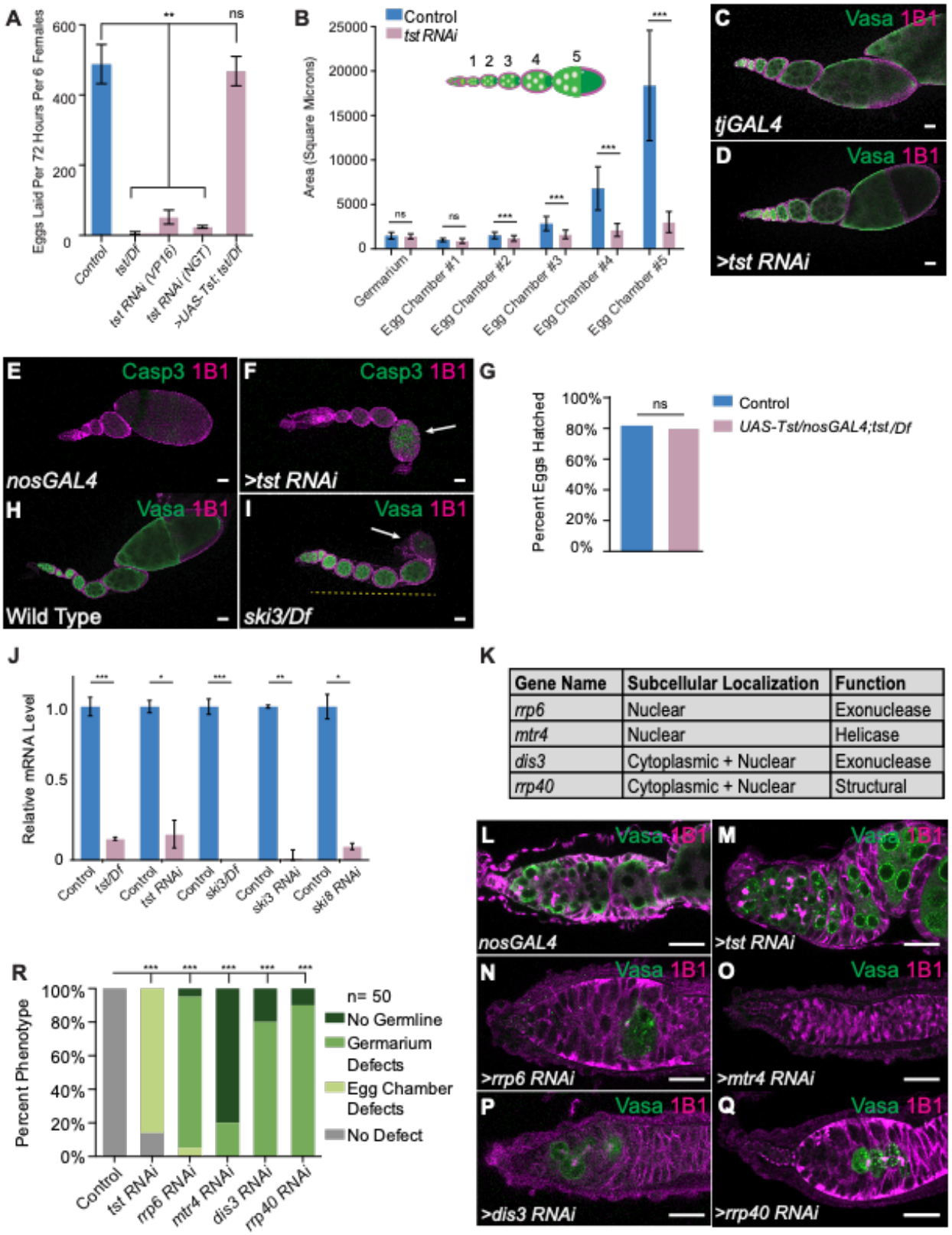
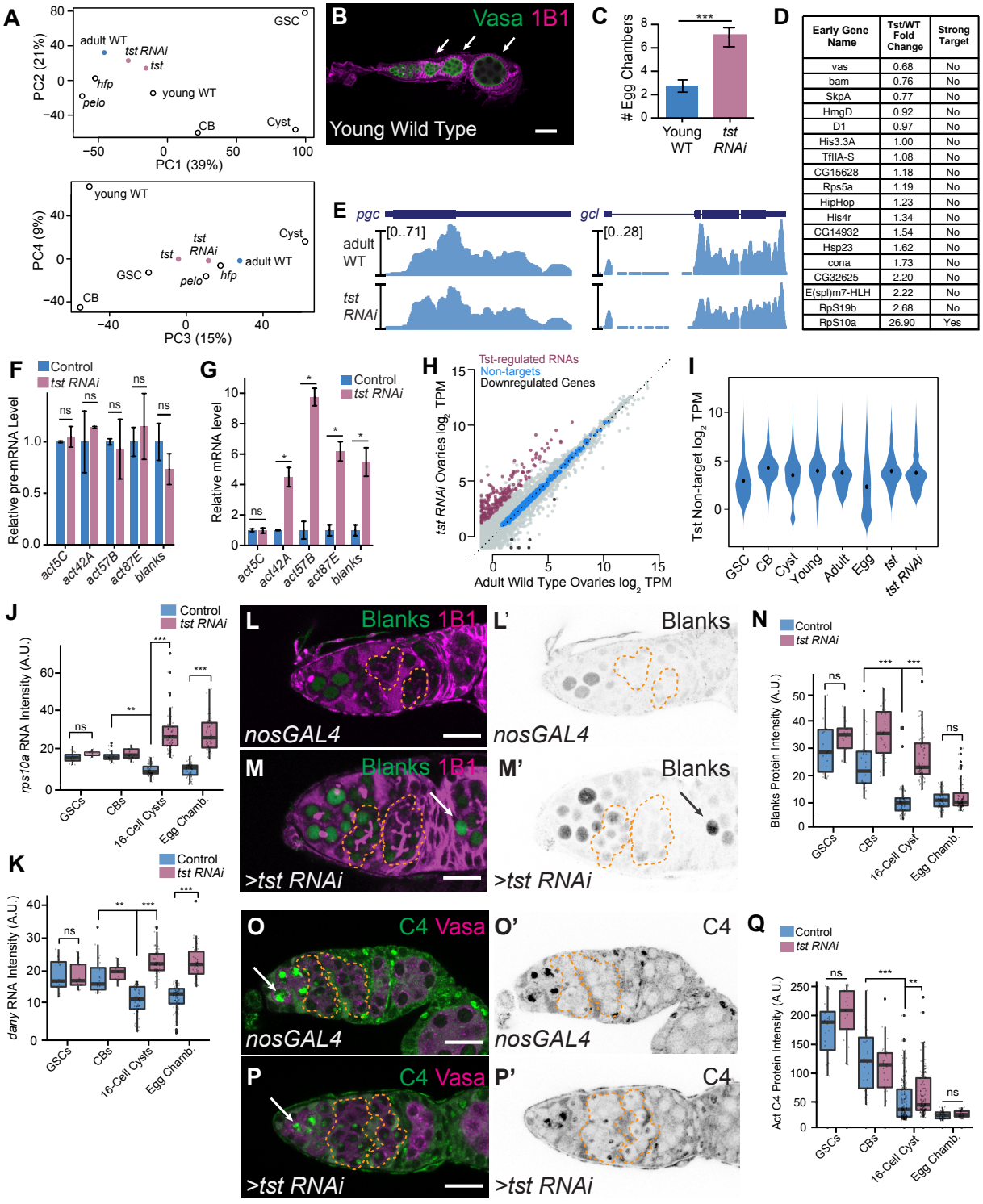


Gene Name	Subcellular Localization	Function
rrp6	Nuclear	Exonuclease
mtr4	Nuclear	Helicase
dis3	Cytoplasmic + Nuclear	Exonuclease
rrp40	Cytoplasmic + Nuclear	Structural

Figure S1. Ski complex components are required for successful oogenesis, related to Figure 1. (**A**) Egg laying assay after germline RNAi knockdown of Ski complex components

indicating a loss of fertility compared to driver control (n=2-545 eggs). ** = $p < 0.01$, Tukey's post-hoc test after one-way ANOVA, $p < 0.001$. Error bars are standard deviation (SD). (**B**) Quantification of the area of the germarium and egg chambers #1- #5 comparing control and *tst RNAi*, showing that egg chambers do not grow upon loss of *tst* (n=35 ovarioles). *** = $p < 0.001$, Student's t-test. (**C**) *tjGAL4* soma driver control and (**D**) *tst* soma *RNAi* ovarioles stained with Vasa (green) and 1B1 (magenta) do not exhibit a phenotype. (**E**) *nosGAL4* driver control and (**F**) *tst RNAi* ovarioles stained with cleaved Caspase 3 (green) and 1B1 (magenta) indicating dying egg chamber (arrow). (**G**) Viability assay showing there is no significant difference in eggs hatching between control and *UAS-Tst,nosGal4;tst*/Df (ns, $p>0.05$, df = 1, n=233-237 eggs, Chi-squared test). (**H**) WT control and (**I**) *ski3* mutant ovarioles stained with Vasa (green) and 1B1 (magenta) indicating egg chambers that do not grow in size (yellow dashed line) and dying egg chambers in *ski3* mutant (arrow). (**J**) qRT-PCR assaying the RNA levels of *tst*, *ski3* and *ski8* in their respective mutant background or germline RNAi normalized to control and indicating successful knockdown (n=3). