = 1 mm.
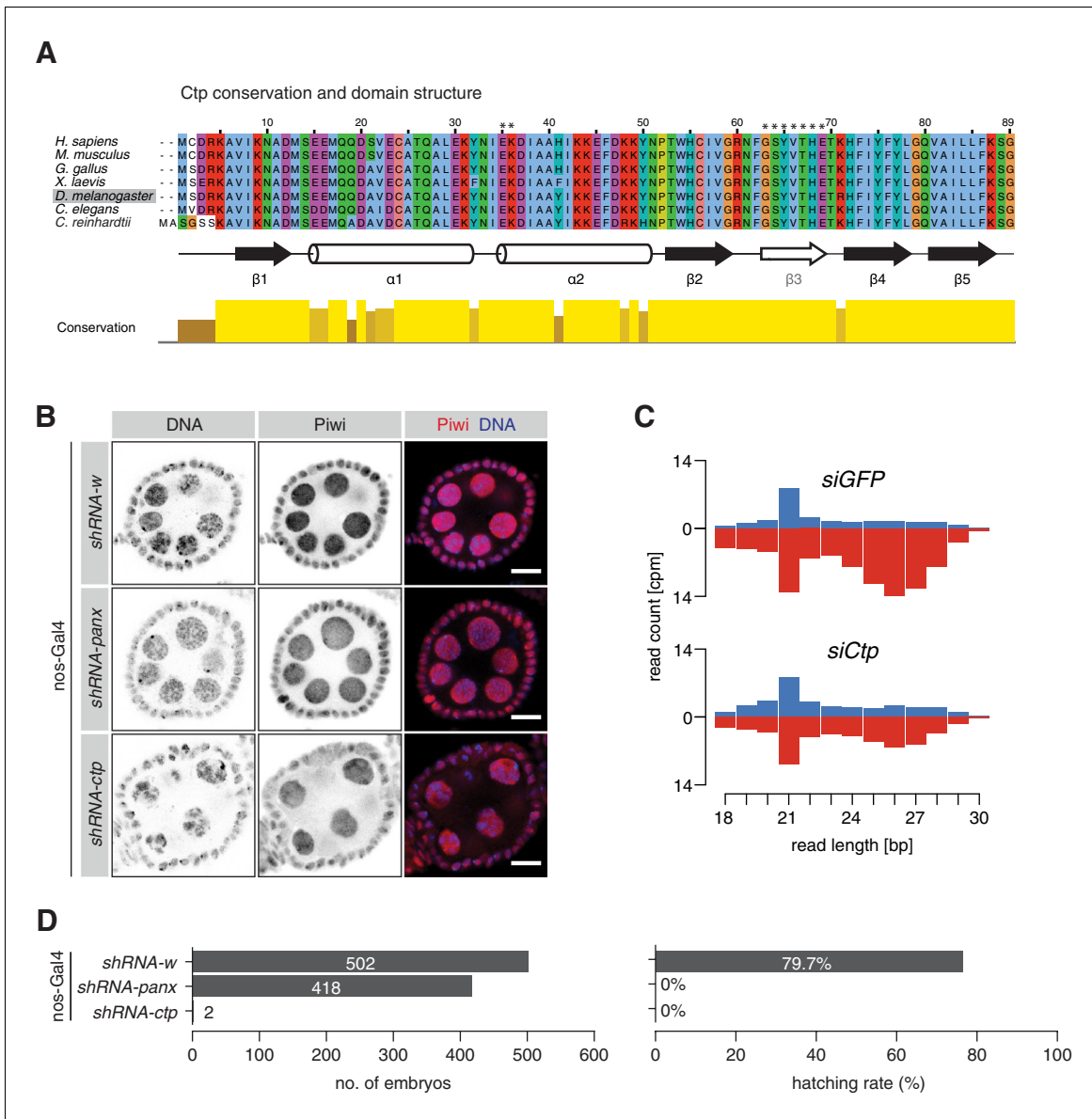
Figure 1—figure supplement 1. Ctp is highly conserved and essential for germline transposon repression. (**A**) Protein sequence alignment of Ctp orthologs across the indicated species. Known secondary structural elements are shown below and asterisks indicate residues that form the main contacts with the TQT motif. (**B**) Immunofluorescence images showing Piwi localisation in ovaries upon germline knockdown of the indicated gene. Scale bar = 10 µm. (**C**) Bar graphs showing the size distribution of transposon-mapping small RNAs from ovaries upon germline knockdown of the indicated gene (n = 2). Sense and antisense reads are shown in blue and red, respectively. (**D**) Bar graphs showing the number of eggs laid by six females over 3.5 days (left) and of those the percentage that hatched (right) for the indicated germline knockdown.
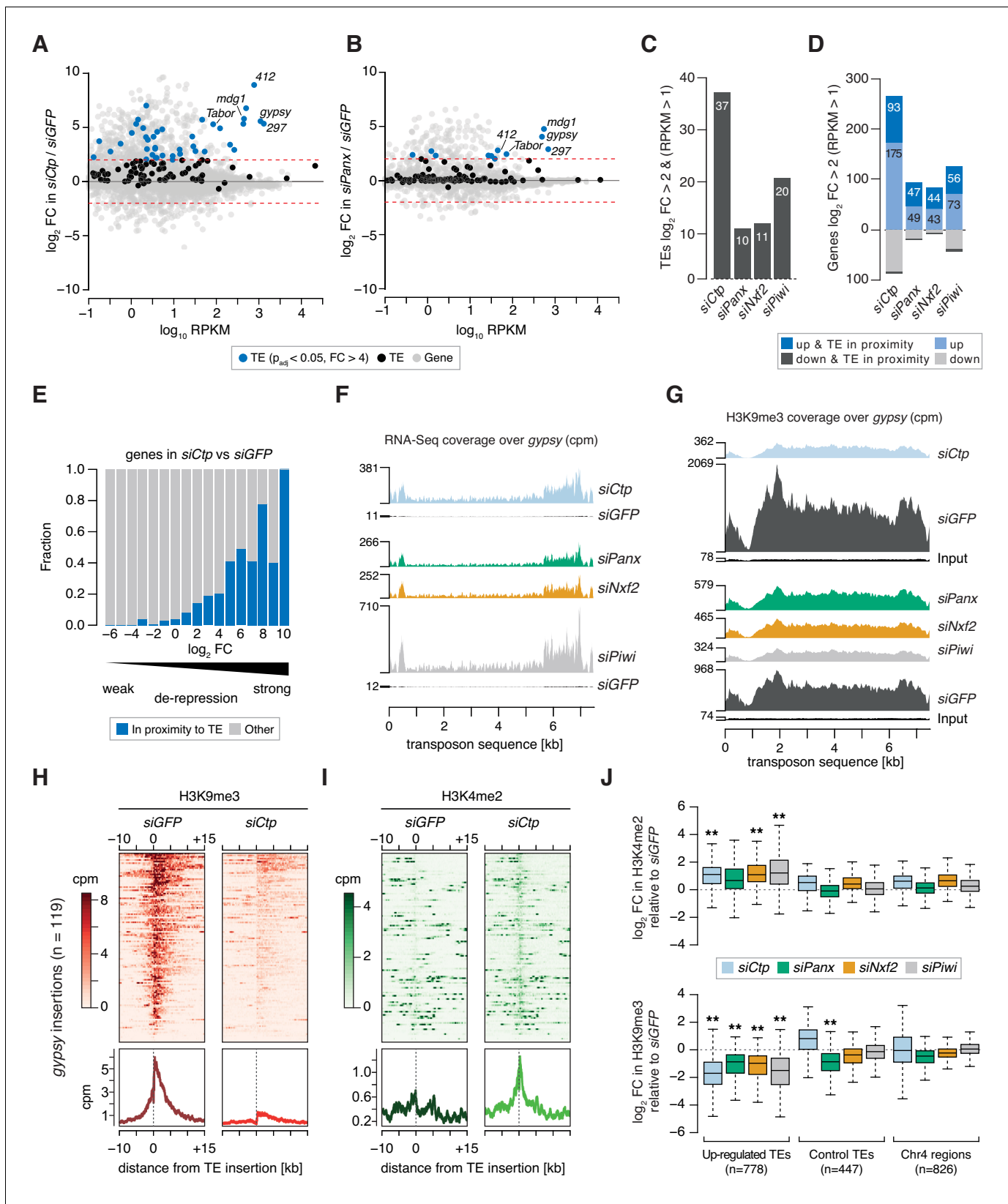
Figure 2. Ctp is required for transposon silencing and H3K9me3 deposition at transposon loci in ovarian somatic cells. (A) MA plot showing expression level against fold-change (FC) for genes and transposons in *siCtp* vs *siGFP*. Red dotted lines indicate $\log_2 FC = 2$. TE, transposable element. (B) As in A

Figure 2 continued on next page

Figure 2 continued

but comparing *siPanx* vs *siGFP*. (**C**) Bar graph showing the number of transposable element (TE) families de-repressed more than fourfold in the indicated knockdowns (RPKM >1). (**D**) Bar graph showing the number of mis-expressed genes in the indicated knockdowns, highlighting those with their promoters in proximity to a *gypsy*, *mdg1*, *297*, *blood* or *412* insertion (RPKM >1). (**E**) Bar graph showing the proportion of genes with promoters in proximity to a *gypsy*, *mdg1*, *297*, *blood* or *412* insertion according to their fold-change in *siCtp* vs *siGFP* (RPKM >1). TE, transposable element. (**F**) Coverage plots showing normalised reads from RNA-seq over the *gypsy* transposon consensus sequence for the indicated OSC knockdowns. (**G**) As in F but showing coverage plots of H3K9me3 ChIP-seq reads for