Supplementary Figure Legends, Okamura et al, "R2D2 organizes small regulatory RNA pathways in *Drosophila*"

Figure S1. Effect of *r2d2* or *ago2* mutation in small RNA loading to AGO2

Small RNAs matching to mature miRNAs, miRNA* strands, TEs and overlapping transcripts (cis-NATs) were plotted. Each point shows normalized read counts in WT (Y-axes) against in heterozygous *r2d2* mutant, homozygous *r2d2* mutant or *ago2* mutant (X-axes). The left three columns and the right three columns show results of total RNA libraries and oxidized libraries, respectively.

Figure S2. Verification of RNAi mutants and recombinant proteins

(A) siRNA-directed target cleavage activity of embryo lysates. *ago2*, *dcr-2* and *r2d2* mutant lysates were defective in cleaving an siRNA target. *loqs* knockout flies rescued by *loqs-PB* transgene fully supported siRNA mediated target cleavage. FLAG-AGO2 transgene fully rescued RNAi activity of *ago2* mutant lysate, indicating the functionality of the transgene. (B) Activity of recombinant proteins. Purified AGO2 protein could be loaded with siRNA in the presence of recombinant R2D2/Dcr-2 complex. Recombinant AGO2 protein also rescued the RNAi defect of *ago2* mutant lysate. (C) Small RNA expression in *loqs* knockout flies rescued by *loqs-PB* transgene. The accumulation of miR-8 was normal due to presence of loqs-PB, a Dicer-1 cofactor. On the other hand, hp-CG4068B was reduced in this genotype due to absence of loqs-PD, a Dicer-2 cofactor needed for endo-siRNA production.

Figure S3. Association of hp-CG4068G with AGO2 in wild-type

Overexposure of the hp-CG4068G panel in Figure2B shows association of the siRNA with AGO2 complex in wild-type background but not in *r2d2[1]* mutant.

Figure S4. Verification of immunoprecipitation.

Immunoprecipitated samples were subjected to SDS-PAGE and AGO2 (left) or AGO1 (right) proteins were detected by western blotting. AGO2 and AGO1 complexes were successfully precipitated from embryos and adult male flies.

Figure S5. Relative abundance of small RNAs in total RNA from RNAi mutants

(A) The reads that match to TEs, 3' cis-NAT regions, hpRNAs and mature or star strands of miRNA genes were counted and normalized against those in total RNA library from wild-type. (B-I) Small RNAs matching to mature miRNAs (B and F), miRNA* strands (C and G), TEs (D and H) and overlapping transcripts (cis-NATs, E and I) were plotted. Each point shows read counts in WT against in *ago2* (B-F) or *r2d2* mutant (E-I).

Figure S6. AGO1 is not effective in unwinding perfect siRNA duplexes *in vitro*.

The siRNA duplexes with (upper panel) or without (lower panel) 3' 2-nt overhangs were used for *in vitro* sorting assay. A small amount of siRNA duplex co-precipitated with AGO1, but siRNAs were not unwound in the AGO1 complex. Binding of siRNA to AGO1 required 3' overhangs, since a small RNA duplex with blunt ends was not co-precipitated efficiently.

Table S1. Statistics of small RNA libraries analyzed in this study. Adult head datasets were published by the Zamore laboratory (Ghildiyal et al., 2008; Ghildiyal et al., 2010) and subsequently referred to as "Zamore libraries"; the ovary datasets were generated for this study. The first worksheet includes the numbers of raw reads in each category. On the second work sheet, the numbers were normalized by the numbers of total

mapped reads minus 2S reads. The data were used for Figures 1B and 3A.

Table S2. Analysis of miRNA and miRNA* reads in the libraries used for this study. These data were used in Figures S1 (Worksheet "Zamore Libs Normalized") and 3B, C, F, G and S5 B, C, F, G (Worksheet "Ovary Libs Normalized").