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, Student's t-test. Scale bars are 10μm. (**K**) Table of exosome-associated genes, their subcellular localization, and molecular function. (**L**) *nosGAL4* driver control, (**M**) *tst RNAi*, (**N**) *rrp6 RNAi*, (**O**) *mtr4 RNAi*, (**P**) *dis3 RNAi* and (**Q**) *rrp40 RNAi* germaria stained with Vasa (green) and 1B1 (magenta) showing germarium defects and loss of germline in knockdowns of exosome-associated factors but not *tst RNAi*. (**R**) Quantification of phenotypes observed upon germline RNAi of exosome-associated factors (n=50 ovarioles). *** = $p <0.001$, Chi-squared test with Bonferroni correction, df=3).



Early Gene Name	Tst/WT Fold Change	Strong Target
vas	0.68	No
bam	0.76	No
SkpA	0.77	No
HmgD	0.92	No
D1	0.97	No
His3.3A	1.00	No
TfIIA-S	1.08	No
CG15628	1.18	No
Rps5a	1.19	No
HipHop	1.23	No
His4r	1.34	No
CG14932	1.54	No
Hsp23	1.62	No
cona	1.73	No
CG32625	2.20	No
E(spl)m7-HLH	2.22	No
RpS19b	2.68	No
RpS10a	26.90	Yes

Figure S2. Twister post-transcriptionally regulates a cohort of RNAs, related to Figure 2. (**A**) Principal Component Analysis (PCA) comparing RNA-seq data of adult WT, *tst RNAi*, *tst* genomic mutant (*tst*), *half pint* (*hfp*^{9}), *pelota* (*pelo*^{1}), young WT, germline stem cell enriched

(GSC), cystoblast enriched (CB), and differentiating cyst enriched (Cyst), indicating that the *tst* mutant and *tst RNAi* samples cluster closely with adult WT samples. (**B**) Young WT ovariole stained with Vasa (green) and 1B1 (magenta) indicating the presence of only 2-3 egg chambers. Scale bars are 10μm. (**C**) Quantification of the latest-stage egg chambers represented in young WT and *tst RNAi* (n=50 ovarioles). *** = $p < 0.001$, Wilcoxon rank sum test. (**D**) Early germ cell expressed genes identified by Jevitt et al, 2020 and their fold change in *tst* RNA Seq samples compared to WT, only *rpS10a* is