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Supplemental Information

Multidomain Convergence of Argonaute During RISC Assembly Correlates with the Formation of Internal Water Clusters

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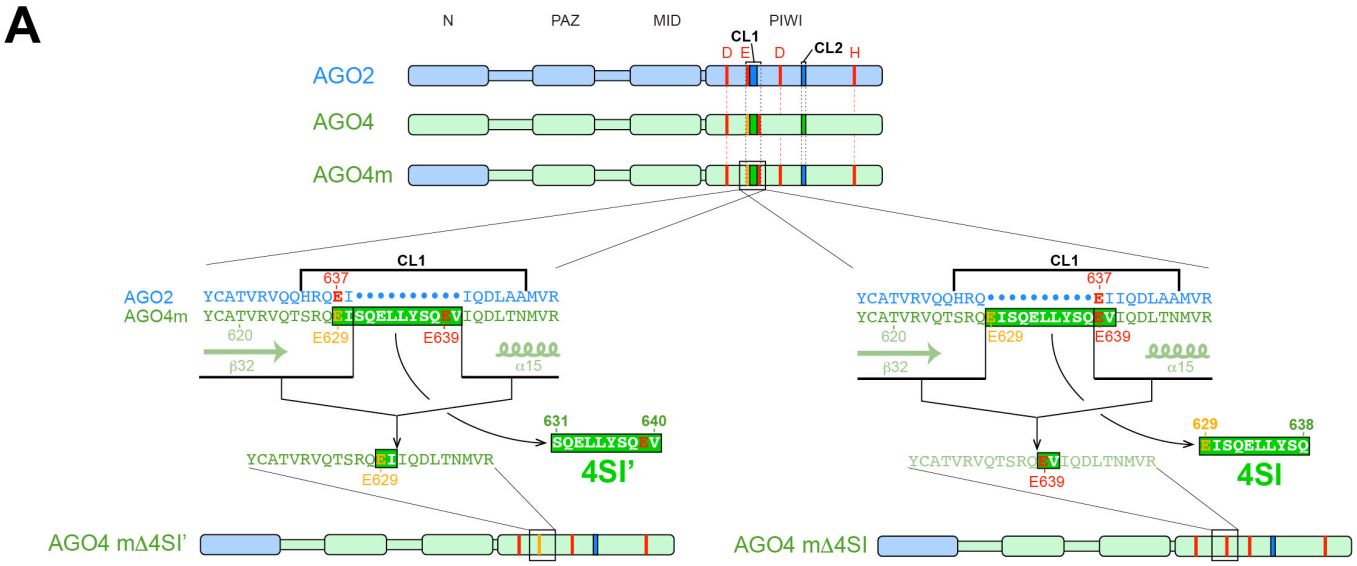
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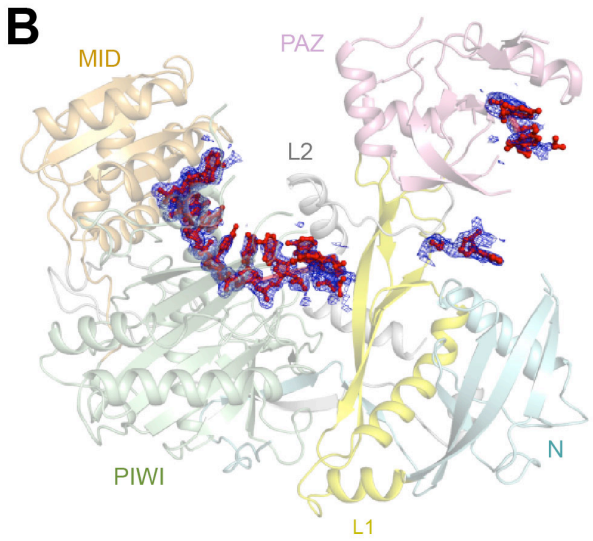
N
PAZ
MID
PIWI
CL1
CL2
AGO2
AGO4
AGO4m
AGO2
AGO4m
637
E629
E639
620
β32
α15
631
640
4SI'
AGO4 mΔ4SI'
629
638
4SI
AGO4 mΔ4SI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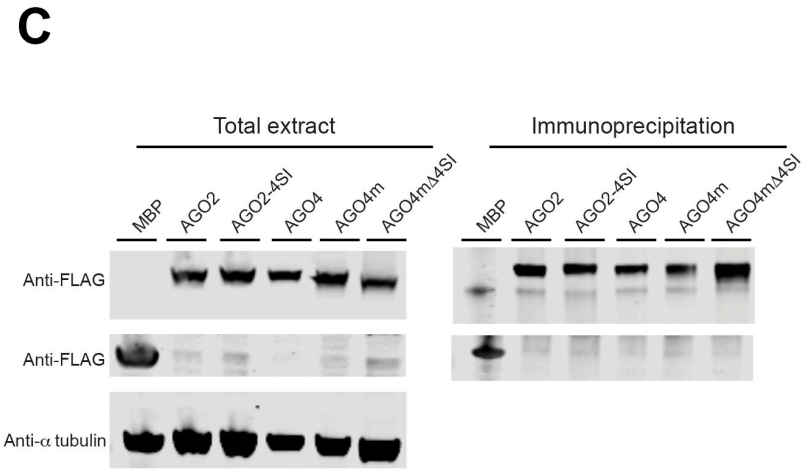
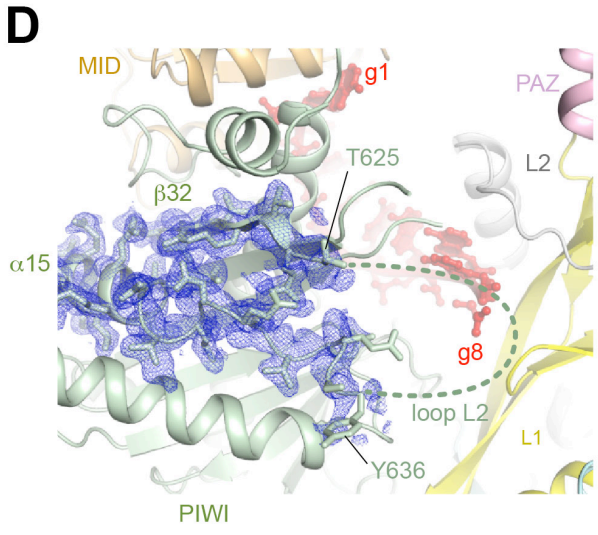
L2
N
PIWI
L1


C
Total extract
Immunoprecipitation
MBP
AGO2
AGO2-4SI
AGO4
AGO4m
AGO4mΔ4SI
Anti-FLAG
Anti-FLAG
Anti-α tubulin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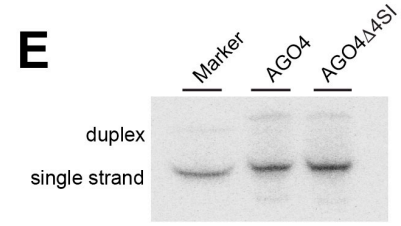
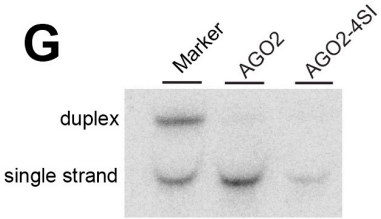
g8
loop L2
L1
Y636
PIWI


E
Marker
AGO4
AGO4Δ4SI
duplex
single strand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Marker
AGO2
AGO2-4SI
duplex
single strand

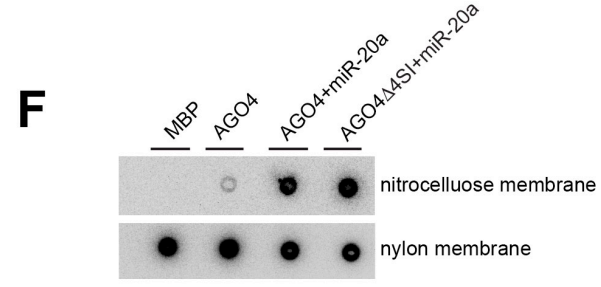

F
MBP
AGO4
AGO4+miR-20a
AGO4Δ4SI+miR-20a
nitrocelluose membrane
nylon membrane

Figure S1. Strategy for making AGO4 into a catalytically active enzyme (Related to Figure 1)

(A) Differences in the construct used by Hauptmann *et al.* (left) and that used in the current study (right), both of which generate a catalytically active AGO4 mutant.

(B) Simulated annealing F_o-F_c omit map contoured at 2.5 σ (blue mesh) around the guide RNA. Colors as in Figure 1B.