the indicated OSC knockdowns. Reads from input libraries are shown below. (**H**) Heatmaps (top) and meta-profiles (bottom) showing H3K9me3 ChIP-seq signal in the 25 kb surrounding 119 *gypsy* insertions in OSCs (sorted for decreasing signal in the *siGFP* control). (**I**) As in H for H3K4me2 signal. (**J**) Boxplot showing the fold-change in H3K9me3 and H3K4me2 signal (cpm) in the indicated knockdown compared to *siGFP* in H3K9me3 and H3K4me2 signal across 1 kb genomic bins surrounding insertion sites of upregulated transposons (TEs; *gypsy*, *mdg1*, *297*, *blood*, and *412*) in OSCs. ** indicates >2 fold difference in median and p<0.001 (Wilcoxon rank sum test).
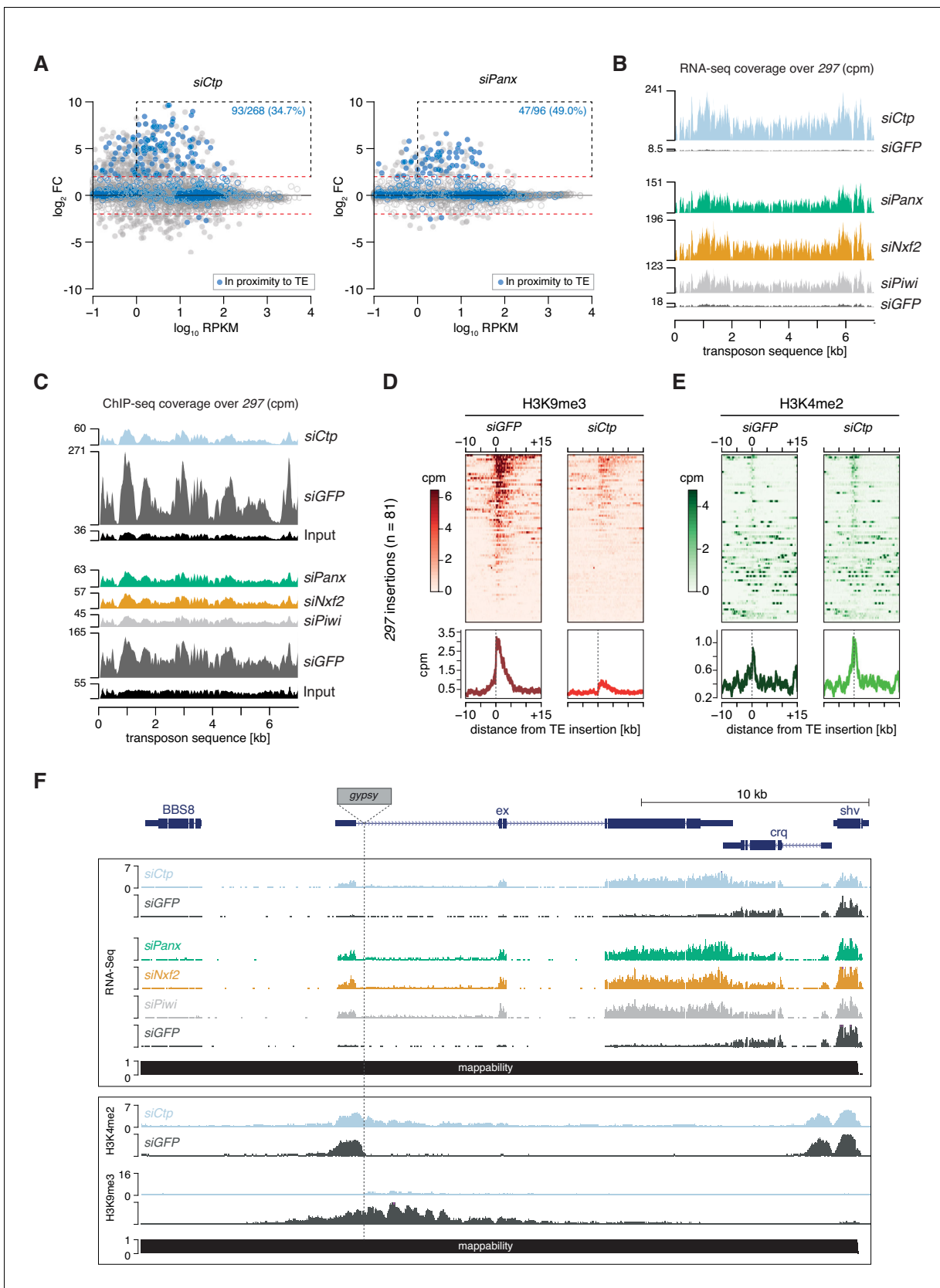
Figure 2—figure supplement 1. Transposon de-repression upon loss of Ctp is associated with changes at the chromatin level. (**A**) MA plots showing expression level against fold-change (FC) for genes in *siCtp* (left) or *siPanx* (right) vs *siGFP*. Red dotted lines indicate log₂FC = 2 and the black dotted

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box indicates overexpressed genes (log$_2$ FC >2, RPKM > 1). Blue dots indicate genes with promoters in proximity to a *gypsy*, *mdg1*, *297*, *blood* and/or *412* insertion used for quantification in **Figure 2D**. (**B**) Coverage plots showing normalised reads from RNA-seq over the *297* transposon consensus sequence for the indicated OSC knockdowns. (**C**) As in B but showing coverage plots of H3K9me3 ChIP-seq reads for the indicated OSC knockdowns. Reads from input libraries are shown below. (**D**) Heatmaps (upper) and meta-profiles (lower) showing H3K9me3 and H3K4me2 ChIP-seq signal in the 25 kb surrounding 81 insertions of the *297* transposon in OSCs (sorted for decreasing signal in the *siGFP* control). (**E**) As in D for H3K4me2 signal. (**F**) Genome browser shot displaying profiles of RNA-seq reads and the density of H3K4me2 and H3K9me3 coverage in OSCs upon the indicated knockdowns. Shown is an euchromatic *gypsy* insertion located within an intron of the 5' UTR of the gene *extended* (*ex*) located on chromosome 2L.