upregulated >4 fold. (**E**) Genome browser tracks of maternal RNA genes *pgc* and *gcl* indicating similar expression in WT and *tst RNAi*. (**F**) qRT-PCR assaying the pre-mRNA levels of several Tst-regulated target genes, including *blanks*, *act42A*, *act57B*, *act87E* and the non-target *act5C* in control and germline *tst RNAi* normalized to control levels and indicating no change in transcription of these genes (n=3). ns = $p > 0.05$, Student's t-test. (**G**) qRT-PCR using random hexamer primed cDNA assaying for the levels of non-target *act5C* and Tst-regulated genes such as *act42A*, *act57B*, *act87E* and *blanks* in control and germline *tst RNAi* showing that increase in Tst-regulated RNA levels are not due to poly A selection (n=3). * = $p < 0.05$, Student's t-test. (**H**) Biplot of RNA-Seq data from adult WT and *tst* germ line RNAi knockdown ovaries in $\log_2$ Transcripts Per Million (TPM) highlighting upregulated RNAs (magenta) and downregulated RNAs (black) that change concordantly in both *tst RNAi* and *tst* genomic mutant ovaries; selected non-target RNAs (see methods) are in blue. (**I**) Violin plot of non-target genes from RNA-seq data sets that include GSCs, CBs, cysts, young WT, adult WT, unfertilized eggs (Egg), *tst* genomic mutant (*tst*), and germline *tst RNAi* (*tst RNAi*) showing that non-targets do not substantially change during the course of development. (**J**) Quantification of *rps10a* RNA expression in GSCs, CBs, cysts and egg chambers of WT control (blue) and *tst RNAi* (magenta) in Arbitrary Units (A.U.) (n=15-54 per cell type). *** = $p < 0.001$, ** = $p < 0.01$, Tukey's post-hoc test after two-way ANOVA, $p < 0.001$. (**K**) Quantification of *dany* RNA expression in GSCs, CBs, cysts and egg chambers of WT control (blue) and *tst RNAi* (magenta) in