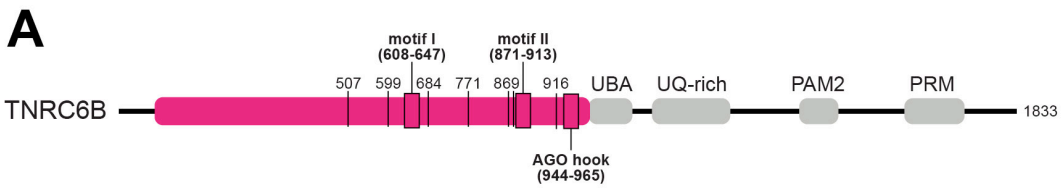
(C) FLAG-AGO2, FLAG-AGO4 and their mutants were expressed in HEK293T cells and detected with anti-FLAG antibody (left). Their protein amounts were adjusted based on the western blot (right) and used for target cleavage assay (Figure 1E).

(D) Simulated annealing F_o-F_c omit map contoured at 2.2 σ (blue mesh) around α15 and β32 of AGO4. The disordered loop L2, including 4SI, is depicted as dotted lines. Colors as in (B).

(E) RISC maturation assays of AGO4 and AGO4Δ4SI (Figure 1H and 1I). The lysates of HEK293T cells expressing either FLAG-AGO4 or FLAG-AGO4Δ4SI were adjusted by western blot so as to include 50 pmol of AGO, followed by incubation with miR-20a duplex. The duplex is composed of a 5′ end labeled miR-20a and a fully complementary passenger strand that lacks a 5' monophosphate. After immunoprecipitation with anti-FLAG beads, the AGO-bound RNAs were extracted and resolved on 10% native gel.

(F) Filter binding assay of MBP, AGO4, and AGO4Δ4SI. The lysates of HEK293T cells expressing either FLAG-MBP, FLAG-AGO4 or FLAG-AGO4Δ4SI were incubated with miR-20a duplexes and then immunoprecipitated with anti-FLAG beads. After the AGOs were eluted from the beads with FLAG peptides, the AGOs were incubated with 60-nt 5′ cap labeled mismatch targets. The resultants were spotted on a nitrocellulose membrane and a nylon membrane. AGO4 without miR-20a was used as a control to subtract the non-specific bound targets (Figure 1J).

(G) RISC maturation assays of AGO2 and AGO2-4SI (Figure 1K). The lysates of HEK293T cells expressing either FLAG-AGO2 or FLAG-AGO2-4SI were adjusted by western blot so as to include 50 pmol of AGO, followed by incubation with miR-20a duplex. The duplex is composed of a 5′ end labeled miR-20a and a fully complementary passenger strand that lacks a 5’ monophosphate. After immunoprecipitation with anti-FLAG beads, the AGO-bound RNAs were extracted and resolved on 10% native gel.


A
motif I
(608-647)
motif II
(871-913)
507
599
684
771
869
916
UBA
UQ-rich
PAM2
PRM
TNRC6B
1833
AGO hook
(944-965)

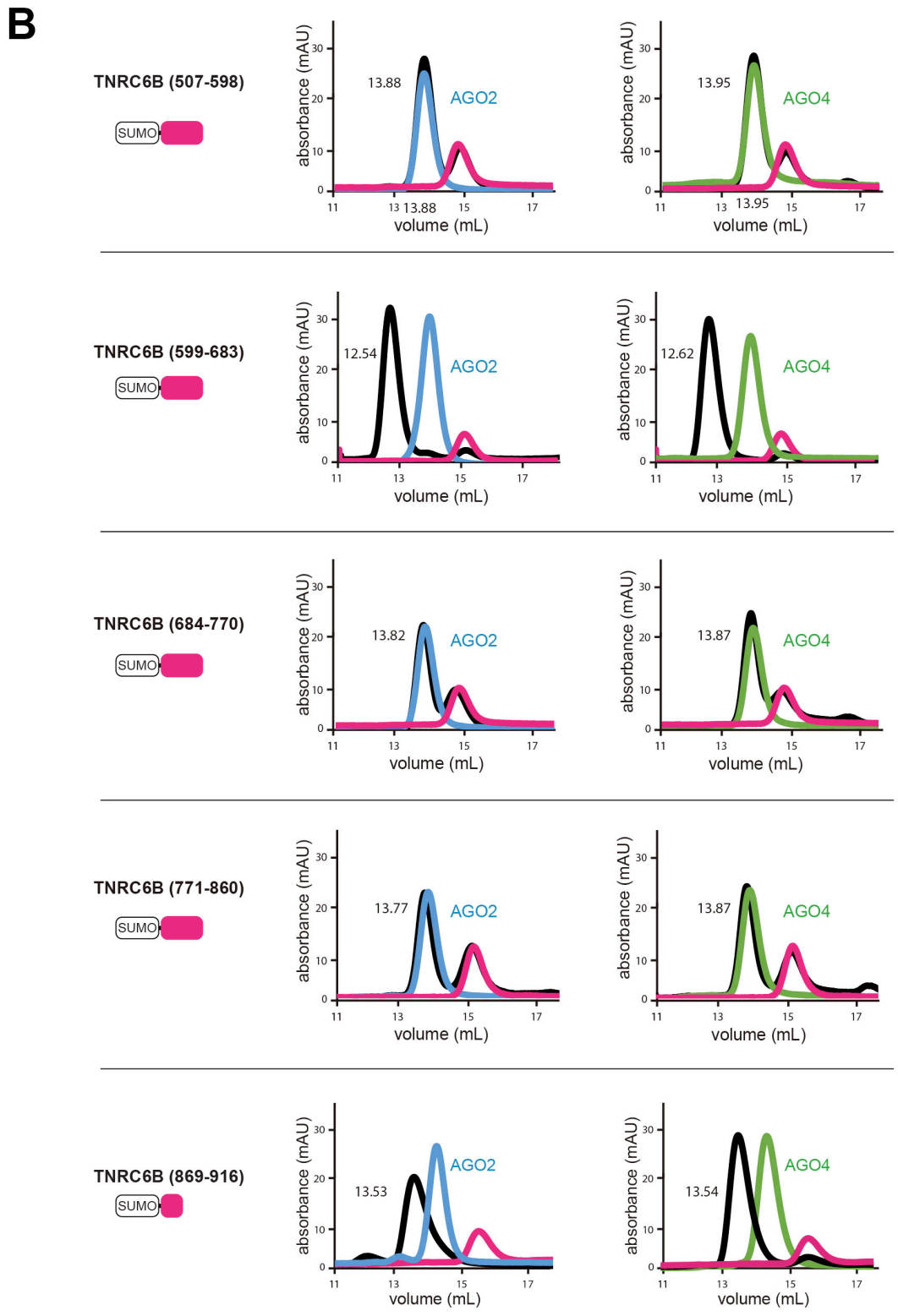
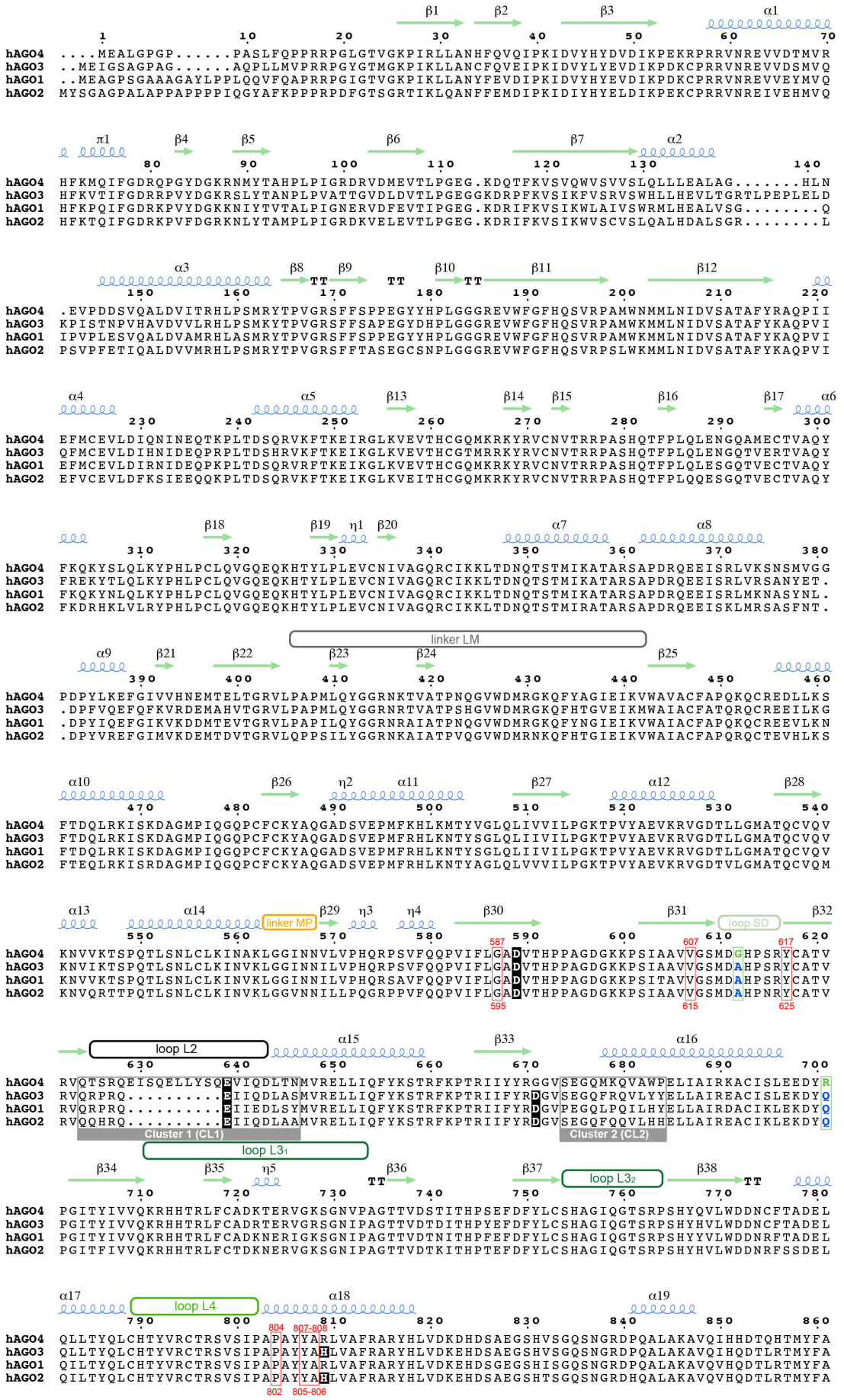