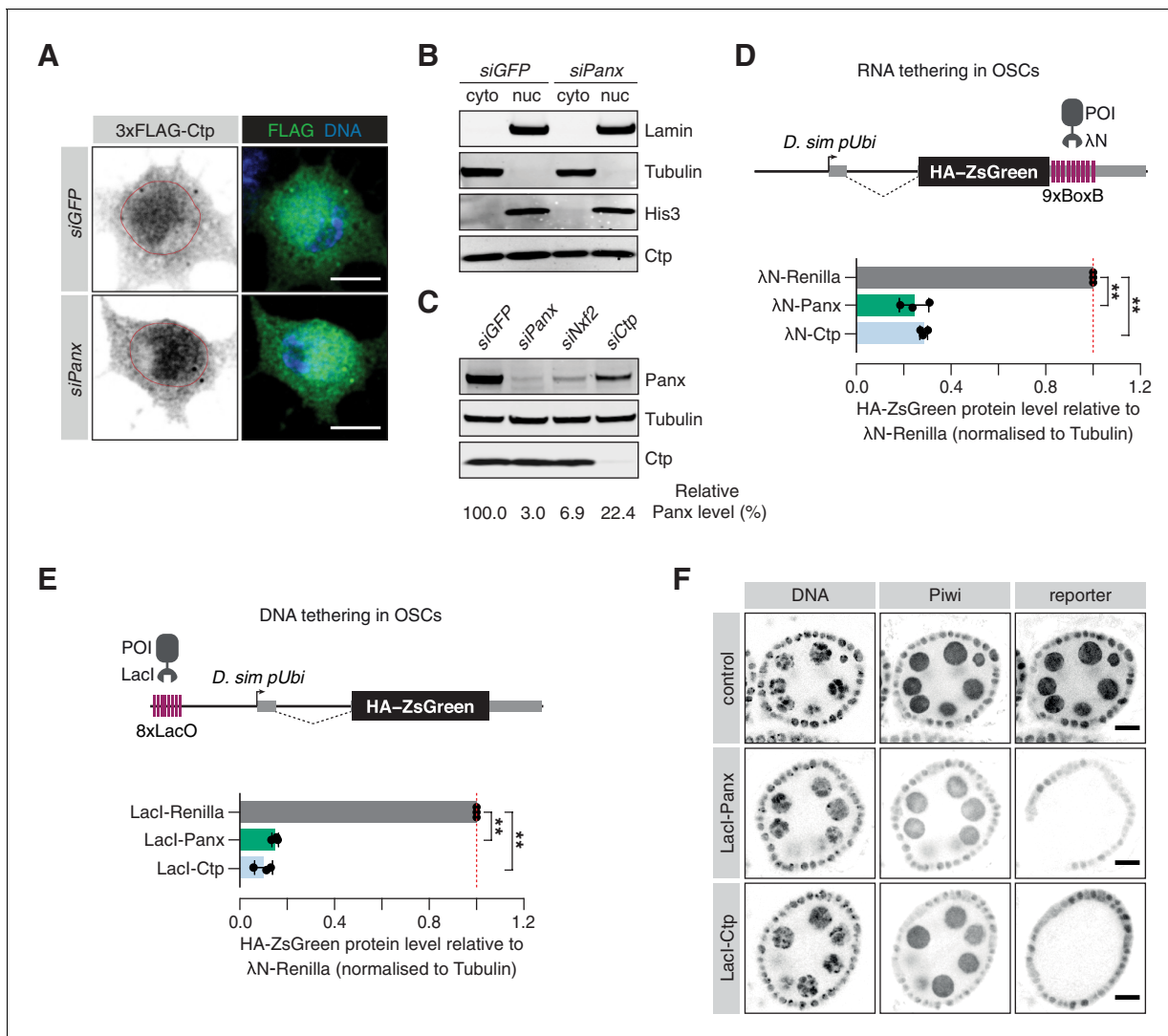
Figure 3. Ctp is involved in co-transcriptional gene silencing of transposons. (**A**) Immunofluorescence images showing the subcellular localisation of 3xFLAG-Ctp transiently transfected into OSCs upon the indicated knockdown. Scale bar = 5 μm. (**B**) Western blot for cytosolic proteins (Tubulin), nuclear proteins (Lamin and His3) and Ctp following subcellular fractionation of OSCs treated with the indicated siRNA. (**C**) Western blot showing Panx and Ctp protein levels in OSCs upon the indicated knockdown. The relative level of Panx protein, compared to *siGFP* and normalised to expression of Tubulin, is shown below. (**D**) Top: schematic showing the RNA tethering reporter system. Proteins of interest (POI) fused to the λN protein are recruited to the reporter mRNA (expressed from the *D. simulans* ubiquitin promoter) via BoxB sites in the 3'UTR. Bottom: bar graphs showing the level of HA-ZsGreen protein relative to the λN-Renilla control upon expression of λN-Panx or λN-Ctp. (******) p<0.001 (unpaired t-test). Error bars indicate standard deviation (n = 3). (**E**) Top: schematic showing the DNA tethering reporter system. Proteins of interest (POI) fused to the LacI DNA binding domain are recruited to the reporter locus via *LacO* sites upstream of the *D. simulans* ubiquitin promoter. Bottom: bar graphs showing the level of HA-ZsGreen protein relative to the LacI-Renilla control upon expression of LacI-Panx or LacI-Ctp. (******) p<0.001 (unpaired t-test). Error bars indicate standard deviation (n = 3). (**F**) Immunofluorescence images showing expression of Piwi compared to expression of the DNA tethering reporter in ovaries upon germline-specific expression of LacI-Panx and LacI-Ctp. Control indicates the parental stock expressing only the reporter. DNA is visualised with DAPI staining. Scale bar = 10 μm.