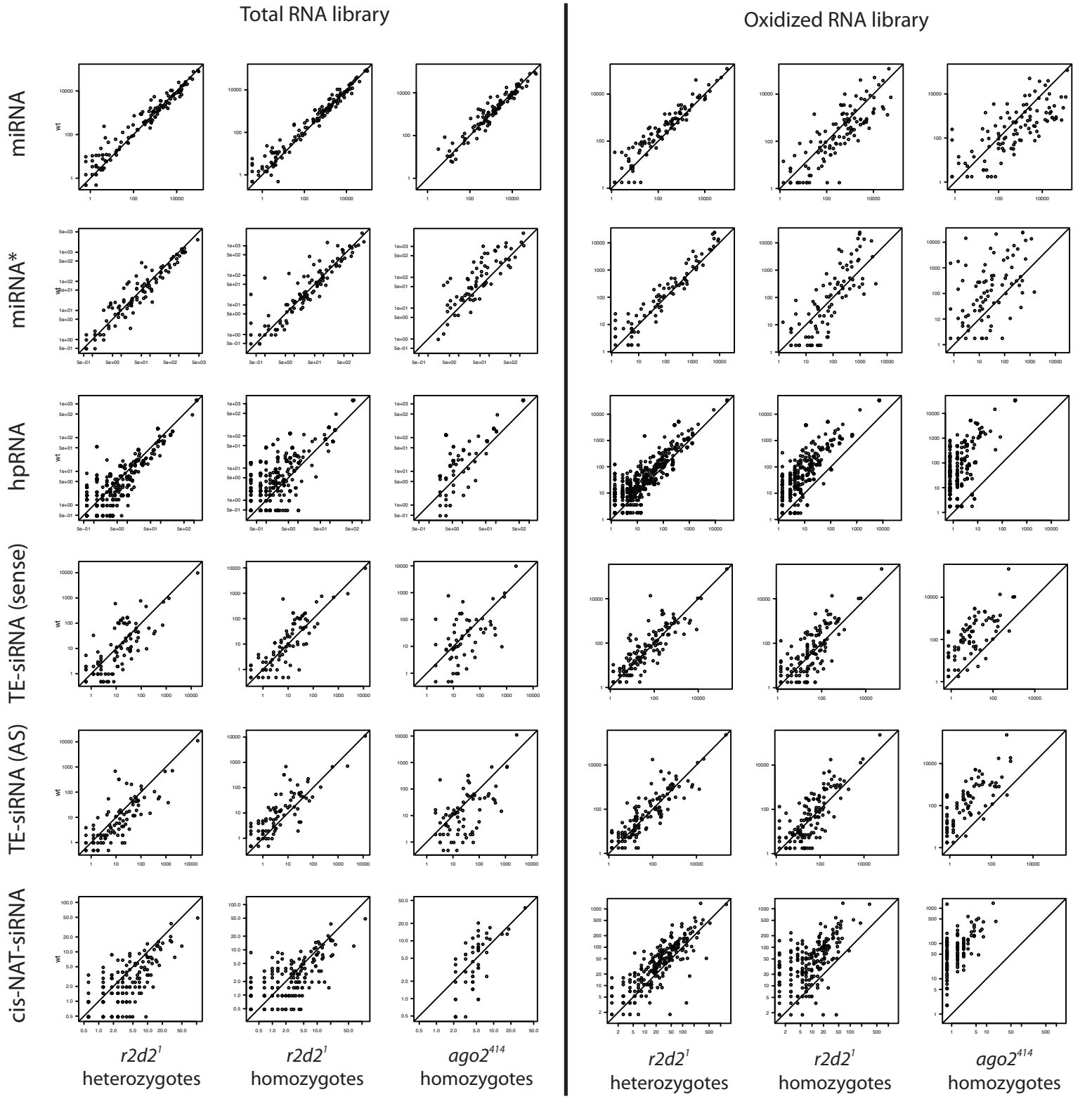
Table S3. Analysis of TE siRNA reads in the libraries used for this study. These data were used in Figures S1 (Worksheet "Zamore Libs Normalized") and 3D, H and S5D, H (Worksheet "Ovary Libs Normalized").

Table S4. Analysis of cisNAT siRNA reads in the libraries used for this study. These data were used in Figures S1 (Worksheet "Zamore Libs Normalized") and 3E, I and S5 E, I (Worksheet "Ovary Libs Normalized").

