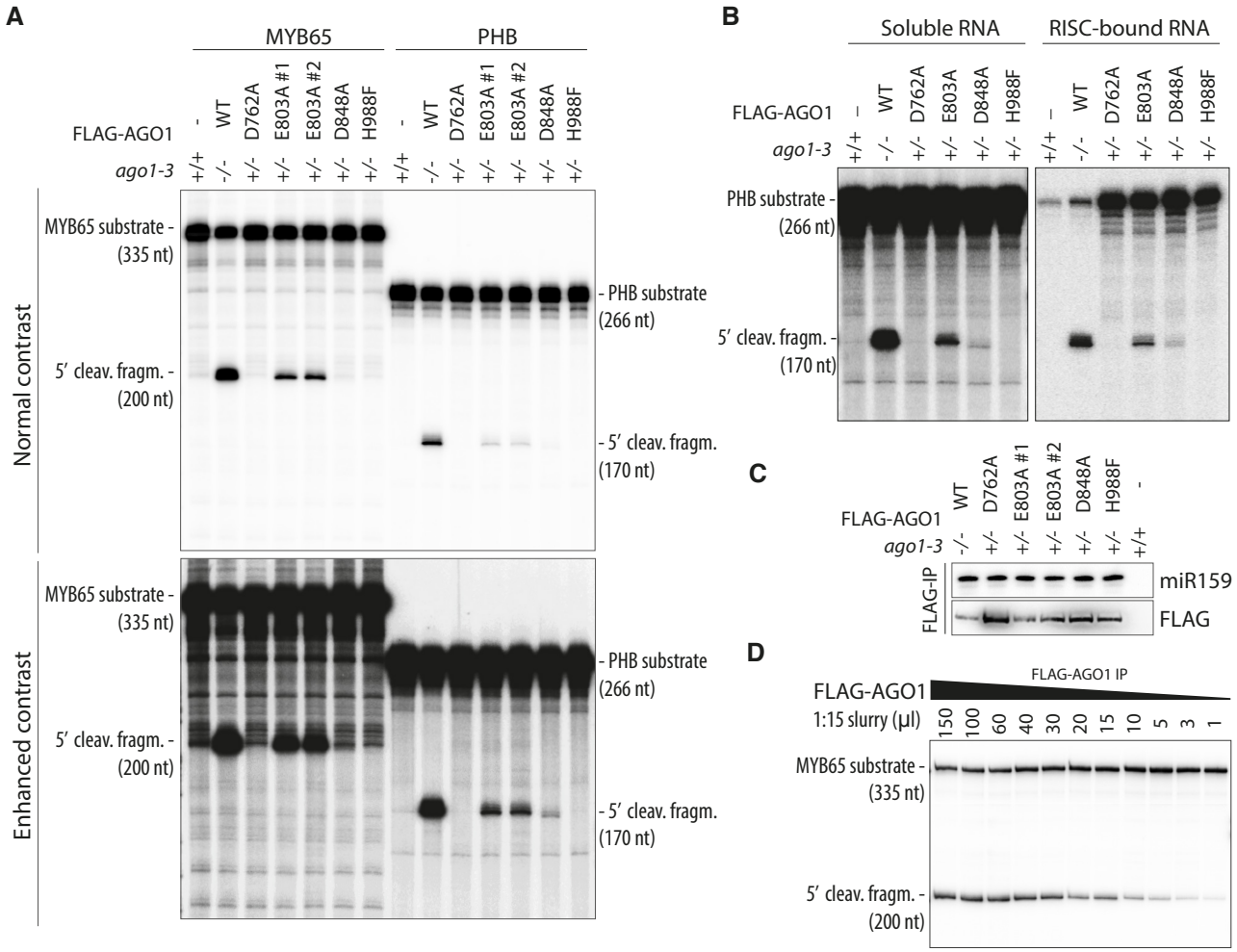


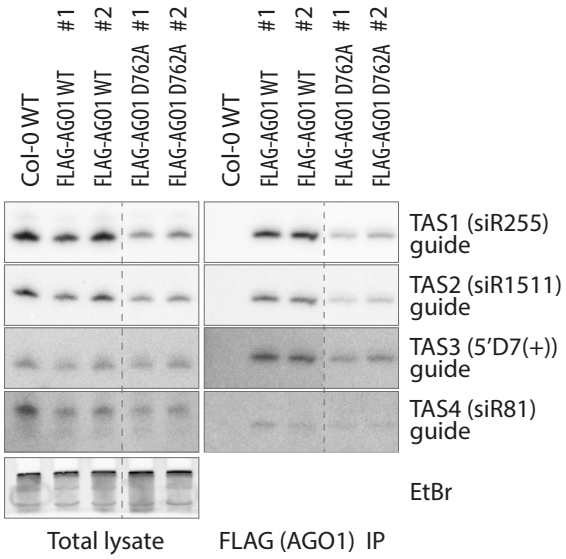
Supplemental Figure 1. Different catalytic roles of the four Mg^{2+}-coordinating active site residues (extended data)

(A) Slicer activity of immuno-affinity purified FLAG-AGO1 from *Arabidopsis* inflorescences of stable transgenic lines expressing WT and slicer-deficient FLAG-AGO1. 350- and 266-nt ^{32}P cap-labeled ssRNAs bearing endogenous miR159 or miR166 target sites were used as substrates. RNA extracted from the whole reaction was analyzed. Cleavage activity is revealed by the accumulation of the corresponding 5' cleavage fragment. FLAG-AGO1^{E803A} extracted from two different tissue batches is shown to verify residual cleavage activity. Col-0 plants without transgene were used to control unspecific binding of endogenous AGO1 during IP (left lanes). The same panel is reproduced with enhanced contrast below to facilitate visualization of the 5' cleavage fragment of the PHB substrate produced by FLAG-AGO1^{D848A}.

