REGULATORY MECHANISM OF THE RNAI PATHWAY

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Dedication

To my parents who gave me endless love and inspiration

REGULATORY MECHANISM OF THE RNAI PATHWAY

by

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DISSERTATION

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REGULATORY MECHANISM OF THE RNAI PATHWAY

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RNA interference (RNAi) is post-transcriptional gene silencing initiated by Dicer, a RNase III that processes double-stranded RNA (dsRNA) precursors into small interfering RNA (siRNA). In *Drosophila*, Dicer2 and R2D2 coordinately recruit duplex siRNA to the effector RNA-induced silencing complex (RISC), wherein single-stranded siRNA guides the endoribonuclease Argonaute (Ago) to catalyze sequence-specific cleavage of complementary mRNA. It remains unclear as to what constitutes holo-RISC, how is RISC assembled and how is RISC regulated.

Here we took a candidate approach to reconstitute for the first time the long double-stranded RNA- and duplex siRNA-initiated RISC activities with the use of recombinant *Drosophila* Dicer-2, R2D2, and Ago2 proteins. We further employed this core reconstitution system to purify a RNAi regulator that we named C3PO (component 3

promoter of RISC), a complex of Translin and Trax. C3PO is a novel Mg^{2+} -dependent endoribonuclease that promotes RISC activation by removing the siRNA passenger strand cleavage products. Similar as *Drosophila* C3PO, human C3PO also degrades passenger strand fragments and facilitates RISC activation.

RISC is a multiple-turnover enzyme, wherein single-stranded (ss)-siRNA guides Ago2 to catalyze sequence-specific cleavage of the target mRNA at the effector step. We employed human minimal RISC reconstitution system to purify antoantigen La as a novel activator of the RISC effector step. Biochemical studies indicated that La promotes the multiple-turnover of RISC catalysis by facilitating the release of RISC cleaved products. Moreover, we demonstrated that La is required for efficient RNAi, antiviral defense, and transposon silencing in mammalian and *Drosophila* cells.

Taken together, our findings of C3PO and La reveal a general concept that regulatory factors are required to remove Ago2-cleaved products to assemble or restore active RISC. The robust reconstitution system establishes a powerful platform for in-depth studies of the assembly, function, and regulation of RISC. Similar to the discovery of C3PO and La, it can be used to identify novel regulators and study post-translational regulations of RNAi, therefore, connecting RNAi to other cellular signaling pathways. As such, these biomedical studies could have a major and lasting impact on the biological understanding and therapeutic application of RNAi.

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Liu Y, Ye X, Jiang F, Liang C, Chen D, Peng J, Kinch L, Grishin N, Liu Q. C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. ***Science*** 5941 (2009) 750-753.

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Liu Y and Liu Q. ATM signals miRNA biogenesis through KSRP. ***Mol. Cell*** 41 (2011) 367-368.

Ye X*, Huang N*, **Liu Y**, Paroo Z, Huerta C, Li P, Chen S, Liu Q, Zhang H. Structure of C3PO and mechanism of human RISC activation. ***Nat. Struct. Mol. Biol.*** 18 (2011) 650-657

Liu Y, Tan H, Tian H, Liang C, Chen S and Liu Q. Autoantigen La promotes RNAi, antiviral response, and transposon silencing by facilitating multiple-turnover RISC catalysis. (2011) Submitted.

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Ago2	Argonaute 2
C3PO	Component 3 promoter of RISC
dsRNA	double-stranded RNA
miRNA	microRNA
ftz	*fushi tarazu*
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNAi	RNA interference
S2 cell	Schneider 2 cell
shRNA	small hairpin RNA
siRNA	small interfering RNA
ss-siRNA	single-stranded siRNA
Trax	Translin-associated factor X
TRBP	TAR RNA-binding protein

Chapter 1 : Background Introduction

1.1 Discovery of RNA interference

The discovery of RNA interference (RNAi) is one of the most significant biomedical breakthroughs in recent history. Multiple classes of previously unidentified small regulatory RNAs play important roles in many fundamental biological and disease processes. Not only a powerful genetic tool, RNAi also holds great promise for developing novel therapeutics for human diseases.

The first documented phenomenon of RNAi, initially named co-suppression, was discovered when plant biologists Jorgensen and coworkers attempted to introduce chalcone synthase into petunia to enrich flower pigmentation (Napoli et al., 1990). Instead of the expected dark-purple color, fully or partially white flowers were obtained (Figure 1-1). Further investigation suggested that the mRNA levels of both endogenous and transgenic genes were decreased. However, the detailed mechanism by which the transgene silences endogenous genes was poorly understood.