Arbitrary Units (A.U.) (n=13-68 per cell type). *** = $p < 0.001$, ** = $p < 0.01$, Tukey's post-hoc test after two-way ANOVA, $p < 0.001$. Confocal images of (**L-L'**) *nosGAL4* and (**M-M'**) *tst RNAi* germaria stained for 1B1 (magenta) and Blanks protein (green and grayscale) showing expanded Blanks expression in *tst RNAi* cysts (orange dashed lines) and sporadic expression in early egg chambers (arrow). (**N**) Quantification of Blanks protein expression in GSCs, CBs, cysts and egg chambers of WT control (gray) and *tst RNAi* (magenta) (n=16-92 per cell type) expressed in A.U. *** = $p < 0.001$, Tukey's post-hoc test after two-way ANOVA, $p < 0.001$. Confocal images of (**O-O'**) *nosGAL4* and (**P-P'**) *tst RNAi* germaria stained for Vasa (magenta) and nuclear Actin (C4) antibody (green and grayscale) showing expanded nuclear Actin expression in *tst RNAi* cysts (orange dashed line). Scale bars are 10μm. (**Q**) Quantification of C4 levels in GSCs, CBs, cysts and egg chambers of control (gray) and *tst RNAi* (magenta) expressed in A.U. (n=36-178 per cell type). *** = $p < 0.001$, ** = $p < 0.01$, Tukey's post-hoc test after two-way ANOVA, $p < 0.001$.

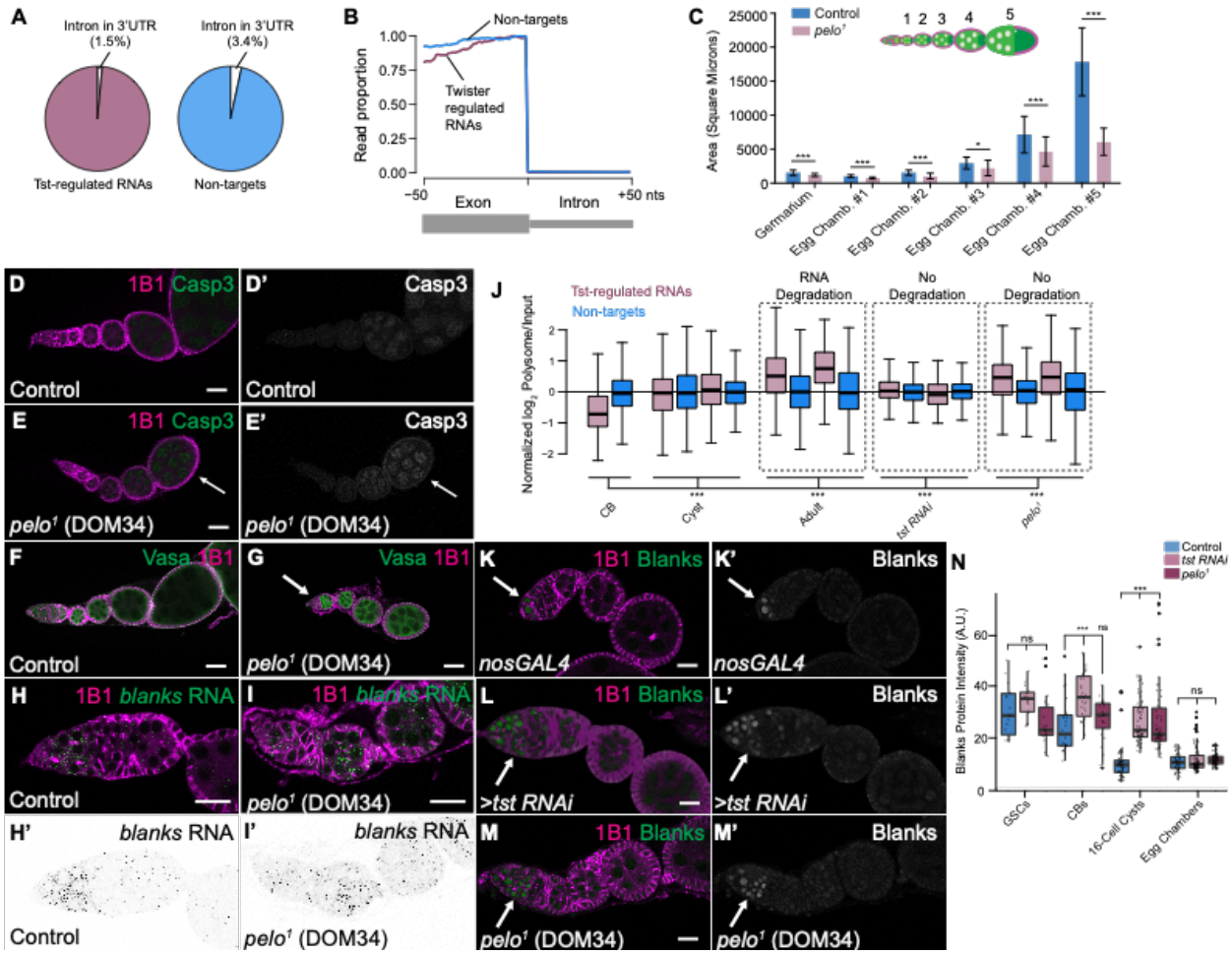


Figure S3. Twister-regulated RNAs exhibit hallmarks of NGD, but not NMD, related to Figure 3. (**A**) Pie charts showing that introns occur in the 3'UTRs of Tst-regulated RNAs (magenta) and non-targets (blue) at only low frequency. (**B**) Metaplots showing the proportion of RNA-Seq coverage mapping to exon-intron boundaries for both Tst-regulated targets (magenta) and non-targets (blue) indicating that Tst-regulated RNAs are spliced correctly. (**C**) Quantification of the area of the germarium and egg chambers #1- #5 comparing control and *pelo*^{1}, showing that egg chambers do not grow upon