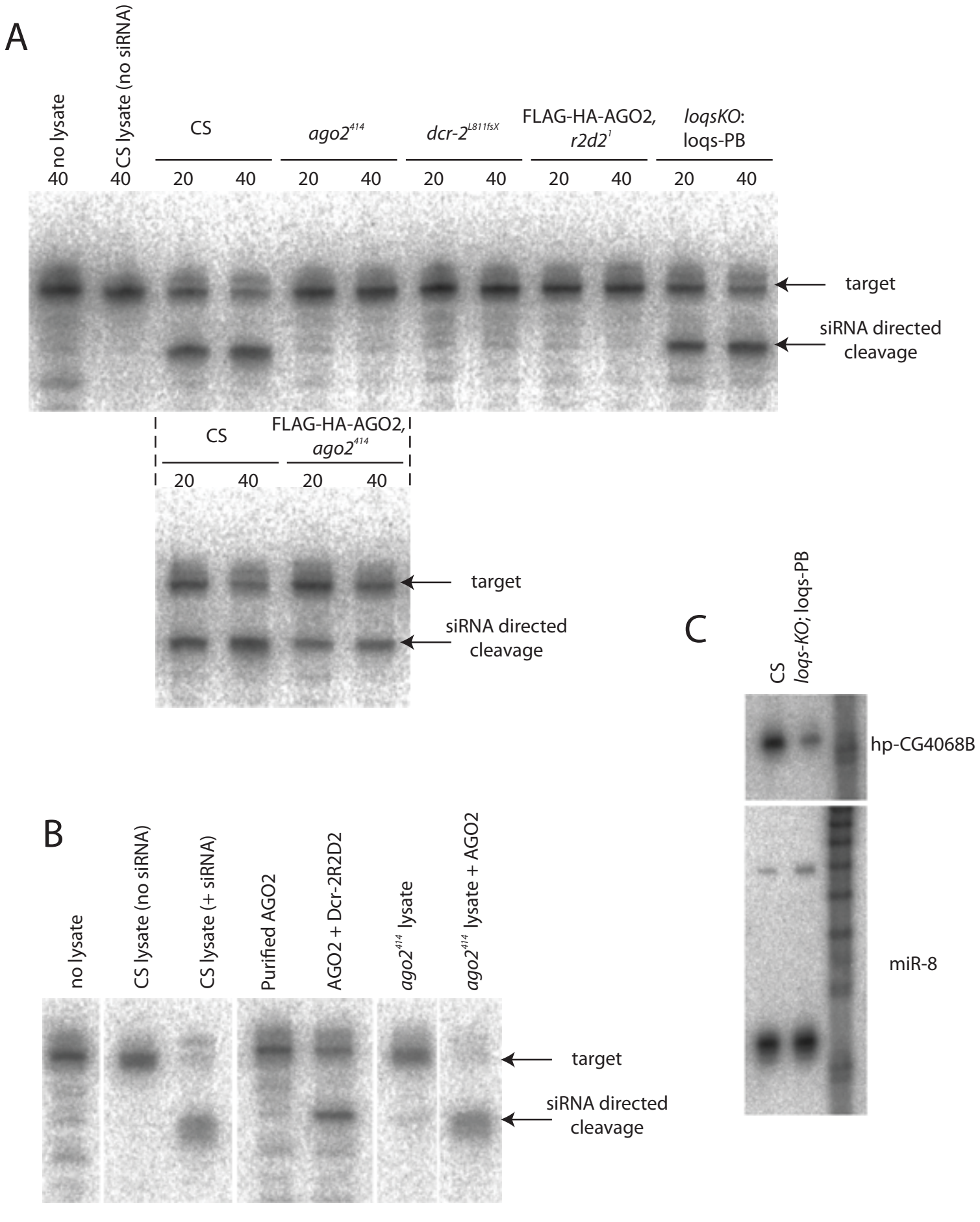
Table S5. Analysis of hp-siRNA reads in the libraries used for this study. hp-siRNA reads were grouped by their 5' ends. These data were used in Figures 1B (Worksheet "Zamore Libs Normalized") and 3J-K (Worksheet "Ovary Libs Normalized").