(B) Reproduction of Figure 1B with enhanced contrast to facilitate visualization of the 5'-cleavage fragment produced by FLAG-AGO1^{D848A}.

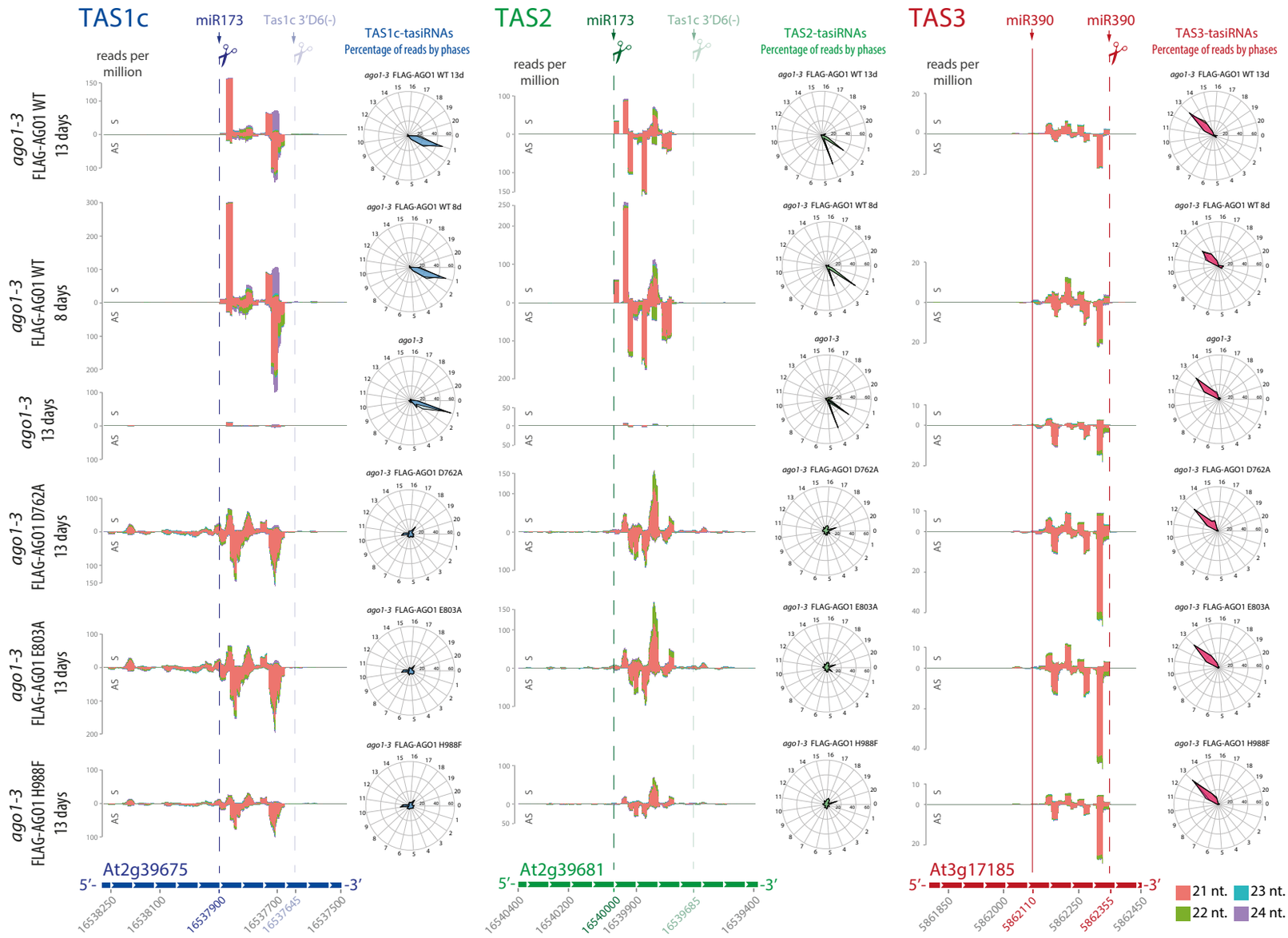
(C) Top, RNA gel blot of miR159 co-purified with FLAG-AGO1 in the IP used in the slicer assay shown in (A). Bottom, protein gel blot with FLAG antibody of the same IP.

(D) Slicer assay as described in (A) with decreasing amounts of FLAG-AGO1 WT immobilized on FLAG beads. The amount of FLAG-AGO1 indicates the volume of 1:15 slurry of FLAG beads in PBS after affinity-purification from tissue in a 10:1 ratio of tissue (mg): beads (µl).