B
TNRC6B (507-598)
SUMO
absorbance (mAU)
13.88
AGO2
volume (mL)
13.95
AGO4
TNRC6B (599-683)
12.54
12.62
TNRC6B (684-770)
13.82
13.87
TNRC6B (771-860)
13.77
13.87
TNRC6B (869-916)
13.53
13.54

Figure S2. Interactions of long TNRC6B fragments with AGO2 and AGO4 (Related to Figure 2)

(A) Domain architecture of TNRC6B. Several long fragments were designed from the AGO-binding regions (pink) and expressed as SUMO-fused proteins.

(B) Gel filtration analyses of AGO2 (blue), AGO4 (green), SUMO-fused TNRC6B fragment (pink), and a mixture of a TNRC6B fragment with either AGO2 or AGO4 (black).


β1 β2 β3 α1
1 10 20 30 40 50 60 70
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hAGO3 ..MEIGSAGPAG......AQPLLMVPRRPGYGTMGKPIKLLANCFQVEIPKIDVYLYEVDIKPDKCPRRVNREVVDSMVQ
hAGO1 ..MEAGPSGAAAGAYLPPLQQVFQAPRRPGIGTVGKPIKLLANYFEVDIPKIDVYHYEVDIKPDKCPRRVNREVVEYMVQ
hAGO2 MYSGAGPALAPPAPPPPIQGYAFKPPPRPDFGTSGRTIKLQANFFEMDIPKIDIYHYELDIKPEKCPRRVNREIVEHMVQ
π1 β4 β5 β6 β7 α2
80 90 100 110 120 130 140
hAGO4 HFKMQIFGDRQPGYDGKRNMYTAHPLPIGRDRVDMEVTLPGEG.KDQTFKVSVQWVSVVSLQLLLEALAG.......HLN
hAGO3 HFKVTIFGDRRPVYDGKRSLYTANPLPVATTGVDLDVTLPGEGGKDRPFKVSIKFVSRVSWHLLHEVLTGRTLPEPLELD
hAGO1 HFKPQIFGDRKPVYDGKKNIYTVTALPIGNERVDFEVTIPGEG.KDRIFKVSIKWLAIVSWRMLHEALVSG........Q
hAGO2 HFKTQIFGDRKPVFDGRKNLYTAMPLPIGRDKVELEVTLPGEG.KDRIFKVSIKWVSCVSLQALHDALSGR........L
α3 β8 β9 β10 β11 β12
TT TT TT
150 160 170 180 190 200 210 220
hAGO4 .EVPDDSVQALDVITRHLPSMRYTPVGRSFFSPPEGYYHPLGGGREVWFGFHQSVRPAMWNMMLNIDVSATAFYRAQPII
hAGO3 KPISTNPVHAVDVVLRHLPSMKYTPVGRSFFSAPEGYDHPLGGGREVWFGFHQSVRPAMWKMMLNIDVSATAFYKAQPVI
hAGO1 IPVPLESVQALDVAMRHLASMRYTPVGRSFFSPPEGYYHPLGGGREVWFGFHQSVRPAMWKMMLNIDVSATAFYKAQPVI
hAGO2 PSVPFETIQALDVVMRHLPSMRYTPVGRSFFTASEGCSNPLGGGREVWFGFHQSVRPSLWKMMLNIDVSATAFYKAQPVI
α4 α5 β13 β14 β15 β16 β17 α6
230 240 250 260 270 280 290 300
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hAGO3 QFMCEVLDIHNIDEQPRPLTDSHRVKFTKEIKGLKVEVTHCGTMRRKYRVCNVTRRPASHQTFPLQLENGQTVERTVAQY
hAGO1 EFMCEVLDIRNIDEQPKPLTDSQRVRFTKEIKGLKVEVTHCGQMKRKYRVCNVTRRPASHQTFPLQLESGQTVECTVAQY
hAGO2 EFVCEVLDFKSIEEQQKPLTDSQRVKFTKEIKGLKVEITHCGQMKRKYRVCNVTRRPASHQTFPLQQESGQTVECTVAQY
β18 β19 η1 β20 α7 α8
310 320 330 340 350 360 370 380
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hAGO1 FKQKYNLQLKYPHLPCLQVGQEQKHTYLPLEVCNIVAGQRCIKKLTDNQTSTMIKATARSAPDRQEEISRLMKNASYNL.
hAGO2 FKDRHKLVLRYPHLPCLQVGQEQKHTYLPLEVCNIVAGQRCIKKLTDNQTSTMIRATARSAPDRQEEISKLMRSASFNT.
linker LM
α9 β21 β22 β23 β24 β25
390 400 410 420 430 440 450 460
hAGO4 PDPYLKEFGIVVHNEMTELTGRVLPAPMLQYGGRNKTVATPNQGVWDMRGKQFYAGIEIKVWAVACFAPQKQCREDLLKS
hAGO3 .DPFVQEFQFKVRDEMAHVTGRVLPAPMLQYGGRNRTVATPSHGVWDMRGKQFHTGVEIKMWAIACFATQRQCREEILKG
hAGO1 .DPYIQEFGIKVKDDMTEVTGRVLPAPILQYGGRNRAIATPNQGVWDMRGKQFYNGIEIKVWAIACFAPQKQCREEVLKN
hAGO2 .DPYVREFGIMVKDEMTDVTGRVLQPPSILYGGRNKAIATPVQGVWDMRNKQFHTGIEIKVWAIACFAPQRQCTEVHLKS
α10 β26 η2 α11 β27 α12 β28
470 480 490 500 510 520 530 540
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hAGO1 FTDQLRKISKDAGMPIQGQPCFCKYAQGADSVEPMFRHLKNTYSGLQLIIVILPGKTPVYAEVKRVGDTLLGMATQCVQV
hAGO2 FTEQLRKISRDAGMPIQGQPCFCKYAQGADSVEPMFRHLKNTYAGLQLVVVILPGKTPVYAEVKRVGDTVLGMATQCVQM
α13 α14 linker MP β29 η3 η4 β30 β31 loop SD β32
550 560 570 580 590 600 610 620
587 607 617
hAGO4 KNVVKTSPQTLSNLCLKINAKLGGINNVLVPHQRPSVFQQPVIFLGADVTHPPAGDGKKPSIAAVVGSMDGHPSRYCATV
hAGO3 KNVIKTSPQTLSNLCLKINVKLGGINNILVPHQRPSVFQQPVIFLGADVTHPPAGDGKKPSIAAVVGSMDAHPSRYCATV
hAGO1 KNVVKTSPQTLSNLCLKINVKLGGINNILVPHQRSAVFQQPVIFLGADVTHPPAGDGKKPSITAVVGSMDAHPSRYCATV
hAGO2 KNVQRTTPQTLSNLCLKINVKLGGVNNILLPQGRPPVFQQPVIFLGADVTHPPAGDGKKPSIAAVVGSMDAHPNRYCATV
595 615 625
loop L2 α15 β33 α16
630 640 650 660 670 680 690 700
hAGO4 RVQTSRQEISQELLYSQEVIQDLTNMVRELLIQFYKSTRFKPTRIIYYRGGVSEGQMKQVAWPELIAIRKACISLEEDYR
hAGO3 RVQRPRQ.........EIIQDLASMVRELLIQFYKSTRFKPTRIIFYRDGVSEGQFRQVLYYELLAIREACISLEKDYQ
hAGO1 RVQRPRQ.........EIIEDLSYMVRELLIQFYKSTRFKPTRIIFYRDGVPEGQLPQILHYELLAIRDACIKLEKDYQ
hAGO2 RVQQHRQ.........EIIQDLAAMVRELLIQFYKSTRFKPTRIIFYRDGVSEGQFQQVLHHELLAIREACIKLEKDYQ
Cluster 1 (CL1) Cluster 2 (CL2)
loop L3₁
β34 β35 η5 β36 β37 loop L3₂ β38
TT TT
710 720 730 740 750 760 770 780
hAGO4 PGITYIVVQKRHHTRLFCADKTERVGKSGNVPAGTTVDSTITHPSEFDFYLCSHAGIQGTSRPSHYQVLWDDNCFTADEL
hAGO3 PGITYIVVQKRHHTRLFCADRTERVGRSGNIPAGTTVDTDITHPYEFDFYLCSHAGIQGTSRPSHYHVLWDDNCFTADEL
hAGO1 PGITYIVVQKRHHTRLFCADKNERIGKSGNIPAGTTVDTNITHPFEFDFYLCSHAGIQGTSRPSHYYVLWDDNRFTADEL
hAGO2 PGITFIVVQKRHHTRLFCTDKNERVGKSGNIPAGTTVDTKITHPTEFDFYLCSHAGIQGTSRPSHYHVLWDDNRFSSDEL
α17 loop L4 α18 α19
790 800 810 820 830 840 850 860
804 807-808
hAGO4 QLLTYQLCHTYVRCTRSVSIPAPAYYARLVAFRARYHLVDKDHDSAEGSHVSGQSNGRDPQALAKAVQIHHDTQHTMYFA
hAGO3 QLLTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVDKEHDSAEGSHVSGQSNGRDPQALAKAVQIHQDTLRTMYFA
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hAGO2 QILTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVDKEHDSAEGSHTSGQSNGRDHQALAKAVQVHQDTLRTMYFA
802 805-806

