Supplementary Information for

A direct role for HSP90 in pre-RISC formation in *Drosophila*

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Methods

Plasmid constructs. To obtain Argonaute 2 (Ago2) cDNA, poly(A)$^+$ RNAs were purified from S2 cells and reverse transcription-polymerase chain reaction (RT-PCR) was carried out using a ProSTAR Ultra-HF RT-PCR system (Stratagene). To produce 3x flag-tagged Ago2, the full-length Ago2 cDNA was subcloned into the pRH3xflag vector[1].

siRNA processing assay. *In vitro* siRNA processing assays were performed as previously described[2]. Geldanamycin (Tokyo Chemical Industry co., LTD) was added to the assay reaction at 100 μM.

siRNA unwinding assay. siRNA unwinding assays were performed essentially as previously described[3]. S2 lysates were pre-incubated with DMSO or geldanamycin (100 μM) for 20 min at 26ºC. After the *luciferase* siRNA duplex was incubated in S2 cell lysates, 10 μL of the reaction mixture was mixed with 2 μL of stop solution (30 mM HEPES-KOH (pH 7.4), 30 mM EDTA, 30% glycerol, 3% SDS, 300 nM siRNA guide strand DNA, 3 mg per mL proteinase K), incubated for 10 min at 37ºC, electrophoresed onto 15 % native polyacrylamide gels at 4ºC.

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RISC formation assay. RISC formation assays were performed essentially as previously described[3]. Briefly, ^{32}P-radiolabeled *luciferase* siRNA (guide strand; 5'-UCG AAG UAU UCC GCG UAC GUG-3') and non-labeled passenger strand (5'-CGU ACG CGG AAU ACU UCG AAA-3') were annealed and the resultant siRNA duplex was incubated in S2 lysates at 26 ºC with DMSO (control) or geldanamycin (100 μM). After incubation, samples were adjusted to 6% (w/v) glycerol and resolved by submarine native agarose gel electrophoresis at 4ºC. Gels were dried under a vacuum onto Hybond-N+ nylon membrane (GE Healthcare) and exposed to an imaging plate of a BAS 2500 (Fuji Film).

Immunoprecipitation. To examine the interaction between Ago2 and Dicer2, lysates of S2 cells expressing Flag-Ago2 were prepared in a NP-40 buffer containing 30 mM HEPES (pH7.4), 150 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 0.1% NP40, 2 μg per mL pepstatin, 2 μg per mL leupeptin, and 0.5% aprotinin. Cell lysates were then incubated with DMSO or geldanamycin (100 μM) for 20 min at 26ºC. The *luciferase* siRNA duplex (0.1 μM) was added to the cell lysates and mmunoprecipitation performed using anti-Flag M2 agarose beads (Sigma-Aldrich). Beads were washed five times with NP-40 buffer. Western blotting was performed as described[4]. Monoclonal antibodies against R2D2 were produced against full-length R2D2 tagged with GST. Anti-Ago1, anti-Ago2 and anti-Dicer2 antibodies were described previously[4,5]. Anti-Hsp90 (rabbit polyclonal) was purchased from Stressgen.

Target RNA cleavage assay. Target RNA cleavage assays were performed essentially as previously described[4]. The sequence of the *luc* target RNA was gggcgaauuggguaccCAAGUACGCGGAAUACUUCGAgaauuccugcagcccgggggauccacu aguucuagagcggccgccaccgcgguggagctccagcuuuuguucccuuuagugagggguuaauu, in which the sequence complementary to *luc* siRNA is indicated by capital letters. Geldanamycin was added to the assay reaction at 100 μM.

Northern blotting. RNAs were isolated from immunoprecipitated fractions using ISOGENE-LS (Nippon Gene). Northern blotting was performed as described previously[1], except that the RNAs were run on 15% acrylamide-denaturing gels, transferred onto Hybond-N membrane (GE Healthcare) and cross-linked with

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1-ethyl-3-(3-dimethylaminopropyl) carbodiimide[6]. The DNA Oligo used as a siRNA guide strand probe was: 5'-CAC GTA CGC GGA ATA CTT CGA-3'.

Photo-crosslinking assay. 5-iodide-uracil[7] siRNA photo-crosslinking assays were performed using a modified version of a previously reported method[8]. S2 cell lysates were prepared as in the RISC formation assays and then incubated with DMSO, geldanamycin or Radicicol (Sigma-Aldrich) for 20 min at 26ºC. *luciferase* siRNA duplexes were added to the lysates and then irradiated by 312 nm UV for 10 min and resolved on 7.5% SDS-polyacrylamide gels. The band signal in each lane (10, 30, 60 or 90 min) in the siRNA1 and siRNA2 panels was compared with that of '0 (min)' lane. In the siRNA3 geldanamycin - panel (the second from the right hand side), the band signal in each lane (0, 10, 30 or 60 min) was compared with that in '90 (min)' lane. The band signal in the siRNA3 with geldanamycin panel (far right) was compared with that in the '90 (min)' lane in the siRNA3 without geldanamycin panel.