Supplemental Figure 2. Trimming of siRNAs bound to slicer-deficient AGO1 does not occur in seedlings

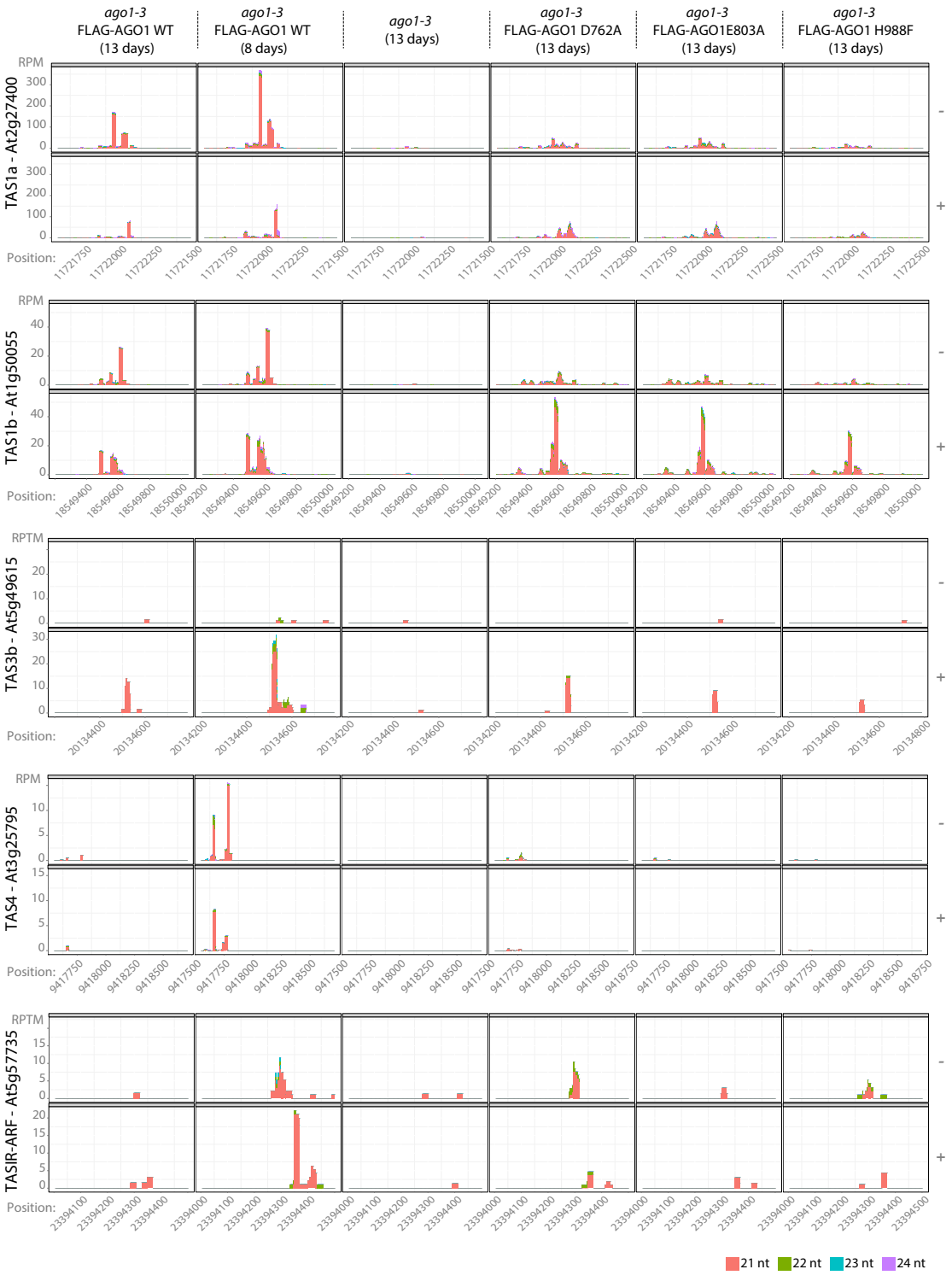
RNA gel blot analysis of tasiRNAs in total RNA or RNA bound to immuno-affinity purified FLAG-AGO1 from seedlings of stable transgenic lines expressing WT or slicer-deficient FLAG-AGO1. The same membranes were used to re-hybridize with different siRNA probes. Ethidium bromide (EtBr) staining of the upper part of the gel is used as loading control. Dashed lines indicate removal of lanes on the original gel image with samples not relevant for the present study.