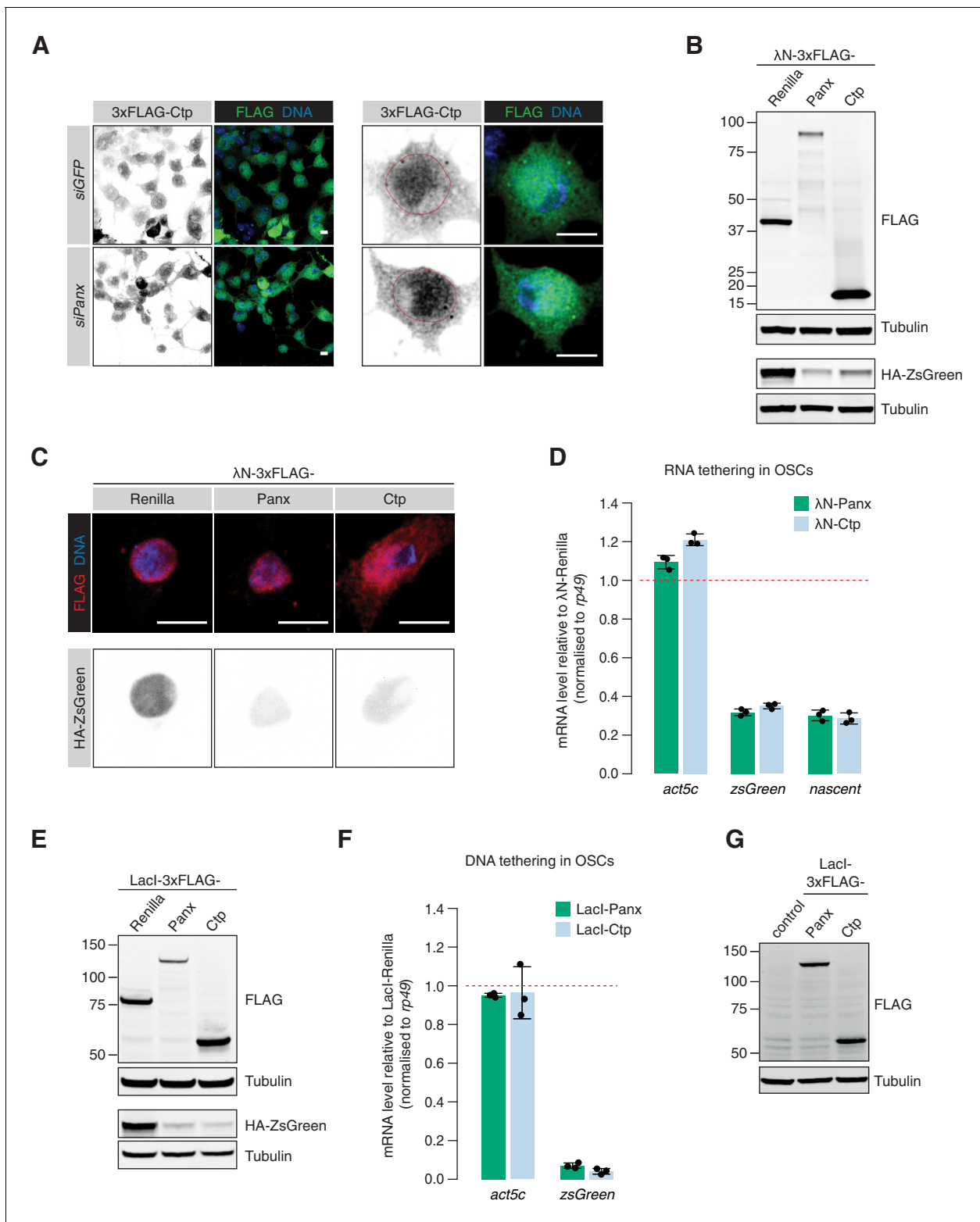
Figure 3—figure supplement 1. Ctp is a TGS factor. (A) Immunofluorescence images showing the localisation of 3xFLAG-Ctp in OSCs with the indicated knockdown. Scale bar = 5 μm. (B) Western blot analyses from OSC lysates showing the expression of the indicated λN-tagged construct and the level of the HA-ZsGreen protein used for the quantification in *Figure 3D*. Tubulin was used as a loading control. (C) Immunofluorescence images showing the localisation of the indicated λN-tagged construct in OSCs. The expression level and localisation of HA-ZsGreen is shown. Scale bar = 5 μm. (D) Bar graph showing the levels of *act5c* and *zsgreen* mRNA compared to λN-Renilla upon tethering of λN-Panx or λN-Ctp to the RNA reporter in

Figure 3—figure supplement 1 continued

OSCs. Primers that anneal in the reporter intron were used to quantify nascent transcript levels. Error bars indicate standard deviation (n = 3). (**E**) As in B for the indicated LacI-tagged construct in OSCs, showing the level of HA-ZsGreen protein for the quantification in *Figure 3E*. (**F**) As in D for the DNA tethering reporter in OSCs. (**G**) Western blot analyses from ovary lysates showing the expression of the indicated LacI-tagged construct.
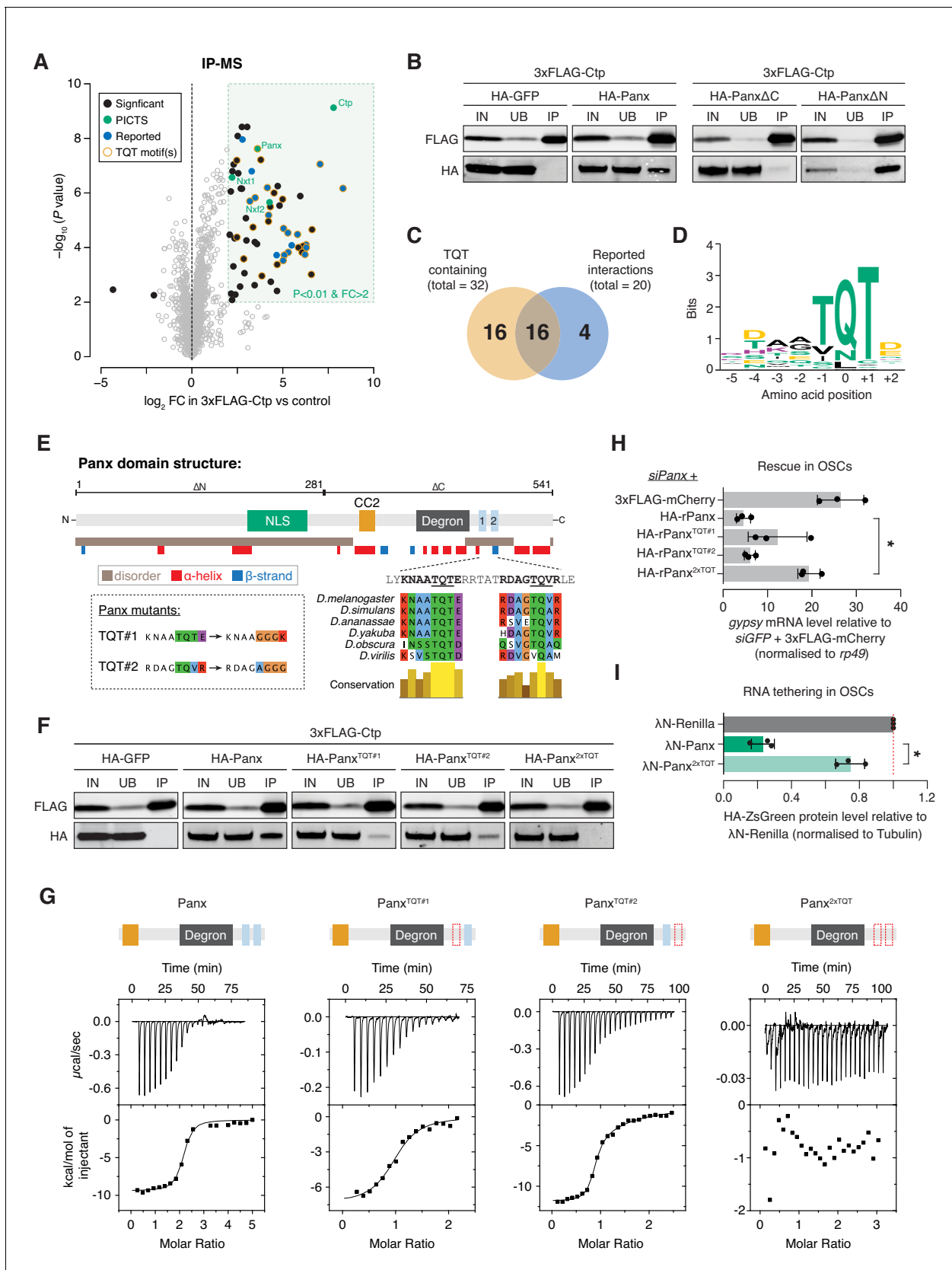
Figure 4. Ctp associates with PICTS via two highly conserved motifs in the carboxy-terminal region of Panx. (**A**) Volcano plot showing enrichment against significance for proteins identified by mass spectrometry that co-purify with 3xFLAG-Ctp from OSC lysates compared to the 3xFLAG-mCherry

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Figure 4 continued

control (n = 3). (**B**) Western blot analysis for 3xFLAG-Ctp and indicated HA-tagged constructs following FLAG immunoprecipitation from OSCs. IN=input (1x), UB=unbound (1x), and IP=immunoprecipitate (10x). (**C**) Venn diagram for proteins significantly enriched in the Ctp IP-MS indicating the overlap between previously reported Ctp interactors and TQT motif-containing proteins. (**D**) Sequence logo representing the most common Ctp recognition motif found in proteins co-purifying with Ctp from OSCs. Letter height represents relative amino acid enrichment and letters are coloured according to amino acid property (positive charge=purple, negative charge=yellow, polar=green and hydrophobic=black). (**E**) Schematic