A direct role for Hsp90 in pre-RISC formation in *Drosophila*

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Heat-shock proteins (Hsps) are molecular chaperones that control protein folding and function. Argonaute 2 (Ago2), the effector in RNA interference (RNAi), is associated with Hsp90; however, its function in RNAi remains elusive. Here we show that Hsp90 is required for Ago2 to receive the small interfering RNA (siRNA) duplex from the RNA-induced silencing complex–loading complex in RNAi, suggesting a model where Hsp90 modifies Ago2 conformation to accommodate the siRNA duplex.

In RNA interference (RNAi), small interfering RNA (siRNA) is associated with Argonaute 2 (Ago2) and guides the protein to its target mRNAs for silencing.¹² In *Drosophila melanogaster*, siRNAs are processed from long double-stranded RNA precursors by Dicer2 (refs. 1, 2). Upon processing, siRNAs are still in a duplex form and are associated with Dicer2 and R2D2, which are major components of the RNA-induced silencing complex (RISC)-loading complex (RLC).¹³ siRNA duplexes are then transferred to Ago2 from the RLC to form the precursor of the RISC, or pre-RISC. It has been shown that pre-RISC formation occurs in an ATP-dependent manner. However, it remains unclear why ATP is needed for this process.

In the pre-RISC, siRNA duplexes are ‘unwound’ by Ago2 endonuclease or Slicer activity; the passenger strand of the duplexes is cleaved by Slicer and displaced from Ago2 (refs. 5, 6). This step is known to occur in an ATP-independent manner. The guide strand of the duplex remains associated with Ago2. The resultant Ago2–siRNA complex is termed RISC and is now active and ready to bind and cleave target RNAs. In this way, genes targeted by the RNAi machinery are effectively silenced.¹³ Ago2 frequently co-purifies with heat-shock protein (Hsp) 90 (refs. 8–13), a chaperone whose activity depends on ATP. Hsp90 is not necessary for Ago2 Slicer activity once the RISC has been formed without Hsp90 inhibition. Luc, luciferase.

Thus, Hsp90 may have a role in RNAi. However, the functional involvement of Hsp90 at the molecular level in RNAi remains unclear.

To determine for which step Hsp90 is required in RNAi, we dissected the RNAi pathway into multiple steps and performed assays for the individual steps in the presence or absence of geldanamycin, the specific Hsp90 inhibitor that mimics ATP binding with the protein.¹⁴ In siRNA processing assays, we incubated Flag-tagged Dicer2 isolated from S2 cells with ³²P-labeled dsRNA precursors with or without geldanamycin. The ability of Dicer2 to excise siRNA duplexes from the precursors was not affected by geldanamycin (Fig. 1a). Thus, Hsp90 is not necessary for siRNA excision from the precursor.

We next examined whether the siRNA-unwinding step in RNAi requires Hsp90 function. We incubated siRNA duplexes, which were labeled with ³²P at the 5' end of the guide siRNA within the duplexes, in S2 lysates with or without geldanamycin. In the presence of geldanamycin, siRNA duplexes remained as duplexes even after 1 h incubation (Fig. 1b). DMSO, an organic solvent used for dissolving geldanamycin.

**Figure 1** Hsp90 is required for RISC formation in *Drosophila* RNAi. (a) siRNA processing assays showed that Hsp90 is not necessary for excising siRNA duplexes from the precursor. Geld, geldanamycin; IP, immunoprecipitation. (b) siRNA duplex unwinding assays showed that Hsp90 is required for siRNA unwinding in RNAi. (c) RISC formation assays show that Hsp90 is necessary to convert the RLC into the RISC. (d) Hsp90 is not necessary for Ago2 Slicer activity once the RISC has been formed without Hsp90 inhibition. Luc, luciferase. (e) Hsp90 does not affect the association between Dicer2 and Ago2 in *Drosophila*.

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in the lysates, did not affect the activity (Fig. 1b). Thus, Hsp90 is required for unwinding siRNA duplexes into single-stranded siRNAs.