In 1998, Andrew Fire, Craig Mello and co-workers discovered that double-stranded RNA (dsRNA) was significantly more effective in silencing the endogenous gene than either strand alone when injected into nematode *C. elegans* (Fire et al., 1998). This dsRNA-induced gene silencing, which was then named RNAi, was shown to be heritable and accompanied by the degradation of target mRNA (Fire et al., 1998). RNAi is an evolutionarily conserved gene silencing mechanism, which has been discovered in

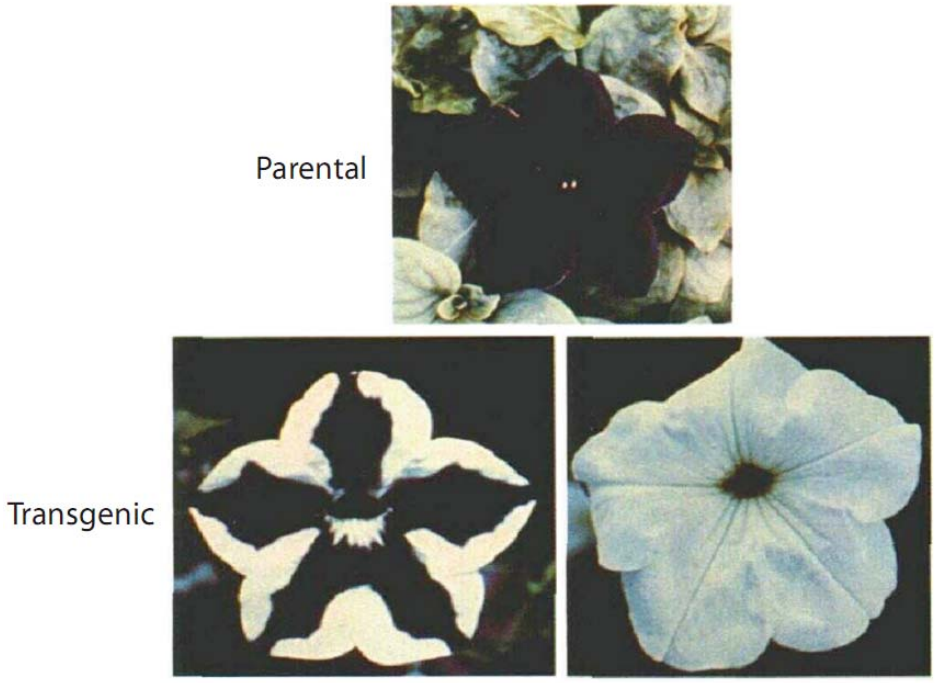


Figure 1-1 The first observed RNAi phenotype

Phenotypes of parental (top) and transgenic (bottom) petunia flowers expressing an ectopic chalcone synthase gene. Instead of the dark-purple flowers, partially or fully white flowers were obtained (Napoli et al., 1990). Adapted from *The Plant Cell*, Vol. 2, 279-289 © 1990 American Society of Plant Physiologists.

various organisms besides plants and nematodes, including fungi, fish, fly, mice and human (Zamore et al., 2000).

1.2 siRNA

To understand the detailed mechanism of RNAi, Tuschl and coworkers developed a cell-free system to recapitulate the dsRNA-initiated gene silencing *in vitro*. They successfully showed that addition of dsRNA against luciferase transcript into *Drosophila* embryo lysate led to the repression of luciferase reporter gene and the degradation of its transcript (Tuschl et al., 1999). Furthermore, Zamore et al. modified this *in vitro* system to directly monitor the fate of a radiolabelled target mRNA and showed that addition of dsRNA triggers sequence-specific cleavage of the target mRNA (Zamore et al., 2000). They further uncovered that 21-23 nucleotide (nt) small RNAs, named small interfering RNAs (siRNAs), were generated from long dsRNA as intermediate products (Zamore et al., 2000).

Subsequent studies demonstrated that synthetic 21-22nt siRNA with 3'overhang efficiently induced the cleavage of homologous target RNA (Elbashir et al., 2001b). These synthetic siRNA could also effectively repress the endogenous gene when introduced into mammalian cells (Elbashir et al., 2001a), making RNAi a powerful reverse genetic tool and a potential therapeutic for human diseases.