Supplemental Figure 3. AGO1 slicer activity is required for phasing, but not for production of tasiRNAs (extended data)

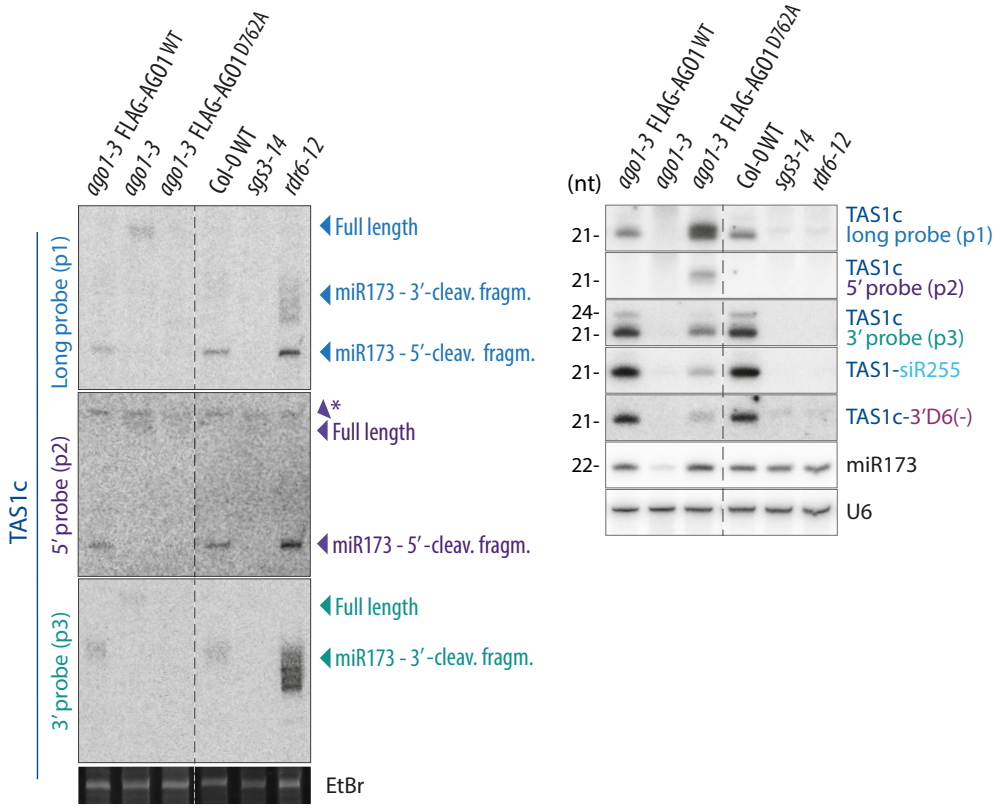
Reads per million and percentage of reads by phases of tasiRNAs mapping to TAS1c, TAS2 and TAS3a transcripts. RNA was extracted from 8- or 13-d-old seedlings of *Arabidopsis ago1-3*, and stable transgenic lines expressing WT or slicer-deficient FLAG-AGO1 (D762A, E803A, and H988F) in the *ago1-3* null background. Abscissae, TAIR9 coordinates. S indicates sense siRNAs, AS indicates antisense siRNAs, relative to the TAS precursor transcripts. Percentage of reads by phases from the two biological replicates of every genotype are depicted on different shades of color and overlaid on the diagrams showing percentage of reads by phases.

The figure is an extension of Figures 3A and 3B as it includes data for the mutants FLAG-AGO1^{E803A} and FLAG-AGO1^{H988F}, and 8-d-old FLAG-AGO1^{WT} seedlings in addition to the samples shown in the main figure panels.



