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Supplementary Information for

Human Argonaute2 and Argonaute3 are catalytically activated by different lengths of guide RNA

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Extended Methods

Guide RNAs

14-nt and intact guides:

let-7a

14 nt: pUGAGGUAGUAGGUU, 21 nt: pUGAGGUAGUAGGUUGUAUAGU

miR-16

14 nt: pUAGCAGCACGUAAA, 22 nt: pUAGCAGCACGUAAAUAUUGGCG

miR-19b

14 nt: pUGUGCAAAUCCAUG, 23 nt: pUGUGCAAAUCCAUGCAAAACUGA

miR-17

14 nt: pCAAAGUGCUUACAG, 23 nt: pCAAAGUGCUUACAGUGCAGGUAG

miR-18a

14 nt: pUAAGGUGCAUCUAG, 23 nt pUAAGGUGCAUCUAGUGCAGAUAG

miR-19a

14-nt: pUGUGCAAAUCUAUG, 23 nt: pUGUGCAAAUCUAUGCAAAACUGA

miR-27a

14 nt: pUUCACAGUGGCUAA, 21 nt: pUUCACAGUGGCUAAGUUCCGC

miR-92a

14 nt: pUAUUGCACUUGUCC, 22 nt: pUAUUGCACUUGUCCCGGCCUGU

Cloning, expression, and purification of recombinant AGO proteins

The genes of AGO1, AGO2, AGO3, AGO4, and FLAG-AGO3 were cloned in pFastBac-HTB (Invitrogen). Their recombinant proteins were purified from insect cells as previously reported (1, 2).

In vitro cleavage assay

1 μM recombinant AGO protein was incubated for 1 hour with 100 nM 5′ phosphorylated synthetic single-stranded guide RNA for RISC assembly in 1× Reaction Buffer (25 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 0.2 mM EDTA, 0.05 mg/mL BSA (Ambion),

and 5 U/μL RiboLock RNase Inhibitor (Thermo Scientific)), followed by addition of 100 nM 5′ cap-labeled target RNA (1). The reaction was directly mixed with 2× urea quenching dye (7 M urea, 1 mM EDTA, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue, 10% (v/v) phenol). The cleavage products were resolved on a 7 M urea 16% (w/v) polyacrylamide gel.

RISC assembly assay

10 μg of pCAGEN vector encoding FLAG-AGO were transfected into HEK293T cells, and after 48 hours, the cells were harvested. Based on the western blot analysis, 50 pmol of AGO protein were incubated for 1 hour with 5 pmol of the p14ss or the p23ds for RISC assembly. The RISCs were immunoprecipitated by 50 μL of anti-FLAG M2 beads (Sigma-Aldrich) for 2 hours at room temperature. The immunoprecipitated RISCs were washed 8 times with IP Wash Buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, and 0.05% NP-40) and 2 times with Cleavage Buffer (25 mM HEPES-KOH pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, and 2 mM EDTA). The AGO-bound RNAs were extracted with 200 μL of phenol-chloroform and followed by ethanol precipitation overnight. The extracted RNAs were resolved on a 10% native gel to separate the single-stranded guide RNA from the duplex (*2*).

In vitro cleavage assay using FLAG-AGO programmed in the cell lysate

10 μg of pCAGEN vector encoding FLAG-AGO2, FLAG-AGO3, or FLAG-AGO3 (E638A) were transfected into HEK293T cells. The amount of FLAG-AGO proteins in the cell lysate was normalized based on the western blot result. FLAG-AGO proteins in the cell lysate were quantified by using a standard curve generated with known amounts of recombinant FLAG-AGO3 (*2*). The lysate was incubated for 1 hour with the 14ss or the 23ds, followed by immunopurification with anti-FLAG M2 beads. The beads were washed 8 times with IP Wash Buffer, 2 times with Cleavage Buffer, and then the cap-labeled 60-nt target RNAs were added for target cleavage reaction. The reactions were quenched with 2× urea quenching dye and resolved on a 7 M urea 16% (w/v) polyacrylamide gel.

Validation of modified 14-nt miR-20a

1 μM recombinant AGO3 was incubated with the 14ss, the 14md, or the 23ss for 1 hour at 37 ºC in 1× Reaction Buffer, followed by target cleavage as described above. The reaction was stopped with 2× urea quenching dye and resolved on a 7 M urea 16% (w/v) polyacrylamide gel.

In vitro cleavage assay using FLAG-AGO programmed within the cell

10 μg of pCAGEN vector encoding FLAG-AGO2 or FLAG-AGO3 were transfected into HEK293T cells. After 24 hours, the 14ss, the 14md, or the 23ds was transfected. 24 hours later,

the cells were harvested and sonicated. FLAG-AGO proteins in the cell lysate were quantified based on a standard curve generated with known amounts of recombinant FLAG-AGO3 (2). The overexpressed FLAG-AGOs were immunoprecipitated with 50 μL of anti-FLAG M2 beads, washed with IP Wash Buffer, and incubated with 100 nM cap-labeled 60-nt target RNA**.**

SI References

1. M. S. Park *et al.*, Human Argonaute3 has slicer activity. *Nucleic Acids Res* 45, 11867-11877 (2017).
2. M. S. Park *et al.*, Multidomain Convergence of Argonaute during RISC Assembly Correlates with the Formation of Internal Water Clusters. *Mol Cell* (2019).