1.3 Dicer

To understand how the 21-22nt duplex siRNA can be generated from long dsRNA, the feature of siRNA products were characterized, and revealed a RNase-III like

pattern of endonucleolytic cleavage (Bass, 2000; Elbashir et al., 2001b). Bernstein and coworkers then used a candidate approach to ectopically express different RNase III enzymes in *Drosophila* S2 cells and found that immunoprecipitates of CG4792, which they named Dicer-1, could process dsRNA into siRNA *in vitro* (Bernstein et al., 2001). However, a subsequent study purified another RNase III enzyeme CG6493 (named Dicer-2) after following the siRNA-generating activity from S2 cell extract through a six-step chromatographic purification (Liu et al., 2003). *Drosophila* genome encodes two functional Dicer enzymes, Dicer-1 and Dicer-2. Genetic studies indicated that Dicer-2 is required for siRISC assembly whereas Dicer-1 is critical for microRNA (miRNA)-mediated gene silencing (Lee et al., 2004). Subsequent biochemical analysis further supported this idea by showing that Dicer-2 took dsRNA as its substrate, whereas Dicer-1 preferentially processed the precursor of miRNA (Jiang et al., 2005).

The family of Dicer contains an ATPase/helicase domain, a DUF283 domain, a PAZ domain and two RNase III domains (Figure 1-2A). The RNase III domains of Dicer protein form an intramolecular dimer to cut the opposing strands of dsRNA. The PAZ domain of Dicer recognizes the terminus of dsRNA. Therefore, the distance between PAZ domain and RNase III domain of Dicer determines the size of small RNA products (Figure 1-2B) (Macrae et al., 2006; Zhang et al., 2004), making Dicer itself a molecular ruler.

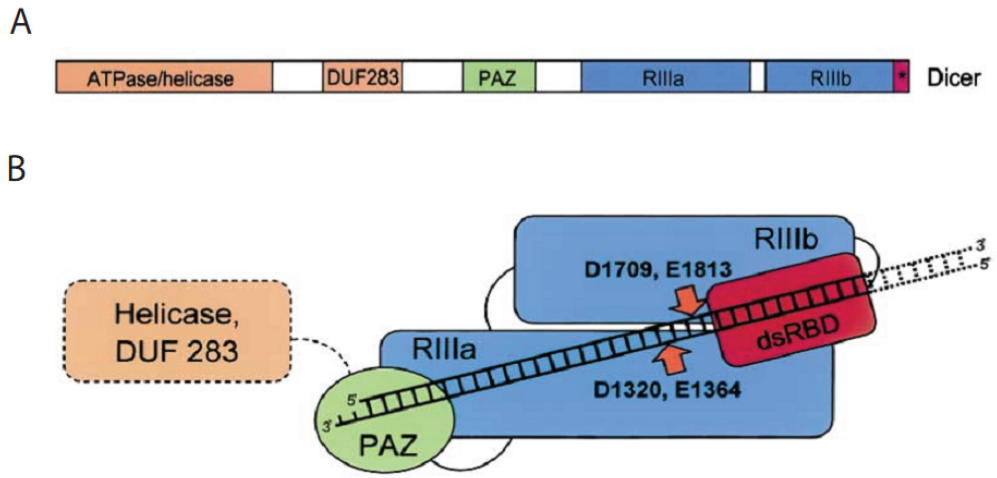


Figure 1-2 Molecular Mechanism of Dicer function

(A) The human Dicer contains an ATPase/helicase domain, a domain of unknown function (DUF) 283, a PAZ domain, two RNase III domains and a double strand RNA-binding domain (indicated by *). (B) The PAZ domain of Dicer recognizes the terminus of siRNA. Two RNase III domains form intramolecular dimer to cut the opposing strand of duplex siRNA, generating 2-nt overhang at the 3'end. The size of siRNA is determined by the distance between PAZ domain and RNase III domain (Zhang et al., 2004). Adapted by permission from Elsevier, *Cell* © 2004 (Paroo et al., 2007).

1.4 RISC

In an attempt to develop a cell free system for large scale biochemical purification of additional components in the RNAi pathway, Hammond et al. cultured Drosophila Schneider 2 (S2) cells in large-scale suspension culture (Hammond et al., 2000). They further showed that pre-loading of S2 cell with dsRNA prior to the preparation of cell extract recapitulated the mRNA cleavage activity *in vitro*. Chromatographic fractionation identified a ribonuclease complex comprised of both proteins and small RNA, which catalyzed sequence-specific cleavage of target mRNA (Hammond et al., 2000). This ribonuclease complex was then named RNA-induced silencing complex (RISC).