Figure S3. Sequence alignment of human AGO paralogs (Related to Figure 2; Figure 3; Figure 4)

Residue number and secondary structure of AGO4 are shown above the sequence. The four catalytic residues are highlighted with black columns. AGO4-specific residues in Trp Pocket-2 and -3 are boxed (green). Residues involved in van der Waals interactions between two PIWI subdomains are boxed (red). Domain linkers and loops forming LAKE1 and LAKE2 are labeled above the sequence. CL1 and CL2 are boxed (gray).

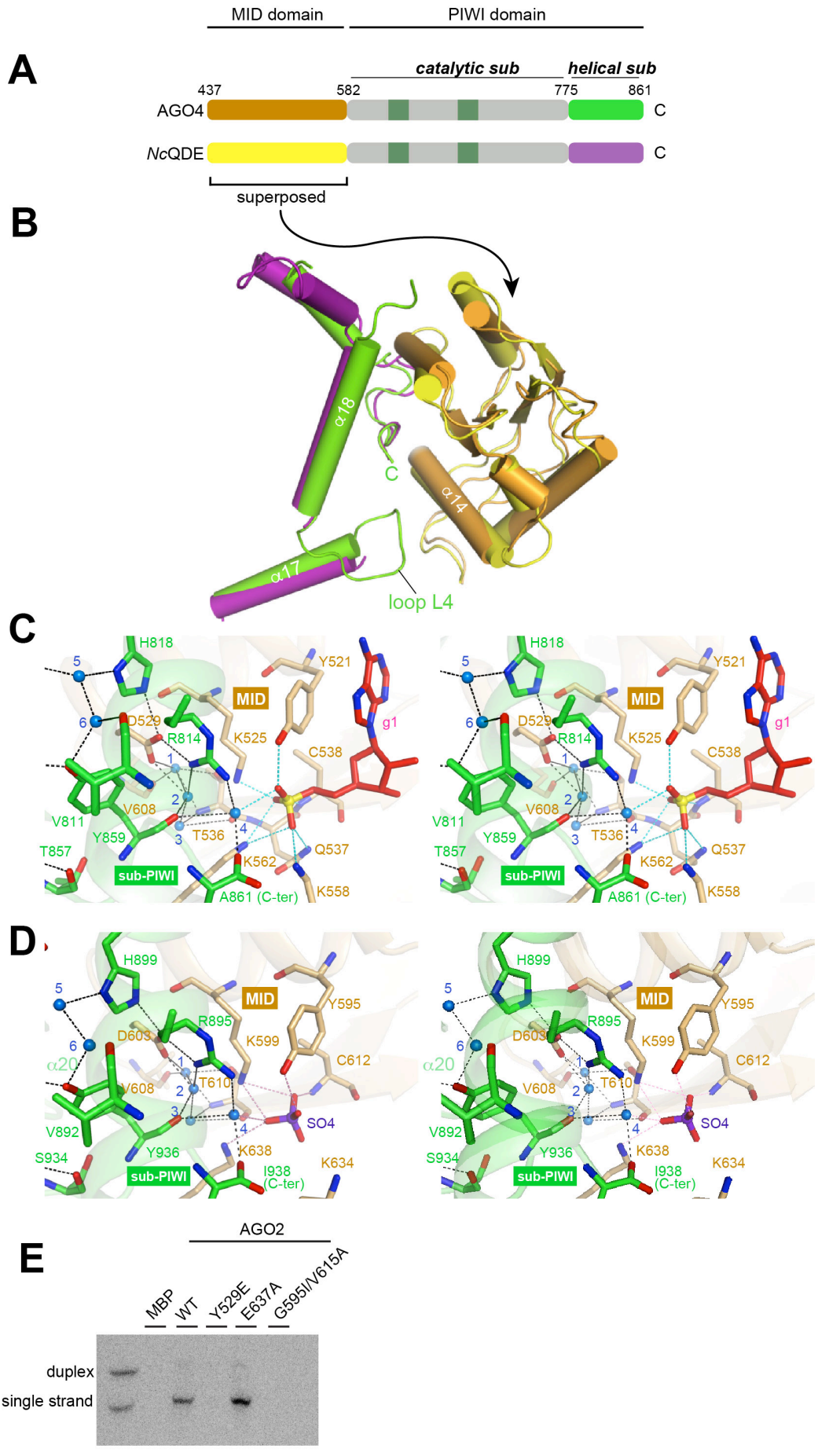

MID domain
PIWI domain
A
catalytic sub
helical sub
437
582
775
861
AGO4
C
NcQDE
C
superposed
B
α18
C
α14
α17
loop L4
C
H818
Y521
MID
D529
R814
K525
g1
C538
V608
V811
Y859
T536
T857
sub-PIWI
K562
Q537
A861 (C-ter)
K558
D
H899
Y595
R895
D603
K599
C612
α20
T610
SO4
V892
S934
Y936
K638
K634
I938
(C-ter)
E
AGO2
MBP
WT
Y529E
E637A
G595I/V615A
duplex
single strand