We then speculated that the RISC would not be assembled in S2 lysates when Hsp90 was inhibited. To test this, we performed RISC formation assays. Whereas DMSO alone did not show any obvious effect, geldanamycin severely inhibited RISC formation (Fig. 1c). RLC formation was not affected by geldanamycin treatment (Fig. 1c). These results indicate that Hsp90 has an important role in the particular step(s) necessary for converting RLC into RISC. A recent study has shown that geldanamycin significantly reduces the levels of Argonaute proteins in mammalian cells and thus reduces the programming of the RISC12. However, the stability of Ago2 was barely changed in S2 lysates even after we added geldanamycin (Supplementary Fig. 1a). Thus, the reduced RISC-forming activity was likely not due to reduced Ago2 stability.

siRNA duplexes remained as duplexes in the presence of geldanamycin (geld). Ago2 association with the siRNA duplexes was slightly affected. Dicer2 association with the duplexes was moderately altered by Hsp90 inhibition (Fig. 1d). Radicicol, another Hsp90 inhibitor, also interfered with siRNA-protein interaction experiments using three siRNA duplexes, siRNA1, siRNA2 and siRNA3, each of which was composed of the same sequence but contained an iodine-uridine at different positions within the duplex (Fig. 2a). We individually incubated siRNA duplexes in S2 lysates for the time periods indicated in Figure 2a, and then the mixtures were exposed to UV light to cross-link the siRNA duplexes with the proteins physically associated with them. Previous studies have indicated that Dicer2 and R2D2, the second known component of the RLC, are mainly cross-linked at the 5' end of the guide and passenger strands of the duplex, respectively17,18. Ago2 was shown to be cross-linked predominantly at the 5’ end of the guide strand of the duplex. We confirmed that the main bands observed in the left, center and right panels of Figure 2a corresponded to R2D2, Dicer2 and Ago2, respectively (Supplementary Fig. 1b). We found that the association of R2D2 and Dicer2 with siRNA1 and siRNA2, respectively, was not significantly altered by Hsp90 inhibition (Fig. 2a).

Hsp90 functions in displacing the siRNA duplex from the RLC, whereas the second is that Hsp90 is required for Ago2 to receive the siRNA duplex from the RLC. After the siRNA duplex has been properly displaced from the RLC. To examine which model is correct, we performed siRNA-protein interaction experiments using three siRNA duplexes, siRNA1, siRNA2 and siRNA3, each of which was composed of the same sequence but contained an iodine-uridine at different positions within the duplex (Fig. 2a). We individually incubated siRNA duplexes in S2 lysates for the time periods indicated in Figure 2a, and then the mixtures were exposed to UV light to cross-link the siRNA duplexes with the proteins physically associated with them. Previous studies have indicated that Dicer2 and R2D2, the second known component of the RLC, are mainly cross-linked at the 3' end of the guide and passenger strands of the duplex, respectively17,18. Ago2 was shown to be cross-linked predominantly at the 5’ end of the guide strand of the duplex. We confirmed that the main bands observed in the left, center and right panels of Figure 2a corresponded to R2D2, Dicer2 and Ago2, respectively (Supplementary Fig. 1b). We found that the association of R2D2 and Dicer2 with siRNA1 and siRNA2, respectively, was not significantly altered by Hsp90 inhibition (Fig. 2a). However, Hsp90 inhibition drastically affected siRNA duplex association with Ago2; in the presence of geldanamycin, Ago2 barely interacted with siRNA3 (Fig. 2a, right). We observed the inhibitory effect when we added geldanamycin at a concentration of 10 µM or higher (Supplementary Fig. 1c). Radicicol, another Hsp90 inhibitor, also interfered with siRNA duplex association with Ago2 (Supplementary Fig. 1d). A siRNA duplex mutant (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 similarly to siRNA3 (Supplementary Fig. 1e). In addition, we showed that Hsp90 was present in immunoprecipitated Ago2 complex (Supplementary Fig. 1f). These results suggest that Hsp90 is required for Ago2 to receive siRNA duplexes from the RLC in the RNAi pathway. In siRNA-Ago2 binding assays, the guide siRNAs were barely associated with Ago2 when we added geldanamycin first to the lysates prior to the addition of geldanamycin.
of the siRNA duplexes (Fig. 2b). These results further support the idea that Hsp90 is needed for Ago2 to bind the siRNA duplex. Structural analysis of the eubacterial Ago protein has revealed that the cavity in the Ago protein that accommodates the siRNA duplex is too small to be bound by an RNA molecule, and a conformational change in the Ago protein would be required for binding. In the current study, our results suggest that Hsp90 acts as the driving force for changing the conformation of Ago2, likely by hydolyzing ATP as an energy source, as geldanamycin is known to inhibit the ATPase activity of Hsp90 by occupying the N-terminal ATP binding pocket of the protein, enabling it to accommodate siRNA duplex from the RLC (Fig. 2c). This step is crucial in RNAi, and so, without Hsp90, the pre-RISC is not formed and RNAi is impaired.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS
T.M. and A.T. conducted experiments; T.M., A.T., H.S. and M.C.S. designed experiments, interpreted data and prepared the manuscript.

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The authors declare no competing financial interests.

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