Table S6. Statistical test for AU contents at each position of cis-NAT siRNAs. For each position, we tested if the percentage of AU was higher than a normal distribution of percentages whose parameters were computed from all the other positions. For each of the libraries, position 1 and 2 were significantly different than the other positions, with p-values < 0.05.

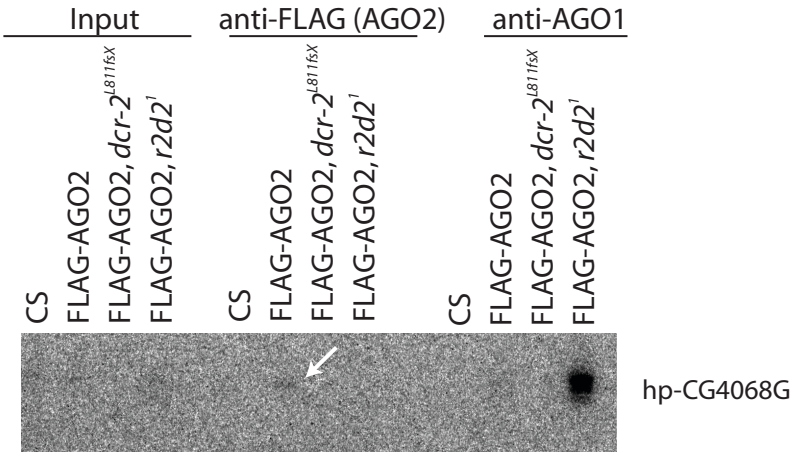
Table S7. Sequences of oligonucleotides used for this study.