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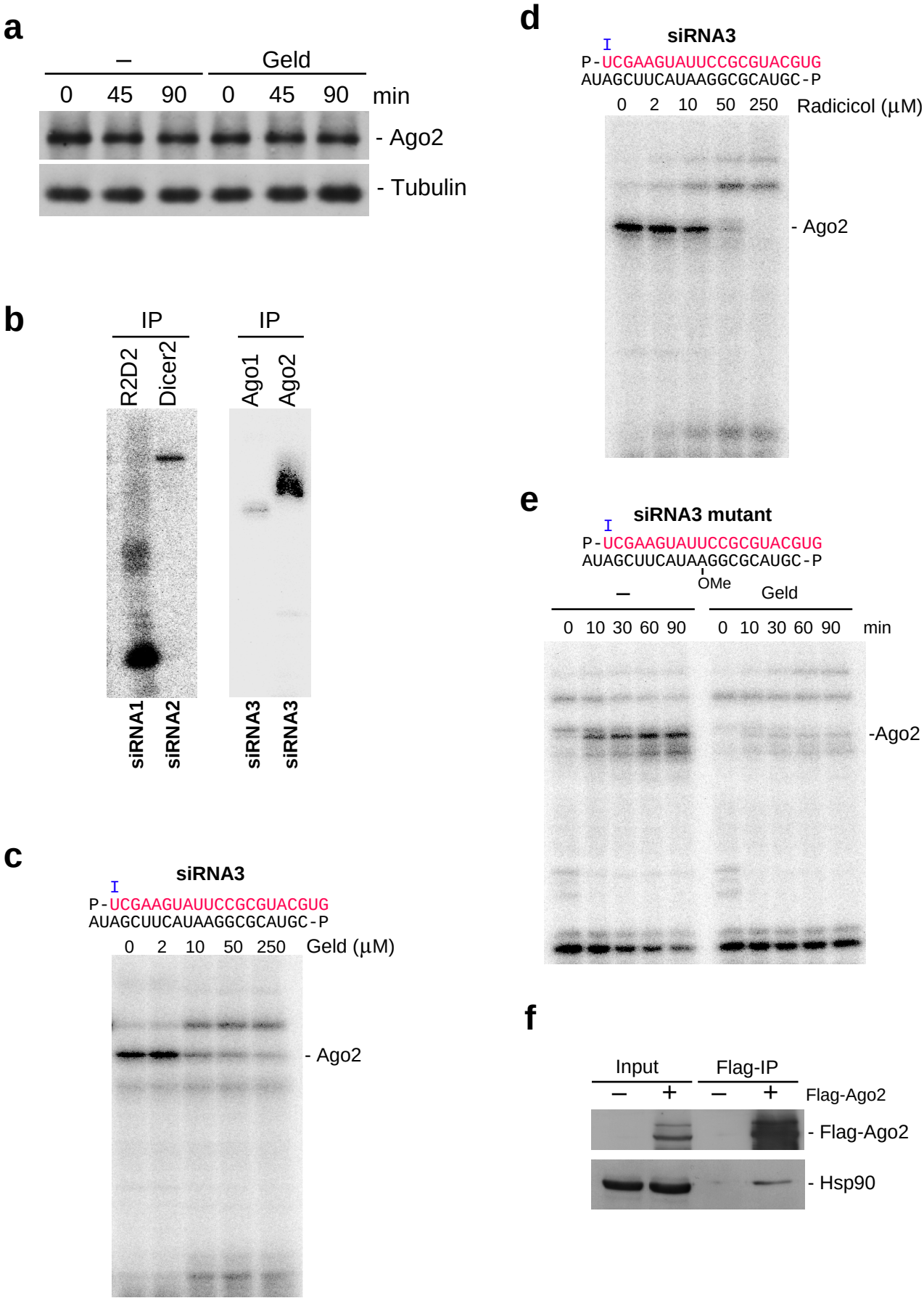
a
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Geld
0 45 90 0 45 90 min
- Ago2
- Tubulin
b
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IP
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Dicer2
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siRNA1
siRNA2
siRNA3
siRNA3
c
siRNA3
P-UCGAAGUAUUCCGCGUACGUG
AUAGCUUCAUAAGGCGCAUGC-P
0 2 10 50 250 Geld (μM)
- Ago2
d
siRNA3
P-UCGAAGUAUUCCGCGUACGUG
AUAGCUUCAUAAGGCGCAUGC-P
0 2 10 50 250 Radicicol (μM)
- Ago2
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- Flag-Ago2
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Supplementary Figure 1 Geldanamycin inhibitory effect on RNAi

Supplementary Figure 1. (**a**) Western blot analysis revealed that addition of geldanamycin to S2 cell lysates causes little or no instability of Ago2 in the lysates. Tubulin was used as a loading control. (**b**) Immunoprecipitation of Ago1[4] and Ago2[4] was performed from S2 lysates where siRNA3 (see Fig. 2a) was pre-incubated and photocrosslinked. siRNA3 was photocrosslinked to Ago2; however, Ago1 was hardly photocrosslinked with siRNA3. Immunoprecipitation with anti-R2D2 and anti-Dicer2[5] antibodies confirmed the interaction of R2D2 and Dicer2 with siRNA1 and siRNA2, respectively. (**c**) The geldanamycin inhibitory effect was observed when geldanamycin was added at the concentration of 10μM or higher. (**d**) Radicicol, another Hsp90 inhibitor, interferes with siRNA duplex association with Ago2. (**e**) In photocrosslinking assays, siRNA3 mutant, in which the passenger strand was modified with a 2'-*O*-methyl group at the 10th nucleotide from the 5' end and thus interferes with Ago2 Slicer activity, behaved very similarly to siRNA3 (see Fig. 2a). (**f**) The protein-protein interaction assay shows that Ago2 is able to interact with Hsp90 in S2 cells. Flag-tagged Ago2 was expressed in S2 cells by transfection prior to the lysate preparation.