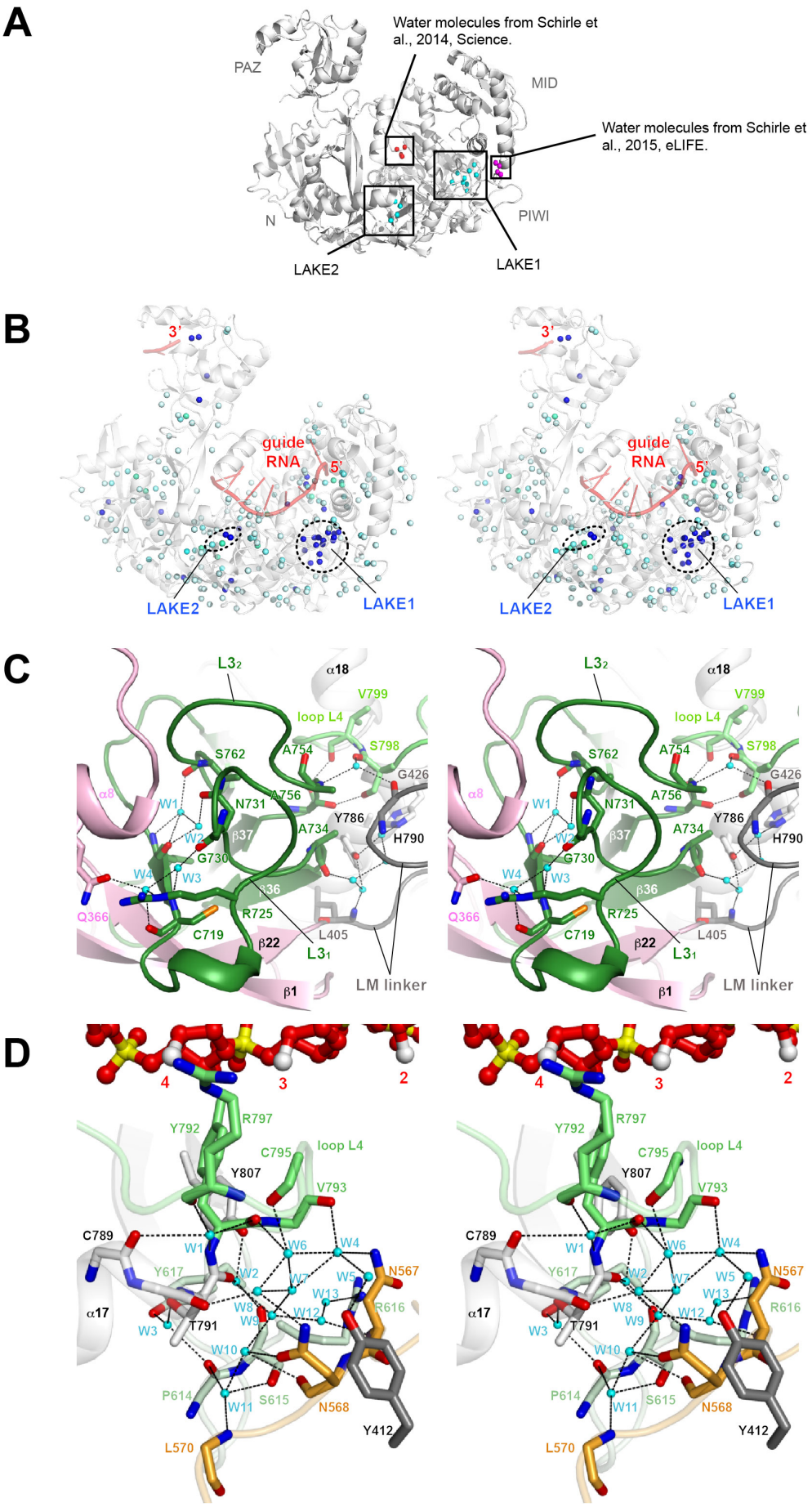
Figure S4. Interactions between MID domain and PIWI-helical subdomain (Related to Figure 3; Figure 4)

(A) MID-PIWI domain architectures of AGO4 and *Nc*QDE2.

(B) Superposition of AGO4 and *Nc*QDE2 on their MID domains results in a good alignment of their PIWI-helical subdomains.

(C-D) Stereoviews of the interactions between the MID domain and PIWI-helical subdomain in AGO4 (C) and *Nc*QDE2 (D). Colors as in (A). Water molecules and hydrogen bonds are depicted as spheres (cyan) and dotted lines, respectively. For clarity, only g1 of the bound guide RNA is shown in (C). The bound sulfate ion is drawn as a stick model (magenta) in (D).

(E) A representative image of RISC maturation assay with siRNA duplex (Figure 3E).


A
Water molecules from Schirle et al., 2014, Science.
PAZ
MID
Water molecules from Schirle et al., 2015, eLIFE.
N
PIWI
LAKE2
LAKE1
B
3'
guide RNA
5'
LAKE2
LAKE1
3'
guide RNA
5'
LAKE2
LAKE1
C
L3₂
α18
V799
loop L4
S762
A754
S798
G426
α8
W1
N731
A756
Y786
W2
β37
A734
H790
G730
W4
W3
β36
Q366
R725
C719
β22
L405
L3₁
β1
LM linker
L3₂
α18
V799
loop L4
S762
A754
S798
G426
α8
W1
N731
A756
Y786
W2
β37
A734
H790
G730
W4
W3
β36
Q366
R725
C719
β22
L405
L3₁
β1
LM linker
D
4
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C795
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Y807
V793
C789
W1
W6
W4
Y617
W2
W7
W5
N567
W13
α17
W8
R616
W12
T791
W3
W9
W10
P614
W11
S615
N568
Y412
L570
4
3
2
Y792
R797
C795
loop L4
Y807
V793
C789
W1
W6
W4
Y617
W2
W7
W5
N567
W13
α17
W8
R616
W12
T791
W3
W9
W10
P614
W11
S615
N568
Y412
L570

Figure S5. Water-mediated hydrogen-bonding network (Related to Figure 5)

(A) Locations of LAKEs (cyan) and the previously reported water molecules at the active site (red) and the target nucleotide-1 pocket (magenta).

(B) Stereoview of water molecules bound to AGO4. The intensity of the blue color indicates the categories of the water molecule. Dark blue, cyan, and faint blue spheres indicate buried, cleft, and surface water molecules, respectively.

(C-D) Stereoview of the internal water-mediated hydrogen-bonding network in LAKE2 (C) and LAKE1 (D). Water molecules and hydrogen bonds are depicted as cyan spheres and dotted lines, respectively. The bound RNA in (D) is drawn as a ball-and-stick model. Colors as in Figure 4C.