showing the known functional domains of Panx, including the nuclear localisation signal (NLS), Nxf2 interacting region (CC2), degron and TQT motifs, alongside a disorder and secondary structure prediction. TQT motif conservation across *Drosophila* species and mutations made in these sequences are indicated in the inset. (**F**) As in B. IN=input (1x), UB=unbound (1x), and IP=immunoprecipitate (10x). (**G**) Isothermal titration calorimetry thermograms for Ctp with Panx, Panx$^{TQT\#1}$, Panx$^{TQT\#2}$, and Panx^{2xTQT}. A schematic showing the Panx fragment (domains as in E) used in each experiment is indicated above. The red dotted outline indicates mutation of the TQT motif. (**H**) Bar graph showing relative fold-changes in OSC mRNA levels of the soma-specific transposon *gypsy* upon knockdown of panx and re-expression of the indicated rescue construct. (*) $p<0.05$ (unpaired t-test). Error bars indicate standard deviation (n = 3). (**I**) Bar graphs showing the level of HA-ZsGreen protein relative to the λN-Renilla control upon tethering of λN-Panx or λN-Panx^{2xTQT} to the RNA reporter in OSCs. (*) $p<0.05$ (unpaired t-test). Error bars indicate standard deviation (n = 3).
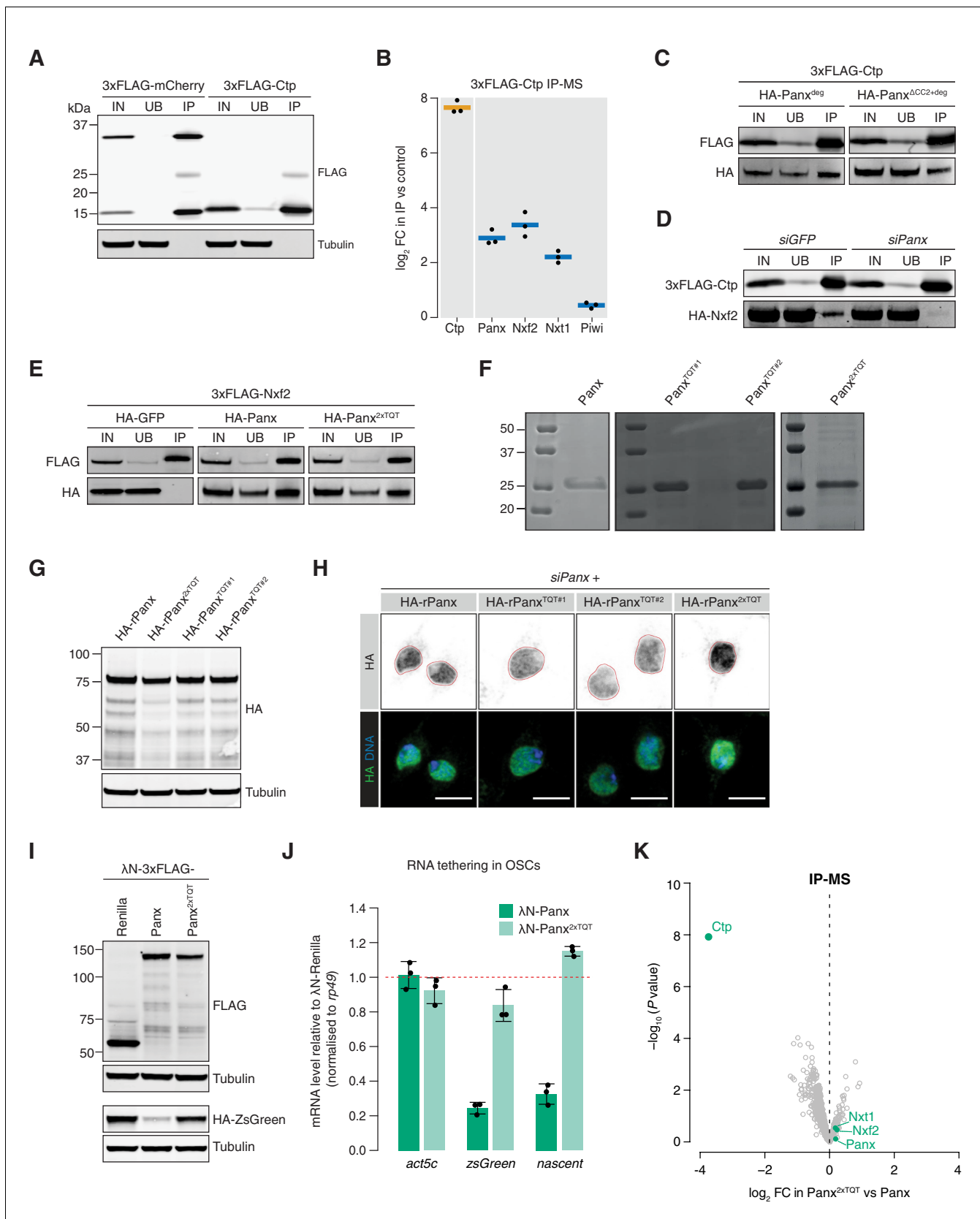
Figure 4—figure supplement 1. Two recognition motifs in the Panx C-terminus interact with Ctp. (A) Western blot analyses for the IP-MS experiment shown in *Figure 4A*. (B) Enrichment plot showing the fold-change of PICTS complex proteins and Piwi in the 3xFLAG-Ctp IP vs control (n = 3). (C)
*Figure 4—figure supplement 1 continued on next page*

Figure 4—figure supplement 1 continued

Western blot analysis for 3xFLAG-Ctp and indicated HA-tagged constructs following FLAG immunoprecipitation from OSCs. IN=input (1x), UB=unbound (1x) and IP=immunoprecipitate (10x). Panx^{deg} indicates mutation of four residues in the degron region that stabilise Panx in the absence of an interaction with Nxf2. (**D**) Western blot analysis for 3xFLAG-Ctp and HA-Nxf2 following FLAG immunoprecipitation from OSCs with the indicated knockdowns. IN=input (1x) UB=unbound (1x) and IP=immunoprecipitate (10x). (**E**) Western blot analysis for 3xFLAG-Nxf2 and indicated HA-tagged constructs following FLAG immunoprecipitation from OSCs. IN=input (1x), UB=unbound (1x) and IP=immunoprecipitate (10x). (**F**) SDS-PAGE analyses of the purified Panx fragments used for ITC