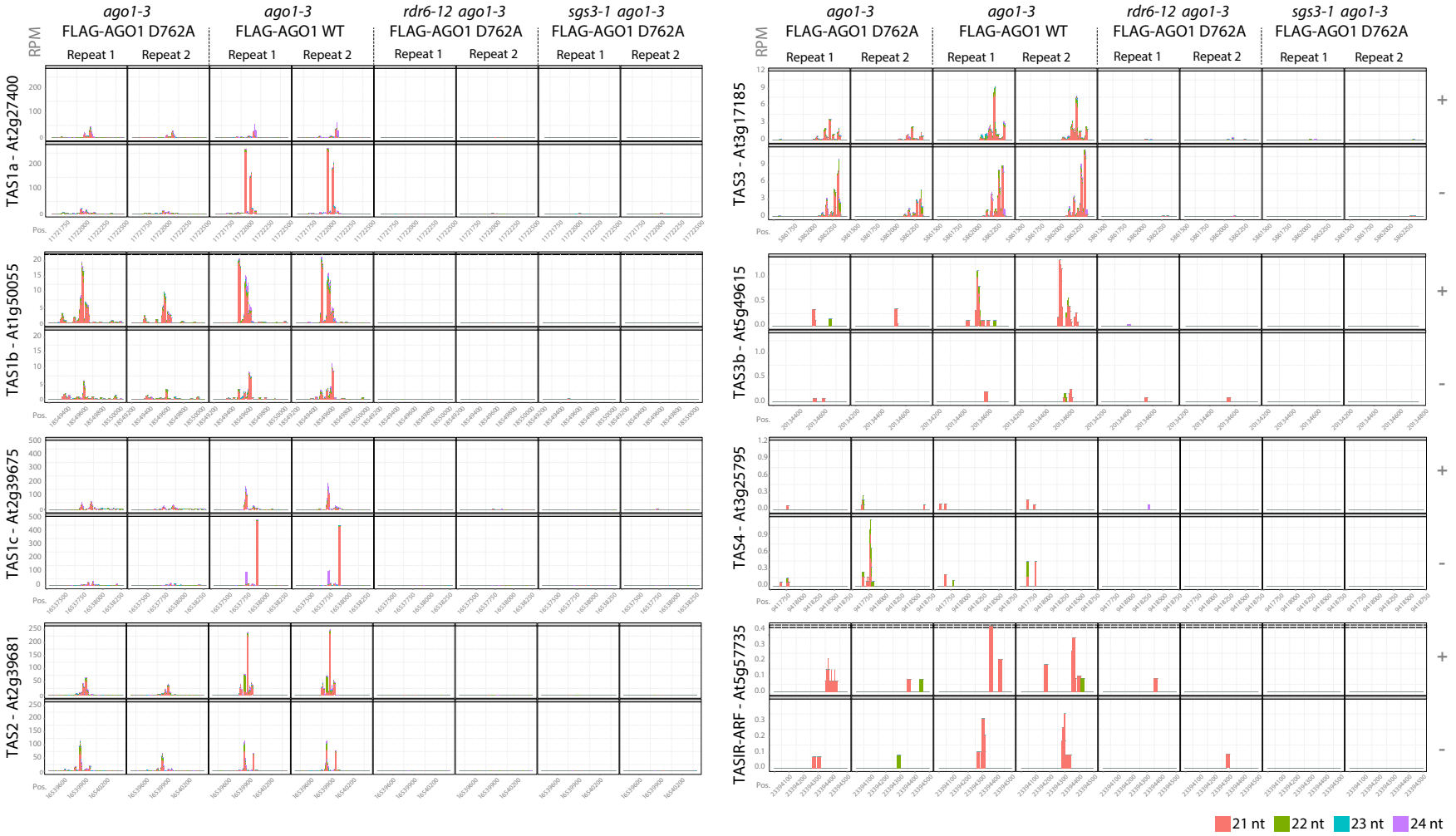
Supplemental Figure 4. AGO1 slicer activity is required for phasing, but not for production of tasiRNAs (extended data)

Reads per million (RPM) or reads per ten million (RPTM) of tasiRNAs mapping to TAS1a, TAS1b, TAS3b, TAS4 and TASIR-ARF transcripts, extracted from 8- or 13-day-old seedlings of *Arabidopsis ago1-3*, and stable transgenic lines expressing WT or slicer-deficient FLAG-AGO1 (D762A, E803A, and H988F) in the *ago1-3* null background. For each transcript, the upper row of graphs corresponds to reads mapping to the reverse DNA strand (minus), while the lower row contains those corresponding to the forward DNA strand (plus).



Supplemental Figure 5. miR173 does not guide TAS1c precursor cleavage in the absence of AGO1 slicer activity (extended data)

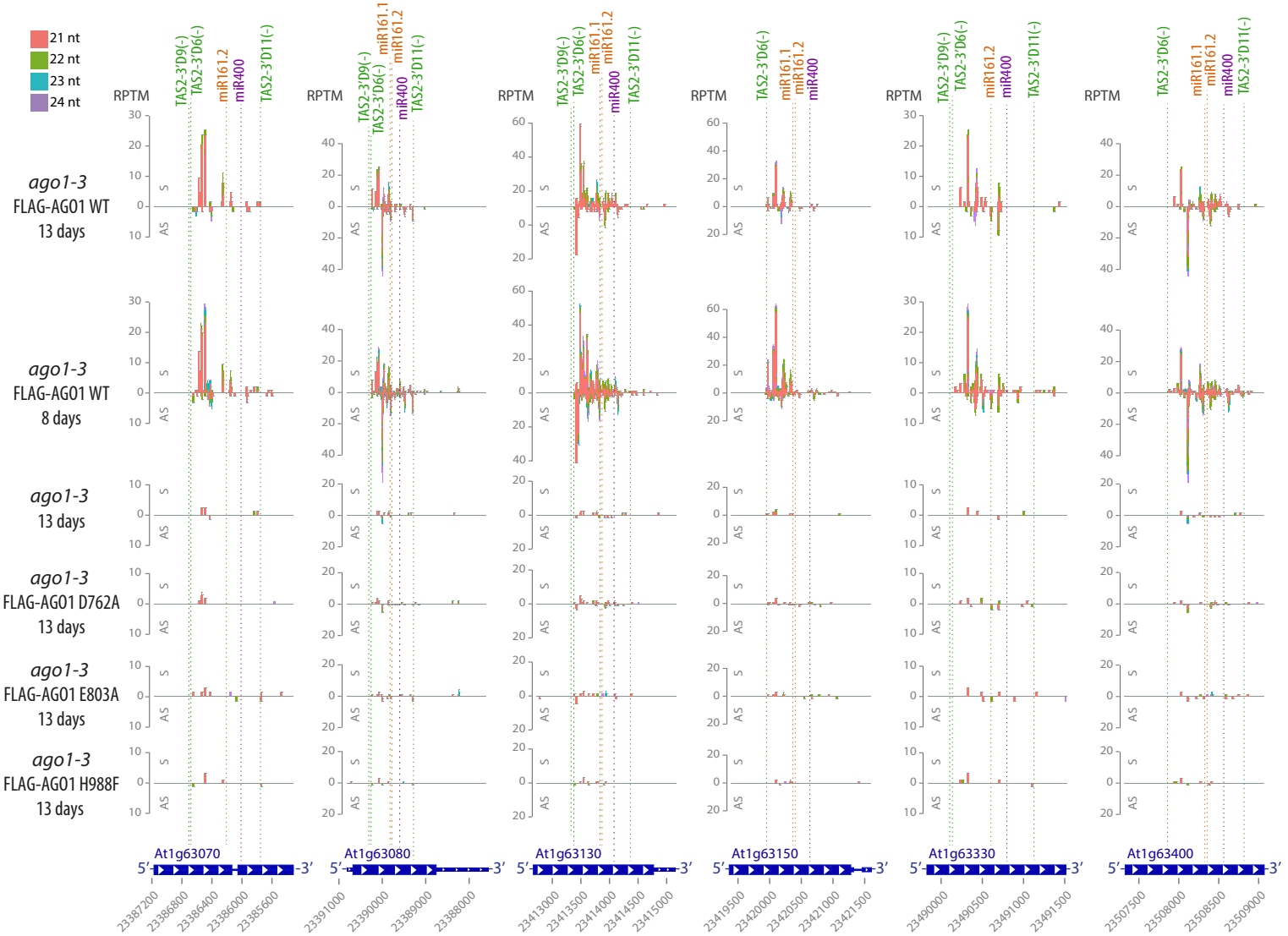
RNA gel blot analyses of TAS1c. Aliquots of the same total RNA preparation were analysed in the left and right panels. Probe positions are shown in Figure 4A. Left panel, analysis of high-molecular weight RNA separated by electrophoresis in a denaturing 5% polyacrylamide gel. The asterisk in the 5' probe (p2) indicates a band produced by unspecific hybridization. Ethidium bromide (EtBr) staining of the lower part of the gel is used as loading control. Right panel, analysis of low-molecular weight RNA separated by electrophoresis in a denaturing 18% polyacrylamide gel. U6 is used as a loading control. Dashed lines indicate removal of lanes on the original gel image with samples not relevant for the present study.