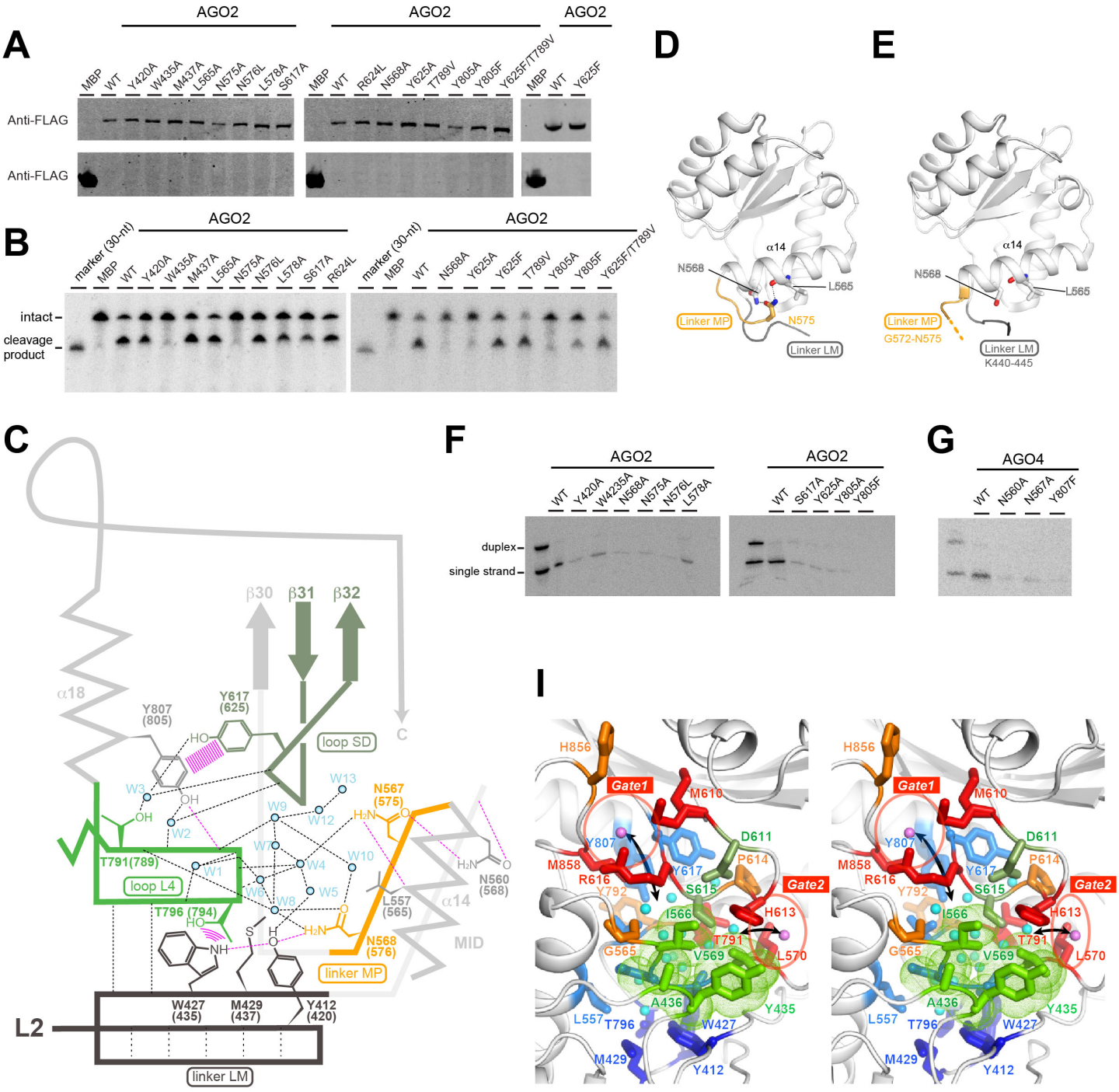
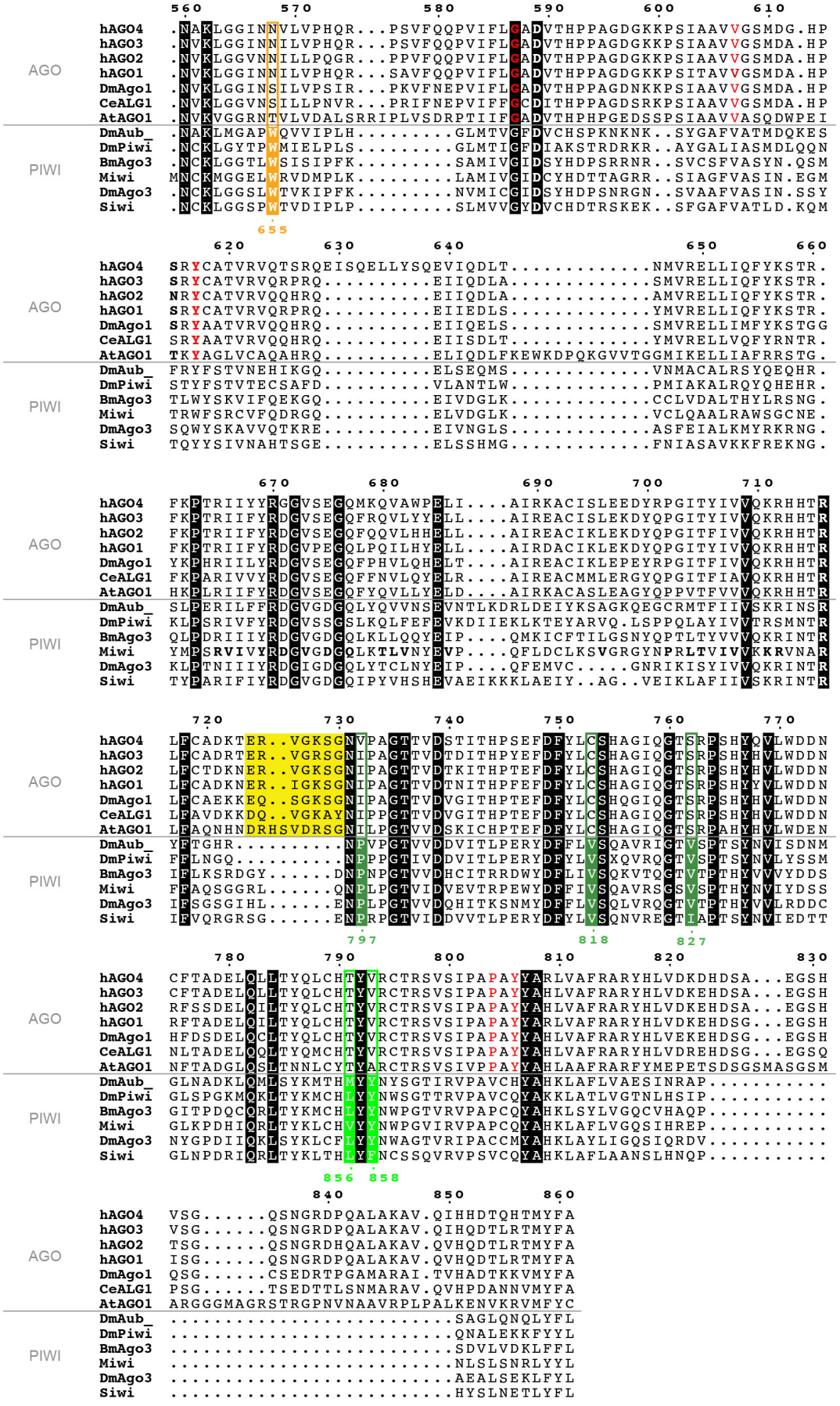


Figure S6. Residues and their interactions required for LAKE1 formation (Related to Figure 5)

(A) A representative image of western blot analysis with anti-FLAG antibody. Expression levels of mutants were tested, and the amount of proteins were adjusted for the target cleavage assay.

(B) A representative image of *In vitro* cleavage assay with miR-20a. All lysates were adjusted by western blots of (A) and the 5 μM AGO proteins were incubated with 50 nM siRNA-like duplex of miR-20a for RISC assembly. 50 nM 5' cap-labeled target RNAs were used for the RNA cleavage reaction.