experiments in *Figure 4G*. Protein was visualised with Coomassie staining. (**G**) Western blot from OSC lysates showing the relative expression level for the indicated HA-tagged rescue constructs. Tubulin was used as a loading control. (**H**) Immunofluorescence images showing the OSC subcellular localisation of the indicated HA-tagged rescue construct. The nuclear outline was defined using Lamin staining. Scale bar = 5 µm. (**I**) Western blot analyses from OSC lysates showing the expression of the indicated λN-tagged construct and the level of the HA-ZsGreen protein used for the quantification in *Figure 4i*. (**J**) Bar graph showing the relative levels of *act5c* and *zsgreen* mRNA upon tethering of λN-Panx or λN-Panx^{2xTQT} to the RNA reporter in OSCs compared to the λN-Renilla control. Primers that anneal in the reporter intron were used to quantify nascent transcript levels. Error bars indicate standard deviation (n = 3). (**K**) Volcano plot showing enrichment against significance for proteins identified by mass spectrometry co-purifying with 3xFLAG-Panx^{2xTQT} compared to 3xFLAG-Panx from OSC lysates (n = 3).
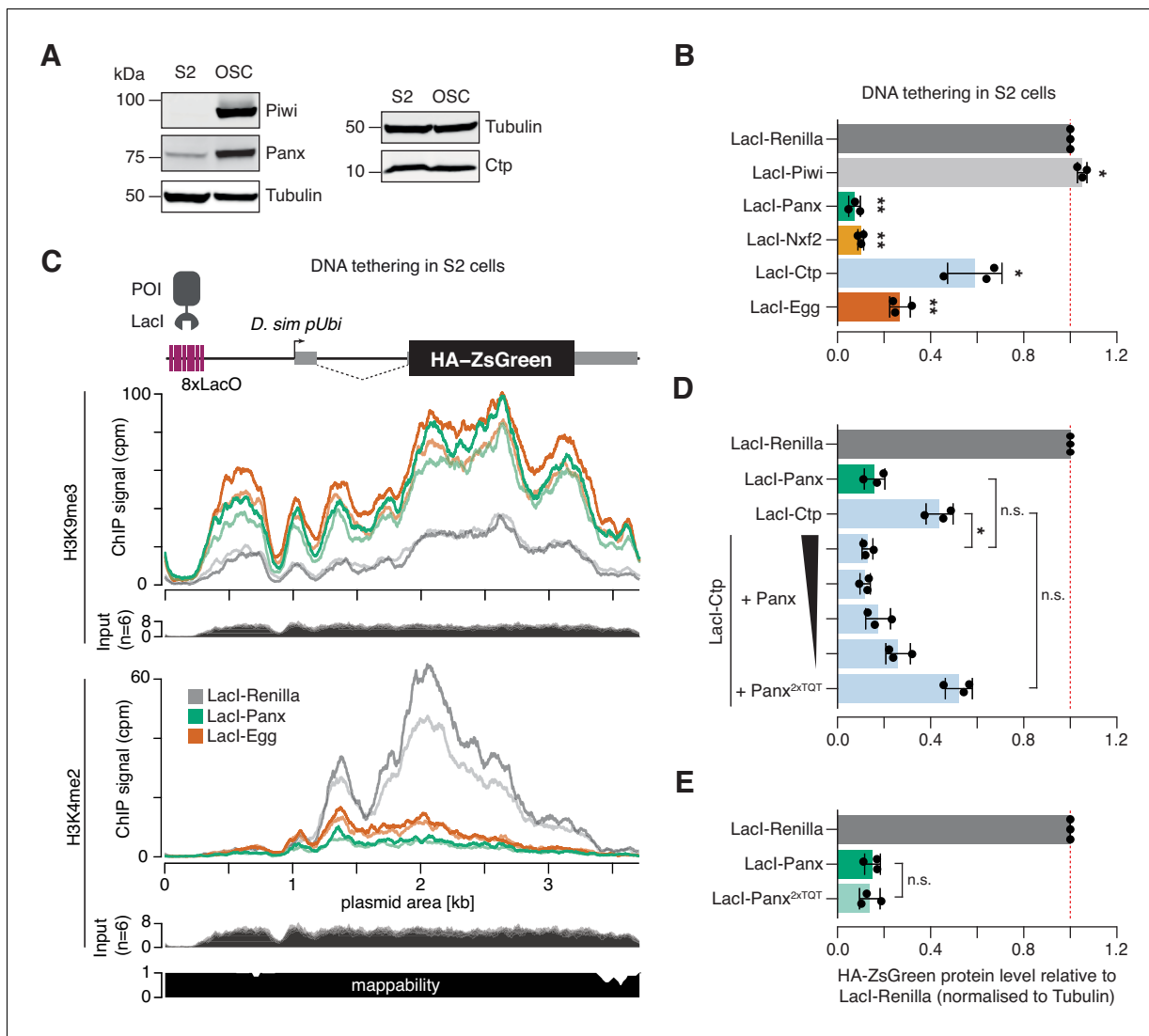
Figure 5. Ctp induces silencing via Panx. (**A**) Western blot analyses showing the relative level of Piwi, Panx, and Ctp in S2 cells compared to OSCs. Tubulin was used as a loading control. (**B**) Bar graphs showing the level of HA-ZsGreen protein relative to the LacI-Renilla control upon tethering of the indicated LacI-tagged protein to the DNA reporter in S2 cells. (*) p<0.05 (**) p<0.001 (unpaired t-test). Error bars indicate standard deviation (n = 3). (**C**) Coverage of H3K9me3 (middle) and H3K4me2 (bottom) ChIP-seq signal with corresponding input across the DNA tethering reporter locus. A schematic of the reporter is shown on the top and a mappability track is show below. (**D**) As in A with overexpression of Panx or Panx[2xTQT] indicated. The ratio of LacI-Ctp to HA-Panx plasmid ranges from 1:1 to 16:1 and is indicated by the black scale. (*) p<0.05 and n.s. = not significant (unpaired t-test). Error bars indicate standard deviation (n = 3). (**E**) Bar graphs showing the level of HA-ZsGreen protein relative to the LacI-Renilla control upon tethering of LacI-Panx or LacI-Panx[2xTQT] to the DNA reporter. n.s. = not significant (unpaired t-test). Error bars indicate standard deviation (n = 3).