loss of *pelo* (n=30 ovarioles). *** = $p < 0.001$, * = $p < 0.05$, Student's t-test. (**D-D'**) WT control and (**E-E'**) *pelo*^{1} ovarioles stained with cleaved Caspase 3 (green) and 1B1 (magenta) indicating dying egg chamber (arrow). (**F**) WT control and (**G**) *pelo*^{1} mutant ovarioles stained with Vasa (green) and 1B1 (magenta) indicating loss of GSC in *pelo*^{1} (arrow). Scale bars are 10µm. (**H-H'**) Confocal images of *in situ* hybridizations against the Tst-regulated RNA *blanks* (green, grayscale) and 1B1 (magenta) in WT control showing *blanks* RNA expression in the undifferentiated cells and decreasing in the cyst stages and (**I-I'**) *pelo*^{1} ovarioles showing *blanks* mRNA expression is expanded to egg chambers. (**J**) Quantification of normalized $\log_2$ polysome/input mRNA of Tst-regulated RNAs (magenta), and non-targets (blue) in CB and replicate cyst, adult WT, *tst RNAi* and *pelo*^{1} samples indicating that ribosome association of Tst-regulated RNAs is dynamic during development relative to non-target RNAs ($p < 2.2e\text{-}16$, two-way repeated-measures ANOVA; *** = $p < 0.001$ by estimated means post hoc tests comparing *tst* target RNAs normalized by non-target levels per condition). (**K**) Control, (**L**) *tst RNAi* and (**M**) *pelo*^{1} mutant ovarioles stained for 1B1 (magenta) and Blanks (green) showing restricted Blanks protein expression restricted to undifferentiated cells in control and expanded expression in *tst*

RNAi and *pelo*^{1} to the cyst stages but not the egg chambers. (**N**) Quantification of Blanks protein expression in GSCs, CBs, cysts and egg chambers of WT control (gray), *tst RNAi* (light magenta) and *pelo*^{1} (dark magenta) in A.U. (n=16-92 per cell type). *** = $p < 0.001$, Tukey's post-hoc test after one-way ANOVA, $p < 0.001$.