(C) A schematic of LAKE1. Conserved residues involved in the LAKE1 formation are shown. The internal water molecules and hydrogen bonds are depicted as spheres (cyan) and dotted lines (black), respectively. Hydrogen bonds not involving water molecules are colored in pink.
(D-E) Interactions between the MID domain and linker MP through two asparagine residues seen in the full-length AGO2 (D) (PDB ID: 4OLA) but not in the isolated AGO2 MID domain (E) (PDB ID: 3LUC).
(F-G) A representative image of RISC maturation assays with siRNA-like duplex of miR-20a (Figure 5H-5J).
(I) Stereoview of the two gates, Gate1 and Gate 2 (red circles), of the AGO4 LAKE1. Water molecules inside LAKE1 are colored in cyan. Two water molecules located just outside the two gates are colored in pink. Residues shaping the AGO4 LAKE1 can be categorized based on their roles; back wall (dark blue), middle wall (marine), gates (red), hydrophobic core (green), backbone (orange).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hAGO3 .NVKLGGINNILVPHQR...PSVFQQPVIFLGADVTHPPAGDGKKPSIAAVVGSMDA.HP
hAGO2 .NVKLGGVNNILLPQGR...PPVFQQPVIFLGADVTHPPAGDGKKPSIAAVVGSMDA.HP
hAGO1 .NVKLGGINNILVPHQR...SAVFQQPVIFLGADVTHPPAGDGKKPSITAVVGSMDA.HP
DmAgo1 .NVKLGGINSILVPSIR...PKVFNEPVIFLGADVTHPPAGDNKKPSIAAVVGSMDA.HP
CeALG1 .NVKLGGVNSILLPNVR...PRIFNEPVIFFGCDITHPPAGDSRKPSIAAVVGSMDA.HP
AtAGO1 .NVKVGGRNTVLVDALSRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEI
AGO
DmAub_ .NAKLMGAPWQVVIPLH.....GLMTVGFDVCHSPKNKNK..SYGAFVATMDQKES
DmPiwi .NCKLGYTPWMIELPLS.....GLMTIGFDIAKSTRDRKR..AYGALIASMDLQQN
BmAgo3 .NCKLGGTLWSISIPFK.....SAMIVGIDSYHDPSRRNR..SVCSFVASYN.QSM
Miwi MNCKMGGELWRVDMPLK.....LAMIVGIDCYHDTTAGRR..SIAGFVASIN.EGM
DmAgo3 .NCKLGGSLWTVKIPFK.....NVMICGIDSYHDPSNRGN..SVAAFVASIN.SSY
Siwi .NCKLGGSPWTVDIPLP.....SLMVVGYDVCHDTRSKEK..SFGAFVATLD.KQM
655
620 630 640 650 660
hAGO4 SRYCATVRVQTSRQEISQELLYSQEVIQDLT.....NMVRELLIQFYKSTR.
hAGO3 SRYCATVRVQRPRQ.....EIIQDLA.....SMVRELLIQFYKSTR.
hAGO2 NRYCATVRVQQHRQ.....EIIQDLA.....AMVRELLIQFYKSTR.
hAGO1 SRYCATVRVQRPRQ.....EIIEDLS.....YMVRELLIQFYKSTR.
DmAgo1 SRYAATVRVQQHRQ.....EIIQELS.....SMVRELLIMFYKSTGG
CeALG1 SRYAATVRVQQHRQ.....EIISDLT.....YMVRELLVQFYRNTR.
AtAGO1 TKYAGLVCAQAHRQ.....ELIQDLFKEWKDPQKGVVTGGMIKELLIAFRRSTG.
AGO
DmAub_ FRYFSTVNEHIKGQ.....ELSEQMS.....VNMACALRSYQEQHR.
DmPiwi STYFSTVTECSAFD.....VLANTLW.....PMIAKALRQYQHEHR.
BmAgo3 TLWYSKVIFQEKGQ.....EIVDGLK.....CCLVDALTHYLRSNG.
Miwi TRWFSRCVFQDRGQ.....ELVDGLK.....VCLQAALRAWSGCNE.
DmAgo3 SQWYSKAVVQTKRE.....EIVNGLS.....ASFEIALKMYRKRNG.
Siwi TQYYSIVNAHTSGE.....ELSSHMG.....FNIASAVKKFREKNG.
PIWI
670 680 690 700 710
hAGO4 FKPTRIIYYRGGVSEGQMKQVAWPELI....AIRKACISLEEDYRPGITYIVVQKRHHTR
hAGO3 FKPTRIIFYRDGVSEGQFRQVLYYELL....AIREACISLEKDYQPGITYIVVQKRHHTR
hAGO2 FKPTRIIFYRDGVSEGQFQQVLHHELL....AIREACIKLEKDYQPGITFIVVQKRHHTR
hAGO1 FKPTRIIFYRDGVPEGQLPQILHYELL....AIRDACIKLEKDYQPGITYIVVQKRHHTR
DmAgo1 YKPHRIILYRDGVSEGQFPHVLQHELT....AIREACIKLEPEYRPGITFIVVQKRHHTR
CeALG1 FKPARIVVYRDGVSEGQFFNVLQYELR....AIREACMMLERGYQPGITFIAVQKRHHTR
AtAGO1 HKPLRIIFYRDGVSEGQFYQVLLYELD....AIRKACASLEAGYQPPVTFVVVQKRHHTR
AGO
DmAub_ SLPERILFFRDGVGDGQLYQVVNSEVNTLKDRLDEIYKSAGKQEGCRMTFIIVSKRINSR
DmPiwi KLPSRIVFYRDGVSSGSLKQLFEFEVKDIIEKLKTEYARVQ.LSPPQLAYIVVTRSMNTR
BmAgo3 QLPDRIIIYRDGVGDGQLKLLQQYEIP....QMKICFTILGSNYQPTLTYVVVQKRINTR
Miwi YMPSRVIVYRDGVGDGQLKTLVNYEVP....QFLDCLKSVGRGYNPRLTVIVVKKRVNAR
DmAgo3 KLPTNIIIYRDGIGDGQLYTCLNYEIP....QFEMVC.....GNRIKISYIVVQKRINTR
Siwi TYPARIFIYRDGVGDGQIPYVHSHEVAEIKKKLAEIY..AG..VEIKLAFIIVSKRINTR
PIWI
720 730 740 750 760 770
hAGO4 LFCADKTER..VGKSGNVPAGTTVDSTITHPSEFDFYLCSHAGIQGTSRPSHYQVLWDDN
hAGO3 LFCADRTER..VGRSGNIPAGTTVDTDITHPYEFDFYLCSHAGIQGTSRPSHYHVLWDDN
hAGO2 LFCTDKNER..VGKSGNIPAGTTVDTKITHPTEFDFYLCSHAGIQGTSRPSHYHVLWDDN
hAGO1 LFCADKNER..IGKSGNIPAGTTVDTNITHPFEFDFYLCSHAGIQGTSRPSHYYVLWDDN
DmAgo1 LFCAEKKEQ..SGKSGNIPAGTTVDVGITHPTEFDFYLCSHQGIQGTSRPSHYHVLWDDN
CeALG1 LFAVDKKDQ..VGKAYNIPPGTTVDVGITHPTEFDFYLCSHAGIQGTSRPSHYHVLWDDN
AtAGO1 LFAQNHNDRHSVDRSGNILPGTVVDSKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDEN
AGO
DmAub_ YFTGHR.....NPVPGTVVDDVITLPERYDFFLVSQAVRIGTVSPTSYNVISDNM
DmPiwi FFLNGQ.....NPPPGTIVDDVITLPERYDFYLVSXQVRQGTVSPTSYNVLYSSM
BmAgo3 IFLKSRDGY.....DNPNPGTVVDHCITRRDWYDFLIVSQKVTQGTVTPTHYVVVYDDS
Miwi FFAQSGGRL.....QNPLPGTVIDVEVTRPEWYDFFIVSQAVRSGSVSPTHYNVIYDSS
DmAgo3 IFSGSGIHL.....ENPLPGTVVDQHITKSNMYDFFLVSQLVRQGTVTPTHYVVLRDDC
Siwi IFVQRGRSG.....ENPRPGTVIDDVVTLPERYDFYLVSQNVREGTIAPTSYNVIEDTT
797 818 827
780 790 800 810 820 830
hAGO4 CFTADELQLLTYQLCHTYVRCTRSVSIPAPAYYARLVAFRARYHLVDKDHDSA...EGSH
hAGO3 CFTADELQLLTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVDKEHDSA...EGSH
hAGO2 RFSSDELQILTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVDKEHDSA...EGSH
hAGO1 RFTADELQILTYQLCHTYVRCTRSVSIPAPAYYARLVAFRARYHLVDKEHDSG...EGSH
DmAgo1 HFDSDELQCLTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVEKEHDSG...EGSH
CeALG1 NLTADELQQLTYQMCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVDREHDSG...EGSQ
AtAGO1 NFTADGLQSLTNNLCYTYARCTRSVSIVPPAYYAHLAAFRARFYMEPETSDSGSMASGSM
AGO
DmAub_ GLNADKLQMLSYKMTHMYYNYSGTIRVPAVCHYAHKLAFLVAESINRAP.....
DmPiwi GLSPGKMQKLTYKMCHLYYNWSGTTRVPAVCQYAKKLATLVGTNLHSIP.....
BmAgo3 GITPDQCQRLTYKMCHLYYNWPGTVRVPAPCQYAHKLSYLVGQCVHAQP.....
Miwi GLKPDHIQRLTYKLCHVYYNWPGVIRVPAPCQYAHKLAFLVGQSIHREP.....
DmAgo3 NYGPDIIQKLSYKLCFLYYNWAGTVRIPACCMYAHKLAYLIGQSIQRDV.....
Siwi GLNPDRIQRLTYKLTHLYFNCSSQVRVPSVCQYAHKLAFLAANSLHNQP.....
PIWI
856 858
840 850 860
hAGO4 VSG.....QSNGRDPQALAKAV.QIHHDTQHTMYFA
hAGO3 VSG.....QSNGRDPQALAKAV.QIHQDTLRTMYFA
hAGO2 TSG.....QSNGRDHQALAKAV.QVHQDTLRTMYFA
hAGO1 ISG.....QSNGRDPQALAKAV.QVHQDTLRTMYFA
DmAgo1 QSG.....CSEDRTPGAMARAI.TVHADTKKVMYFA
CeALG1 PSG.....TSEDTTLSNMARAV.QVHPDANNVMYFA
AtAGO1 ARGGGMAGRSTRGPNVNAAVRPLPALKENVKRVMFYC
AGO
DmAub_ .....SAGLQNQLYFL
DmPiwi .....QNALEKKFYYL
BmAgo3 .....SDVLVDKLFFL
Miwi .....NLSLSNRLYYL
DmAgo3 .....AEALSEKLFYL
Siwi .....HYSLNETLYFL
PIWI

Figure S7. Sequence alignment of AGO- and PIWI-clade proteins (Related to Figure 7)

Residues highlighted in red participate in the van der Waals interaction between two PIWI subdomains. Residues in colored boxes (linker MP: orange, $L3_1$ and $L3_2$: dark green, loop L4: bright green) have different properties between AGO and PIWI clades. Residue numbers of AGO4 and Siwi are shown above and below the sequence alignment, respectively. AGO-specific insert in $L3_1$ is colored in yellow (see Figure 7C-D).

Table S1. Detailed simulation statistics of LAKE1 and LAKE2 of $AGO2_{cw}$ and $AGO4_{cw}$ (Related to Figure 6)

AGO and Temp	Number of water molecules							Avg. Occup.*	t_1 (ps)	t_2 (ps)
	Cavity	Mean	SD	Variance	max	min	median			
AGO4cw 300 K	LAKE1	10.3	1.8	3.2	18	5	10	10 ± 2	34.6	3.6
	LAKE2	7.3	1.6	2.7	18	3	7	7 ± 2	-	-
AGO4cw 310 K	LAKE1	10.2	1.5	2.3	18	6	10	10 ± 2	31.4	3.2
	LAKE2	6.1	1.1	1.3	11	2	6	6 ± 1	-	-
AGO2cw 300 K	LAKE1	12.7	1.4	2.1	19	8	13	13 ± 1	42.2	3.5
	LAKE2	4.7	0.8	0.6	8	2	5	5 ± 1	-	-
AGO2cw 310 K	LAKE1	12.6	2.0	4.0	23	6	13	13 ± 2	25	2.8
	LAKE2	5.7	0.8	0.7	10	3	6	6 ± 1	-	-

*Correspond to rounded up values of the mean ± sd number of water molecules in columns 3 and 4

Table S2. Details of simulated systems (Related to Simulation systems preparation section of STAR Methods)

System	#	PDB	t_{sim} (ns)	Type	Ensemble	Temp (K)	Start	Size #atoms	Size (nm^3)
AGO4cw	S1a	6OON	1.21	MinEQ	*NpT**	300	------	164,443	12x12x12
AGO4cw	S1b	6OON	100	EQ	*NpT*	300	S1a	164,443	12x12x12
AGO4cw	S1c	6OON	100	EQ	*NpT*	310	S1a	164,443	12x12x12
AGO4ncw	S2a	6OON	1.21	MinEQ	*NpT**	300	------	164,443	12x12x12
AGO4ncw	S2b	6OON	100	EQ	*NpT*	300	S2a	164,443	12x12x12
AGO4ncw	S2c	6OON	61	EQ	*NpT*	310	S2a	164,443	12x12x12
AGO2cw	S3a	4OLA	1.21	MinEQ	*NpT**	300	------	164,230	12x12x12
AGO2cw	S3b	4OLA	100	EQ	*NpT*	300	S3a	164,230	12x12x12
AGO2cw	S3c	4OLA	100	EQ	*NpT*	310	S3a	164,230	12x12x12

Summary of all MD simulations**.** Asterisk (*) denotes simulations that consisted of 5000 steps of minimization, 200 ps of dynamics with the backbone of the protein restrained (k_{bbr} = 1 kcal mol^{-1} $Å^{-2}$), and 1 ns of free dynamics in the *NpT* ensemble (γ = 1 ps^{-1}).