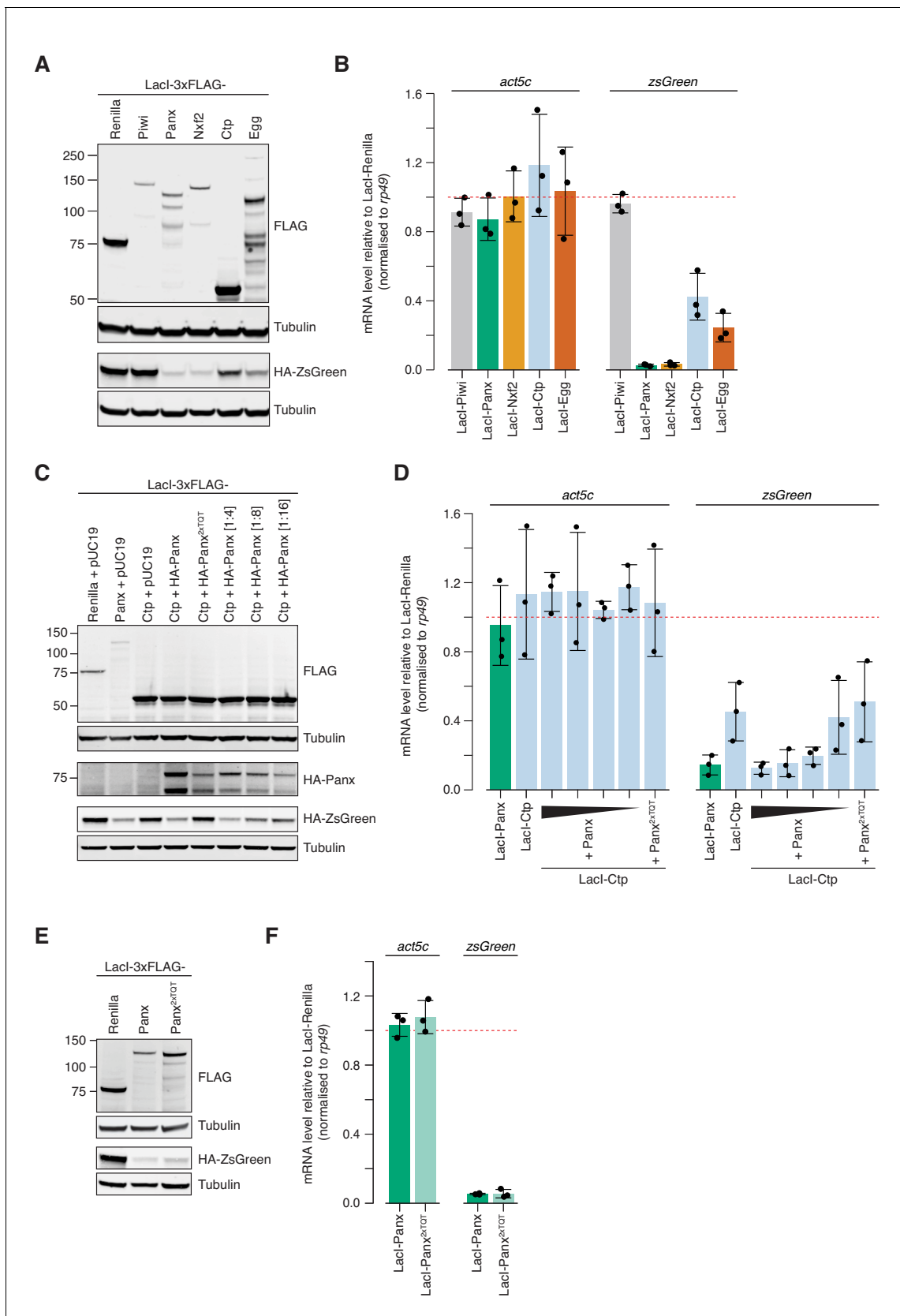
Figure 5—figure supplement 1. Repression by Ctp is mediated via Panx. (A), (C), and (E) western blot analyses from S2 cell lysates showing the expression of the indicated LacI-tagged construct and the level of the HA-ZsGreen protein used for the quantification in *Figure 5B, D and E*,

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respectively. Tubulin was used as a loading control. (**B**), (**D**), and (**F**) Bar graphs showing the relative levels of *act5c* and *zsgreen* mRNA upon tethering of the indicated LacI-construct to the DNA reporter in S2 cells compared to the LacI-Renilla control. Error bars indicate standard deviation (n = 3).
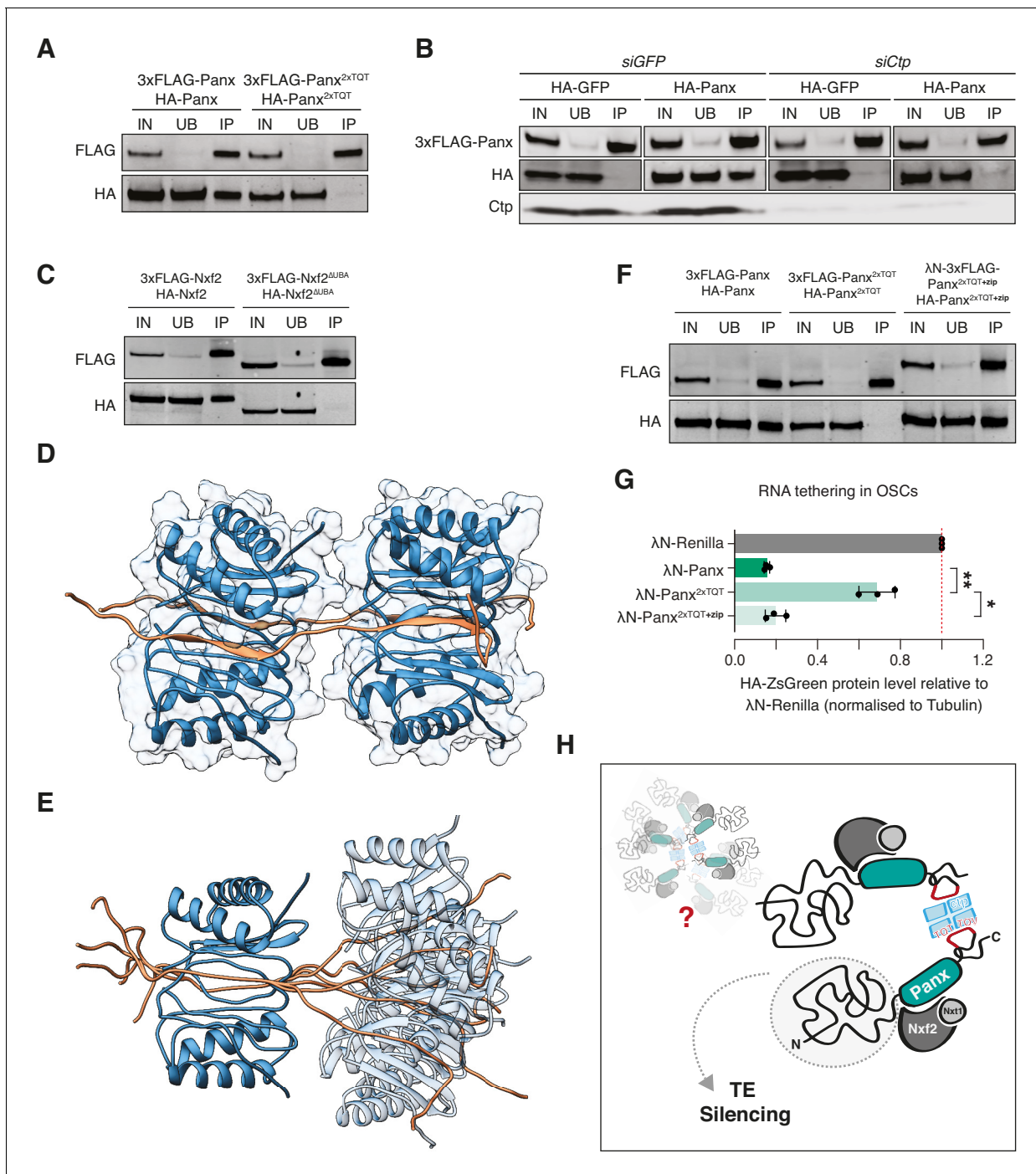
Figure 6. Ctp promotes higher order assembly of PICTS through dimerisation of Panx. (A) Western blot analysis for the indicated FLAG- and HA-tagged constructs following FLAG immunoprecipitation from OSCs. IN=input (1x), UB=unbound (1x), and IP=immunoprecipitate (10x). (B) Western blot analysis for 3xFLAG-Panx and either HA-GFP or HA-Panx following FLAG immunoprecipitation from OSCs treated with either *siGFP* or *siCtp*. IN=input (1x), UB=unbound (1x), and IP=immunoprecipitate (10x). (C) As in A. (D) and (E) Structural models showing two Panx peptides (amino acids 449–485) bound by two Ctp homodimers. For simplicity, a single structure from the ensemble is shown in D. The relative motional freedom of the Panx peptide when aligned