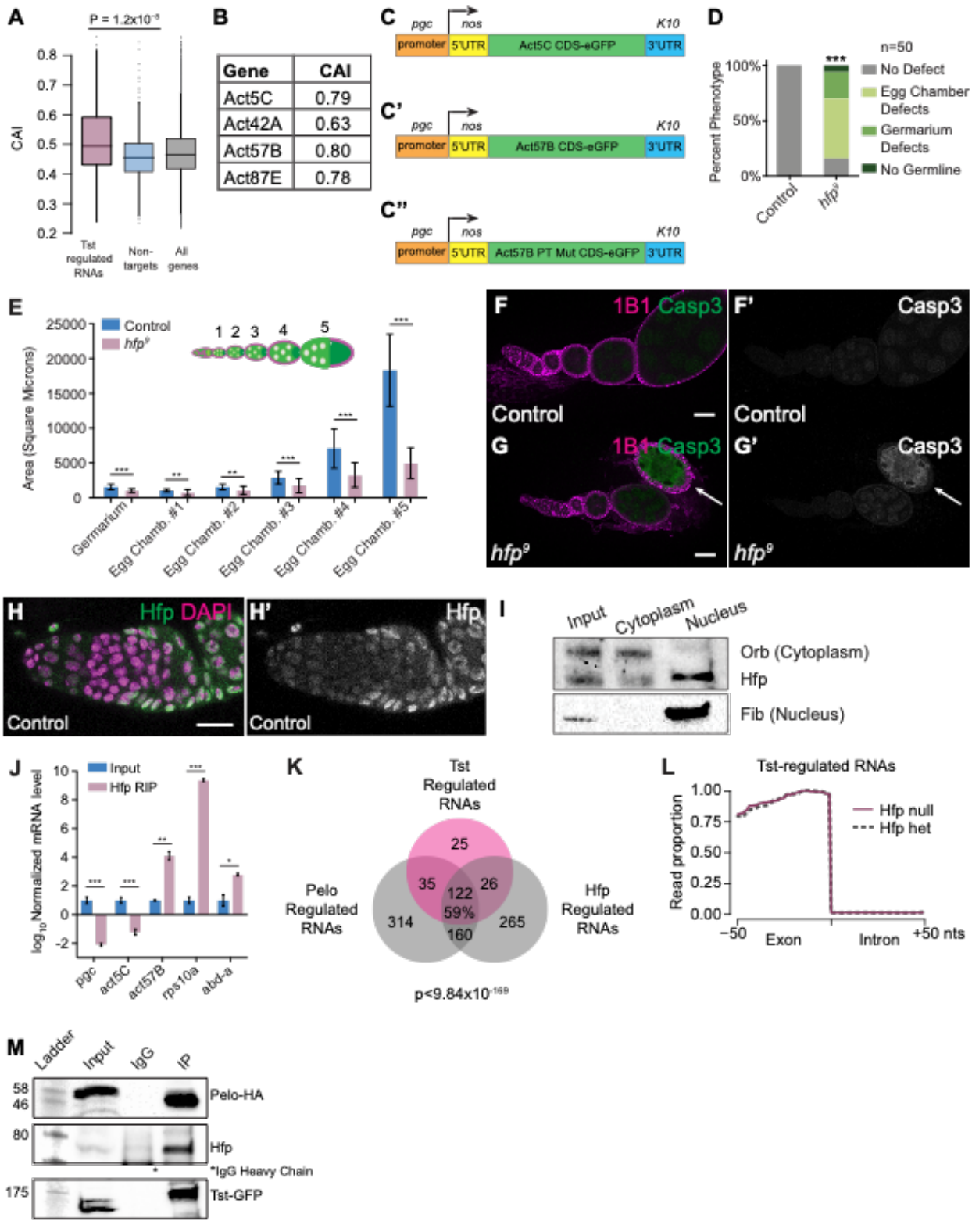


Figure S4. Twister-regulated RNAs are bound by Hfp and do not exhibit suboptimal codon usage, related to Figure 4. (**A**) Codon Adaptation Index (CAI) comparison for Tst-regulated RNAs (magenta) versus non-targets (blue) indicating a higher CAI for Tst-regulated RNAs

(Wilcoxon rank sum test); and all genes (gray). (**B**) Table of the Actin paralog genes indicating that they have similar CAI values. (**C**) Schematic of the *act5C::GFP*, (**C'**) *act57B::GFP* and (**C''**) *act57B PT Mutant::GFP* reporters that are under the control of a germline promoter (*pgc*) and a neutral 5'UTR (*nos*), and 3'UTR (*K10*). (**D**) Quantification of hfp^{9} oogenesis defect phenotypes compared to control (n=50 ovarioles, p<0.001, Chi-squared test with Bonferroni correction, df=3). (**E**) Quantification of the area of the germarium and egg chambers #1-#5 comparing control and hfp^{9}, showing that egg chambers do not grow upon loss of *hfp* (n=25 ovarioles). *** = p < 0.001, ** = p <0.01, Student's t-test. (**F-F'**) WT control and (**G-G'**) hfp^{9} ovarioles stained with cleaved Caspase 3 (green) and 1B1 (magenta) indicating dying egg chamber (arrow). (**H-H'**) Germaria stained for Hfp (green and grayscale) and DAPI (magenta) indicating both cytoplasmic and nuclear Hfp expression during early