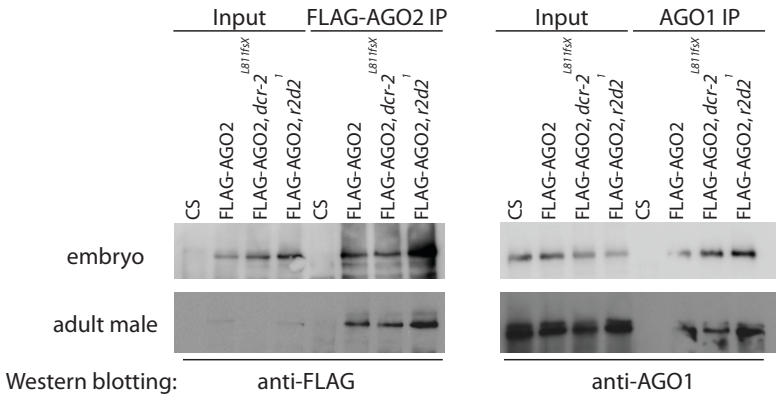
Supplementary Figure 1



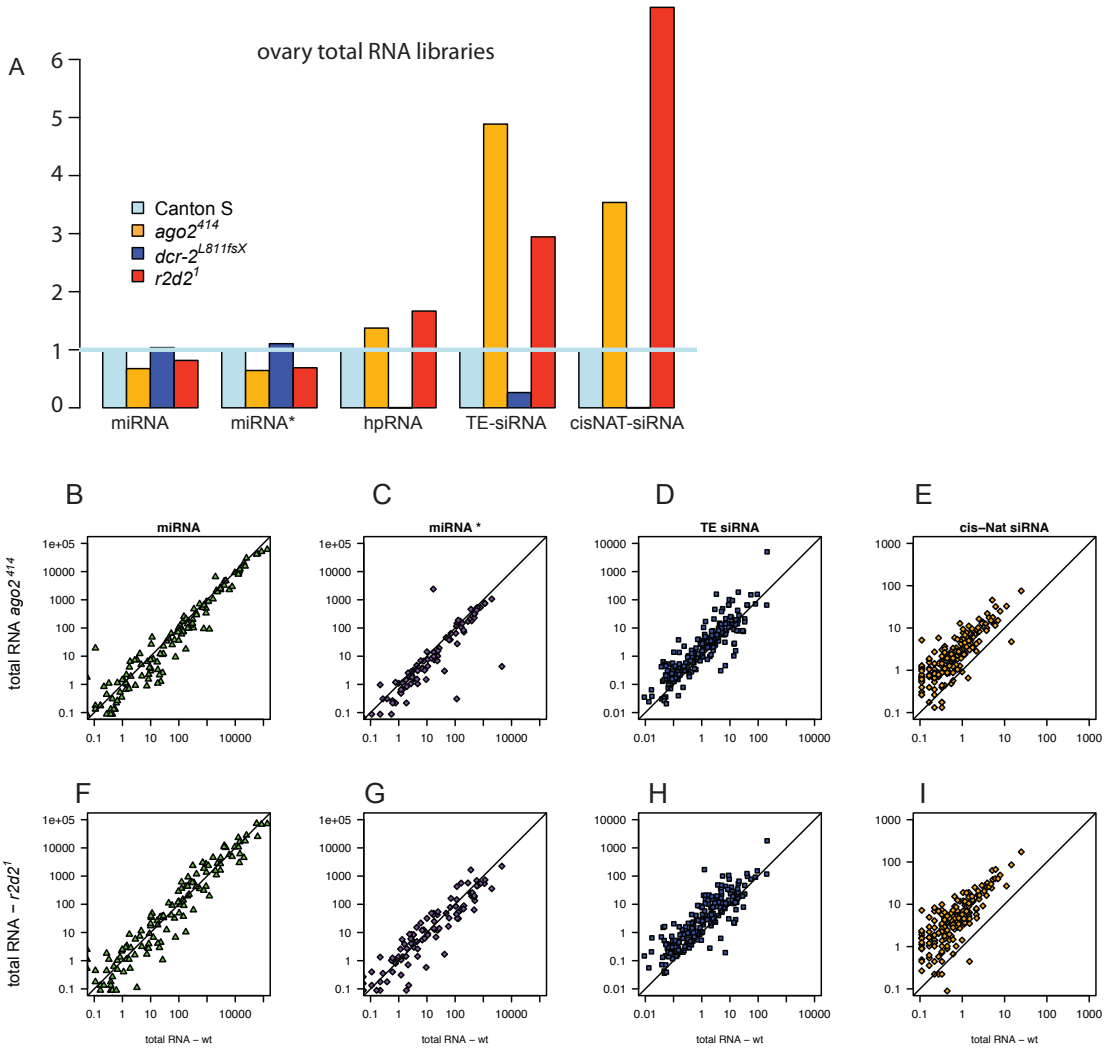
Supplementary Figure 2



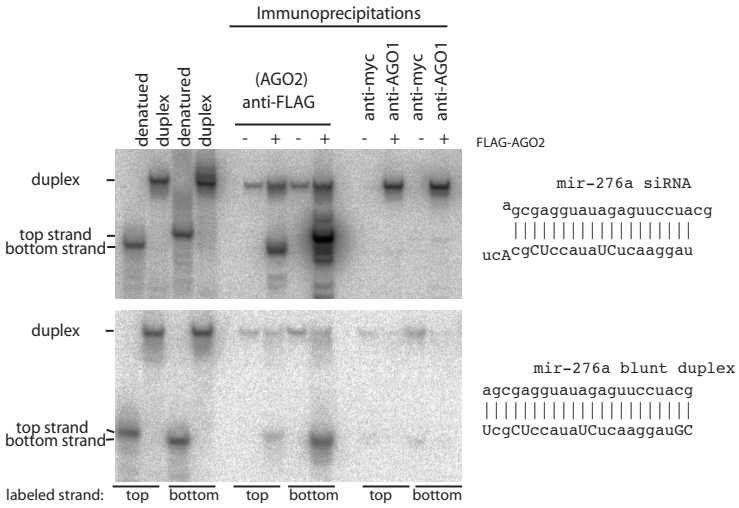
Supplementary Figure 3



Supplementary figure 4



Supplementary FIgure S5



Supplementary Figure S6