on Ctp bound to the first TQT motif is shown in E. (F) As in A. (G) Bar graphs showing the level of HA-ZsGreen protein relative to the λN-Renilla control upon tethering of the indicated λN-Panx construct to the RNA reporter in OSCs. (*) $p<0.05$ (**) $p<0.01$ (unpaired t-test). Error bars indicate standard deviation (n = 3). (H) Model depicting possible PICTS complex organisation in which Ctp drives higher order assemblies of Panx-Nxf2-Nxt1 through dimerisation of the Panx C-terminus.
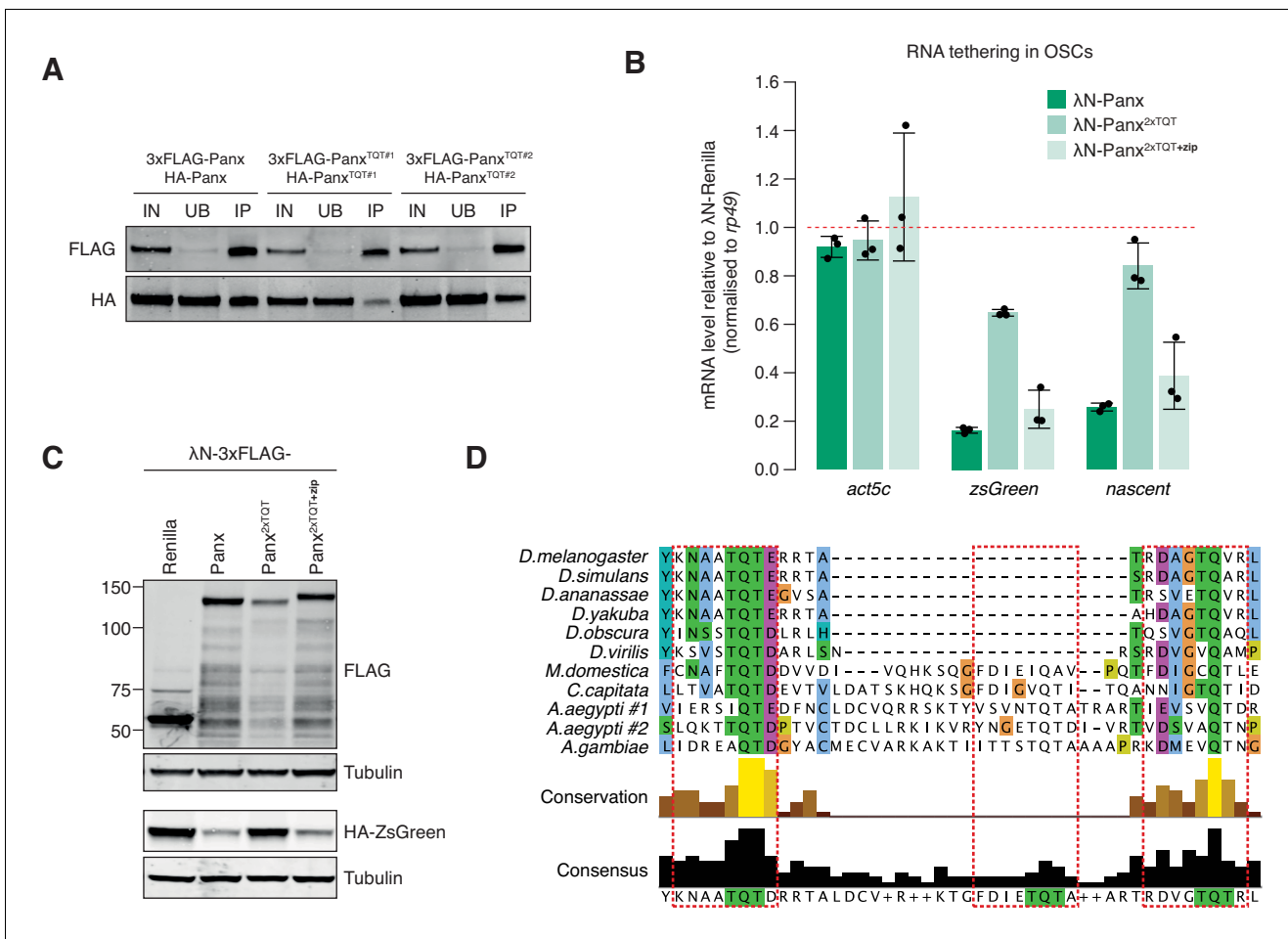
Figure 6—figure supplement 1. Dimerisation of Panx is essential for TGS. (**A**) Western blot analysis for the indicated FLAG- and HA-tagged constructs following FLAG immunoprecipitation from OSCs. IN=input (1x), UB=unbound (1x), and IP=immunoprecipitate (10x). (**B**) Bar graph showing the levels of *act5c* and *zsgreen* mRNA compared to λN-Renilla upon tethering of the indicated λN-Panx construct to the RNA reporter in OSCs. Primers that anneal in the reporter intron were used to quantify nascent transcript levels. Error bars indicate standard deviation (n = 3). (**C**) Western blot analyses from OSC lysates showing the expression of the indicated λN-tagged construct and the level of the HA-ZsGreen protein used for the quantification in *Figure 6G*. Tubulin was used as a loading control. (**D**) Protein sequence alignment of the Panx TQT-containing peptide across insect species. Predicted TQT motifs are highlighted in red boxes.