Table S3. Primer list (Related to Cloning, expression, and purification of AGO proteins and TNRC6B fragment section of STAR Methods)

Oligonucleotides	Source	Identifier
AGO2G595I/V615A-FW-CAGCAGCCCGTCATCTTTCTGATAGCAGACGTCAC, CCATTGCCGCCGTGGCGGGCAGCATGGACGCC	This paper	N/A
AGO2G595I/V615A-RV - GGCGTCCATGCTGCCCGCCACGGCGGCAATGG, GGCGTCCATGCTGCCCGCCACGGCGGCAATGG	This paper	N/A
AGO2Y420A-FW-GCCGCCCTCCATCCTCGCCGGGGGCAGGAATAAAGC	This paper	N/A
AGO2Y420A-RV - GCTTTATTCCTGCCCCCGGCGAGGATGGAGGGCGGC	This paper	N/A
AGO2W435A-FW-CCCTGTCCAGGGCGTCGCGGACATGCGGAACAAGCAGT	This paper	N/A
AGO2W435A-RV - ACTGCTTGTTCCGCATGTCCGCGACGCCCTGGACAGGG	This paper	N/A
AGO2L565A-FW-CAGACCCTGTCCAACCTCTGCGCGAAGATCAACGTCAAGCTGGGA	This paper	N/A
AGO2L565A-RV - TCCCAGCTTGACGTTGATCTTCGCGCAGAGGTTGGACAGGGTCTG	This paper	N/A
AGO2N568A-FW-GTCCAACCTCTGCCTGAAGATCGCCGTC	This paper	N/A
AGO2N568A-RV - CGCCTCCCAGCTTGACGGCGATCTTCAGGCA	This paper	N/A
AGO2N575A-FW-GTCAAGCTGGGAGGCGTGGCCAAC	This paper	N/A
AGO2N575A-RV - CTGGGGCAGCAGGATGTTGGCCACGCCTCCC	This paper	N/A
AGO2 L578A -FW-CTGGGAGGCGTGAACAACATCGCGCTGCCCCAGGGCAGG	This paper	N/A
AGO2 L578A -RV - CCTGCCCTGGGGCAGCGCGATGTTGTTCACGCCTCCCAG	This paper	N/A
AGO2S617A -FW-CCGCCGTGGTGGGCGCCATGGACGCCCACCCC	This paper	N/A
AGO2S617A -RV - GGGGTGGGCGTCCATGGCGCCCACCACGGCGG	This paper	N/A
AGO2Y625A -FW- CGCCCACCCCAATCGCGCCTGC	This paper	N/A
AGO2Y625A -RV - CGCACGGTGGCGCAGGCGCGATTGGG	This paper	N/A
AGO2Y805A-FW-TCCCAGCCAGCATACGCCGCTC	This paper	N/A
AGO2Y805A-RV - GGCCACCAGGTGAGCGGCGTATGCTGGCG	This paper	N/A
AGO2-4SI-FW-TGCAGCAGCACCGGCAGGAGATCTCCCAAGAGCTCCTCTACAGTCAAGA	This paper	N/A
AGO2-4SI-RV - GCGGCCAGGTCTTGTATGATCTCTTGACTGTAGAGGAGCTCTTGG	This paper	N/A

AGO4m-FW-GACTTCCCGGCAGGAGATCTCCCAAGAGCTCCTCTACAGTCAAGA, GACTTCCCGGCAGGAGATCTCCCAAGAGCTCCTCTACAGTCAAGA, GGAGGGGTATCTGAGGGACAATTCCAGCAGGTTCTCCACCACGAGTTGCTGGCCATCCGTG	This paper	N/A
AGO4m-RV -AACCATGTTAGTCAGGTCCTGGATGACCTCTTGACTGTAGAGGAGCTCTTGG, CTTGCCCTAAATGCTACAAGATGGGCATAATATGCAGGGGC, GGCCGGTAATCTTCTTCCAACTTGATACAGGCCTCACGGATGGCCAGCAACT	This paper	N/A
AGO4mΔ4SI-FW-GTGCAGACTTCCCGGCAGGAGGTCATCCAGGACCTGACTAA	This paper	N/A
AGO4mΔ4SI-RV-TTAGTCAGGTCCTGGATGACCTCCTGCCGGGAAGTCTGCAC	This paper	N/A
TNRC6B-599-683-FW-GCGCGCCTCGAGTCAGAGCTCCCCCCATCCAGA	This paper	N/A
TNRC6B-599-683-RV-GCGCGCGGATCCGATTGTCAGGCTGTCTTGCAGA	This paper	N/A
TNRC6B-507-598-FW-GCGCGCGGATCCTCTCAGGGAGAATGGAAACAGC	This paper	N/A
TNRC6B-507-598-RV-GCGCGCCTCGAGTCAAGGATGTGTGGGCCTGTACG	This paper	N/A
TNRC6B-684-770-FW-GCGCGCGGATCCTCAGCCTCTACAGAGTGGAAAGACC	This paper	N/A
TNRC6B-684-770-RV-GCGCGCCTCGAGTCAAGACACAGGTTTACTTGCAGAACTTTC	This paper	N/A
TNRC6B-771-860-FW-GCGCGCGGATCCGGGTGGGGTGAAGGAGGG	This paper	N/A
TNRC6B-771-860-RV-GCGCGCCTCGAGTCACTCCAGCTGGAATTGGAAGG	This paper	N/A
TNRC6B-869-916-FW-GCGCGCGGATCCACTGTGGATAATGGTACTTCAGCATG	This paper	N/A
TNRC6B-869-916-RV-GCGCGCAAGCTTTCAGGGTTCCCCCCAGCTGG	This paper	N/A
TNRC6B-616-636-FW-CAAATTAAGCAGGACACAGTGTGGTGACTCGAGTCTGGTAAAGAAACCG	This paper	N/A
TNRC6B-616-636-RV-CGGTTTCTTTACCAGACTCGAGTCACCACACTGTGTCCTGCTTAATTTG	This paper	N/A
TNRC6B-623-636-FW-ACAGAGAACAGATTGCTGCATCCTGGGGCCAAACTCAAATTAAGC	This paper	N/A
TNRC6B-623-636-RV-GCTTAATTTGAGTTTGGCCCCAGGATGCAGCAATCTGTTCTCTGT	This paper	N/A
TNRC6B-617-634-FW-GCGCGCGGATCCGTGCTCTCAAACACTGGCTGGG	This paper	N/A
TNRC6B-617-634-RV-GCGCGCCTCGAGTCAAATGTCCCACACTGTGTCCTGC	This paper	N/A

TNRC6B-617-634(Q625E)-FW-TCAAACACTGGCTGGGGCGAAACTCAAATTAAGCAGGACACAGTGT	This paper	N/A
TNRC6B-617-634(Q625E)-RV-ACACTGTGTCCTGCTTAATTTGAGTTTCGCCCCAGCCAGTGTTTGA	This paper	N/A
TNRC6B-617-634(K629A)-FW-GCTGGGGCCAAACTCAAGCTAAGCAGGACACAGTGTGGGA	This paper	N/A
TNRC6B-617-634(K629A)-RV-TCCCACACTGTGTCCTGCTTAGCTTGAGTTTGGCCCCAGC	This paper	N/A
TNRC6B-617-634(Q630R)-FW-GGGGCCAAACTCAAATTAAGCGGGACACAGTGTGGGACATTTGA	This paper	N/A
TNRC6B-617-634(Q630R)-RV-TCAAATGTCCCACACTGTGTCCCGCTTAATTTGAGTTTGGCCCC	This paper	N/A