oogenesis. Scale bar is 10µm. (**I**) Western blot analysis of a subcellular fractionation probing input, cytoplasm and nucleus fractions for the cytoplasmic marker Orb, the nuclear marker Fibrillarin, and Hfp. Hfp is present in both the nucleus and cytoplasmic fractions. (**J**) Hfp-HA RIP and qRT-PCR analyses indicating an enrichment of the Tst-regulated targets *act57B*, *rps10a*, and *abd-a* levels compared to non-targets such as *pgc* and *act5C* levels in Hfp RIP samples compared to input (n=3). *** = p < 0.001, ** = p <0.01, * = p < 0.05, Student's t-test. (**K**) 3-Way Venn diagram of Tst-regulated RNAs, Pelo-regulated RNAs and Hfp-regulated RNAs indicating that Tst, Pelo and Hfp co-regulate 59% (122/207) of their target RNAs (p<9.84x10E-169, Hypergeometric test). (**L**) Metaplot of the proportion of RNA-seq coverage mapping to exon-intron boundaries in hfp^{9} mutant and control (heterozygous) RNA-seq data sets for Tst-regulated RNAs (magenta) indicating correct splicing. (**M**) Co-Immunoprecipitation and Western blot of Pelo-HA probed for Hfp and Tst-GFP, indicating that these 3 proteins interact *in vivo*. IgG lane shows Mouse IgG heavy chain signal indicated by (*).

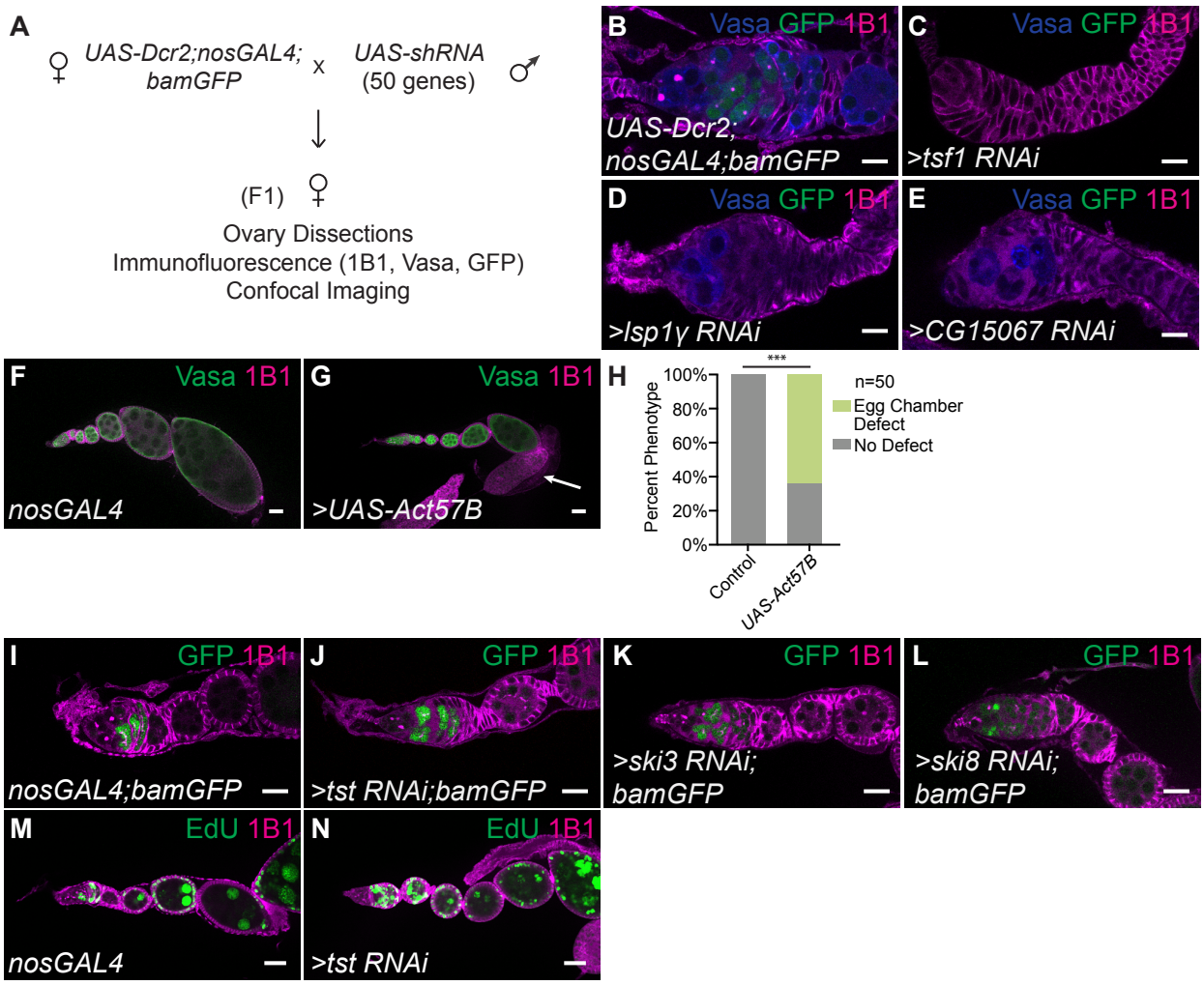