Supplemental Figure 6. RDR6 and SGS3 dependence of tasiRNAs produced in the absence of slicer activity (extended data)

Reads per million (RPM) of small RNAs mapping to TAS1a, TAS1b, TAS1c, TAS2, TAS3, TAS3b, TAS4 and TASIR-ARF transcripts, extracted from 13-day-old seedlings of the indicated transgenic lines. For every transcript, the upper row of graphs corresponds to reads mapping to the forward DNA strand (plus), while the lower row contains those corresponding to the reverse DNA strand (minus).

The figure is an extension of Figure 5, as it includes all the tasiRNAs-generating loci in *Arabidopsis* and biological replicates of each line in addition to the samples shown in the main figure.



Supplemental Figure 7. **AGO1 slicer activity is required for siRNAs spawned by TAS2/miR161/miR400 targets (extended data)**

Reads per ten million (RPTM) of siRNAs mapping to PPR genes in 8- or 13-day-old seedlings of *Arabidopsis ago1-3*, and stable transgenic lines expressing WT or slicer-deficient FLAG-AGO1 (D762A, E803A, and H988F) in the *ago1-3* null background. Abscissae, TAIR9 coordinates. S indicates sense siRNAs, AS indicates antisense siRNAs, relative to mRNAs. Cleavage sites of TAS2 3'D6(-), TAS2 3'D9(-), TAS2 3'D11(-), miR161.1, miR161.2 and miR400 are indicated by dashed lines. The target sites of the couples miR161.1/miR161.2, and TAS2-3'D9(-)/TAS2-3'D6(-) have an overlap of 10-12 nt, and therefore cannot be considered as "double hits" on their own.

The figure is an extension of Figure 6, as it includes data for the mutants FLAG-AGO1^{E803A} and FLAG-AGO1^{H988F}, for 8-day-old FLAG-AGO1^{WT} seedlings, and three additional PPR genes (At1g63070, At1g63080, and At1g3330) in addition to the information shown in the main figure.

Supplemental Methods

AGO1 slicer assay

To produce the RNA substrate, we first cloned 200-300 bp fragments of two endogenous targets, MYB65 and PHB, containing their respective miR159 and miR165/166 target sites, into pGEM-T Easy. Templates for in vitro transcription were obtained by PCR from the target-containing plasmids using the M13 primer combined with the reverse primer used for cloning, as pGEM-T Easy includes a T7 promoter between the M13 sequence and the cloning site. Primer sequences for cloning and template amplification are detailed in Supplemental Data Set 3. Phusion High-Fidelity DNA Polymerase (NEB) was used for PCR to avoid production of 3' overhangs. In vitro transcription was performed using the RiboMAX^{TM} Large Scale RNA Production Systems (Promega). Briefly, 3 µg of purified DNA templates were incubated with T7 Enzyme Mix and rNTPs in T7 Transcription Buffer for 4 hr at 37ºC. The in vitro-transcribed RNA was then extracted with 1 vol of citrate-saturated phenol (pH4.7):chloroform 50:50, followed by a second extraction with 1 vol of chloroform. After standard isopropanol precipitation, the obtained RNA was loaded into a 5% acrylamide:bis 19:1, 7% urea gel in 1x TBE buffer. Bands with the correct size localized by UV shadowing were excised, and the RNA contained in the gel slices was extracted in 300 µl of 250 mM NaOAc, 1 mM EDTA pH5.2, plus 200 µl of Phenol (not Tris-buffered), shaking overnight at RT. The day after, the RNA in solution was extracted with 1 vol of chloroform and precipitated with isopropanol. Pellets were solubilized in 25 µl in nuclease-free H_2O to obtain >50 µg of highly pure RNA.