Figure S5. Some Twister-regulated RNAs are required in the undifferentiated stages for germline maintenance and are detrimental when expressed after differentiation, related to Figure 5. (**A**) Schematic of a screen to test the requirement of Tst-regulated genes in the germline. Using *UAS-Dcr2;nosGAL4* that also carries a *bamGFP* (differentiaton marker) reporter select RNAs were depleted in the germline. F1 ovaries were dissected and phenotypes were assessed by 1B1, Vasa and GFP staining. (**B**) *nosGAL4* driver control, (**C**) *tsf1 RNAi*, (**D**) *lsp1γ RNAi*, and (**E**) *CG15067 RNAi* stained with Vasa (blue), 1B1 (magenta), and BamGFP (green) each exhibiting either a differentiation defect or complete loss of germ line. (**F**) *nosGAL4* driver control ovariole stained with Vasa (green) and 1B1 (magenta) and (**G**) germline *UAS-Act57B* overexpression showing egg chamber defects (arrow). (**H**) Quantification of oogenesis defect phenotypes observed upon germline *UAS-Act57B* overexpression (n=50 ovarioles, $p < 0.001$, Chi-squared test with Bonferroni correction, df=1). (**I**) *nosGAL4;bamGFP* driver control ovariole, (**J**) *>tst RNAi;bamGFP*, (**K**) *>ski3 RNAi;bamGFP*, and (**L**) *>ski8 RNAi;bamGFP* ovarioles stained with 1B1 (magenta) and GFP (green) show correct BamGFP expression. (**M**) *nosGAL4* driver control and (**N**) *>tst RNAi* ovarioles stained for EdU (green) and 1B1 (magenta) showing proper endocycling. Scale bars are 10μm.