Radioactive labeling was performed by addition of α^{32}P-GTP to the 5'-end of the RNA substrate by recombinant guanylyltransferase, to form a 5' cap. For that purpose, we incubated 1 µg of the purified RNA substrate with 5 µg of recombinant guanylyltransferase expressed in *E. coli* (see below), 50 µCi of α^{32}P-GTP (PerkinElmer), 3 nmoles of S-adenosyl methionine (SAM), 0.5 U of Ribolock, and 1x GuTR buffer (50 mM TrisHCl pH8, 6 mM KCl, 12.5 mM $MgCl_2$, 1.25 mM DTT, 0.05 mg/ml BSA) in a total volume of 20 µl, for 1 hr at 37ºC. The labeled substrate was purified in a 5% acrylamide:bis 19:1, 7% urea, 1x TBS gel. A film was exposed on the gel for 2 min to test proper labeling of the substrate and monitor possible degradation. Bands with the correct size localized by UV shadowing were excised, and the RNA was extracted from them in RNA Elution Buffer (300 mM NaOAc, 1mM EDTA and 0.1% SDS) shaking at 4ºC overnight. RNA in solution was precipitated with 2.5 vol of 96% EtOH for 1 hr at -20ºC, washed with 70%EtOH, and dissolved in nuclease-free H_2O for incubation with AGO1. We detected 500-1000 cps from 1 µl of freshly labeled substrate using a bench

Geiger counter.

To perform the enzymatic assay, we added 12.5 ul of 2xTM buffer (133.3 mM KCl, 13.3 mM $MgCl_2$, 16.7 mM DTT, 3.3 mM ATP, 0.7 mM GTP, and 1% (v/v) Ribolock) to 25 µl of FLAG beads in PBS (50:50 slurry) bearing freshly purified FLAG-AGO1 from 125 mg of inflorescences on the beads surface. We added 100-200 cps (or 1-2 Bq/cm^2) of PHB or MYB65 labeled substrate to the reaction, and incubated at 25ºC for 90 min under gentle shaking (250 rpm). The reaction was stopped by addition of 1 ml of TRI Reagent, and RNA extraction was performed as described below, adding 20 µg of glycogen as inert carrier for overnight precipitation at -20ºC.

The recovered RNA was solubilized in 10 µl of RNA Loading Buffer (20 mM HEPES pH7.8, 1 mM EDTA, 50% formamide, 3% glycerol and 0.01% bromophenol blue (BPB)) and denatured for 5 min at 65ºC. 5 µl were loaded into a 0.5 mm sequencing gel (5% acrylamide:bis 19:1, 7% urea, 1x TBS). Once the BPB stain reached the bottom, the gel was vacuum-dried on Whatman 3MM paper at 80ºC for 1 hr, and exposed to a phosphor screen for image analysis.

GuTR expression and purification

A construct containing the subunits D1 and D12 of mRNA guanylyltransferase (GuTR) from *Vaccinia* virus (a kind gift of Stewart Shuman) was introduced into BL21-DE3 (RIL) *E. coli*. Expression driven by the T7 promoter was induced by addition of IPTG (0.2 mM final concentration) to a 1 L culture of cells growing at 0.6 OD_{600nm} in LB media supplemented with 100 mg/L ampicillin and 37 mg/L chloramphenicol. Overexpression of GuTR was carried out overnight at 18ºC shaking at 200 rpm. Bacteria were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 20 mM imidazole, 10% glycerol, 2 mM TCEP, and protease inhibitor cocktail (Roche Complete)). The cells were lysed in a chilled French pressure cell press, and the lysate was cleared by centrifugation at 20000xg for 30 min at 4ºC prior protein purification. In a first purification step, His-tagged D1 was captured on 5 ml Ni-NTA resin (GE Healthcare Life Sciences) allowing co-purification of untagged D12. Fractions containing the D1-D12 complex were pooled and diluted 4 times in heparin loading buffer (50 mM Tris-HCl pH 8.0, 10 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM TCEP, and 1 mM EDTA) before the sample was bound on a HiTrap Heparin HP column (GE Healthcare Life Sciences) for further purification. Highly pure GuTR was eluted in about 30% heparin elution buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM TCEP, and 1 mM EDTA). Following dialysis against 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM TCEP, and 0.1 mM EDTA, the sample volume was reduced to 200 µl using a centrifugal

concentrator (10000 MWCO, Sartorius). The concentrated enzyme was finally diluted 1:1 with 2X enzyme storage buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 50% glycerol, 0.1% Triton X-100, 2 mM TCEP and 1 mM EDTA) for long-term storage at -20ºC. The total yield of GuTR complex was 1.2 mg from 1 L of culture.

RNA extraction

For extraction of total RNA from plant tissues, seedlings or inflorescences were flash-frozen in liquid nitrogen and manually ground to a fine powder using a mortar and pestle. One ml of TRI Reagent (Sigma) was added to 100 mg of powder and immediately vortex until the mixture was totally homogenized. Addition of 0.2 ml of chloroform followed by vigorous shaking allowed separation of two phases by 10 min centrifugation at 4ºC. RNA was then precipitated from the aqueous phase by addition of one volume of isopropanol to the supernatant, and incubation at room temperature for 30 min. RNA pellets obtained by 15 min centrifugation at 4ºC were washed with 70% EtOH prior re-suspension either in H_2O for RNAseq-library preparation, or in 50% formamide for RNA gel blotting.

RNA gel blotting

RNA from total extracts or from affinity-purified FLAG-AGO1 was denatured at 65ºC for 5 min in RNA Loading Buffer, loaded into 18% (for small RNAs) or 5% (for HMW RNA) acrylamide:bis 19:1, 7% urea, 0.5x TBE gel, and run in 0.5x TBE at 100V until the BPB stain reached the bottom of the gel. The RNA contained in the gel was blotted on an Amersham Hybond-NX nylon membrane (GE Healthcare Life Sciences) at 80V for 1 hr in cold 0.5x TBE. After blotting, RNA was retained on the nylon membrane by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) crosslinking (Pall et al., 2007) carried out during 2 hr at 60ºC. Membranes containing RNA were rinsed in H_2O and pre-incubated in PerfectHyb™ Plus Hybridization buffer (Sigma) at 42ºC for 20 min before incubation with the corresponding radioactively labeled probe in the same buffer at 42ºC overnight. After hybridization, membranes were washed with 2x SSC (0.3 M NaCl, 30 mM sodium citrate) 2% SDS at 50ºC for 10 min two times, and exposed to a phosphor screen for image analysis. Membranes were stripped three times with boiling 0.1% SDS for re-hybridization with different probes.

Construction of AGO1P:FLAG-AGO1-AGO1T

The AGO1P:FLAG-AGO1-AGO1T construct was generated by a combination of overlap extension PCR and USER cloning methodologies. First, AGO1P and AGO1+AGO1T gDNA fragments were amplified with primers bearing overlapping overhangs in one end that, fused together, would reconstitute the nucleotide sequence

of the FLAG epitope tag (DYKDDDDK), and XmaI-compatible sites at the other end for insertion into pCAMBIA3300 (LA112-LA113, LA114-LA115). The two overlapping fragments were separately amplified by PCR with Phusion High-Fidelity DNA Polymerase (NEB), mixed, denatured and annealed to generate heteroduplexes that could be extended in a third PCR to generate the full-length construct containing the N-terminal FLAG tag bridging the promoter and the coding sequence of AGO1. Although the full 8.8 Kb fragment was readily visible in a 1% agarose gel stained with EtBr, we failed introducing the construct as such into the XmaI site of pCAMBIA3300 vector. However, we could use this PCR product as a template to amplify a 1209 nt USER-compatible fragment containing the end of the AGO1P, the FLAG tag, and the beginning of the AGO1 coding sequence (primers LA172-173) already fused. This fragment was combined by USER cloning with two contiguous PCR products amplified from the already mentioned AGO1P and AGO1+AGO1T templates in pGEM-T with primers LA34-LA171 and LA174-LA37, to obtain the reconstituted full-length construct directly into the binary vector pCAMBIA3300U. Kanamycin resistant colonies were checked by restriction digestion and sequencing before use for plant transformation. The sequences of all primers can be found in Supplemental Data Set 3.

Protein gel blotting

Plant lysates prepared in the same way as for FLAG-AGO1 affinity purification were heated for 5 min at 85ºC in Laemmli sample buffer (70 mM Tris-HCl pH 6.8, 10% glycerol, 1% LDS, and 0.01% BPB, 100 mM DTT) and subjected to SDS-PAGE. Proteins contained in the gel were blotted on Amersham Hybond PVDF or Amersham Protran Premium nitrocellulose membranes (GE Healthcare Life Science) following the instructions provided by the manufacturer. After blocking in 5% skimmed milk, 0.05% Tween-20 PBS buffer for 30 min, membranes were incubated at 4ºC overnight with monoclonal anti-FLAG M2 HRP-conjugated antibody (Sigma; 1000-fold dilution) for detection of FLAG-AGO1, or anti-PEPC (phosphoenolpyruvate carboxylase) (Rockland) as loading control. Detection of horseradish peroxidase (HRP) conjugated anti-FLAG, or secondary antibody after anti-PEPC (Rockland Immunochemicals 200-4163S, 2000-fold dilution), was performed by enhanced chemiluminescence (ECL).

References

Pall, G.S., Codony-Servat, C., Byrne, J., Ritchie, L., and Hamilton, A. (2007). Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35, e60.