RISC is the catalytic engine of RNAi. To identify the catalytic enzyme within RISC, Hammond et al. fractionated S2 cell extract containing pre-loaded RISC activity and biochemically purified Argonaute2 (Ago2) as the bona fide component of RISC (Hammond et al., 2001). Purified recombinant human Ago2 protein cleaved target mRNA when supplemented with single-stranded (ss)siRNA (Song et al., 2004). Moreover, the structure of Argonaute PIWI domain resembles the structure of RNase H, suggesting that the catalytic center of RISC resides in the PIWI domain (Song et al., 2004). Indeed, mutating the putative RNase H-based catalytic residues completely abolished the RISC cleavage activity *in vitro* (Liu et al., 2004). These structural and biochemical analysis suggested that Argonaute is the catalytic enzyme of RISC.

Members of Argonaute family proteins are evolutionarily conserved and play pivotal roles in RNAi. The Argonaute proteins normally contain three essential domains, PAZ, Mid and PIWI (Figure 1-3A). Structural analysis revealed that the PAZ domain of Argonaute recognizes the 3'overhang of siRNA where as the 5'end of siRNA is anchored in the Mid domain (Figure 1-3B) (Tolia and Joshua-Tor, 2007; Wang et al., 2008a; Wang et al., 2008b). Ago2 is a Mg^{2+}-dependent endoribonuclease. The interaction between siRNA and target mRNA positioned the catalytic center of Argonaute to the scissile phosphate between 9^{th}- and 10^{th}-nt of siRNA (Tolia and Joshua-Tor, 2007; Wang et al., 2008a; Wang et al., 2008b). Two cleaved fragments with 5'phosphate and 3'hydroxyl terminus were generated after RISC cleavage (Martinez and Tuschl, 2004; Schwarz et al., 2004).

1.5 The RNAi pathway

1.5.1 Initiation step

In the initiation step, a long dsRNA that is expressed in or introduced into cells is processed by Dicer into siRNA with 5' phosphate group and 3' hydroxyl group. When biochemically purified from *Drosophila* S2 cells, Dicer-2 strongly associated with another dsRNA-binding protein named R2D2 after sequential columns (Liu et al., 2003). Biochemical studies indicated that Dicer-2/R2D2 complex associated with duplex siRNA once it generated and formed the RISC loading complex (RLC) to facilitate the loading of siRNA onto RISC complex (Liu et al., 2003; Liu et al., 2006; Pham et al., 2004).

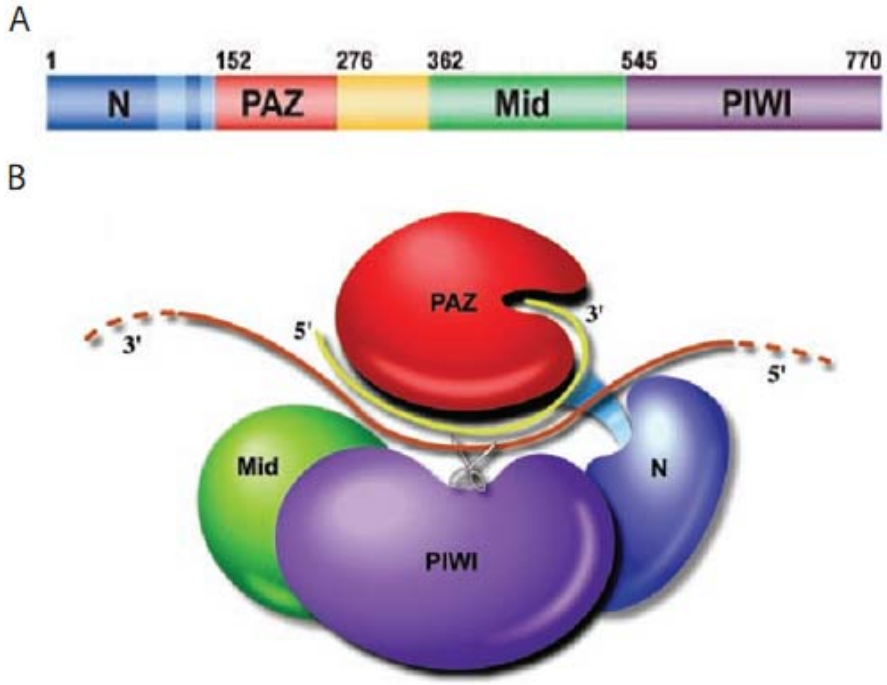


Figure 1-3 Molecular Mechanism of Argonaute function

(A) The Argonaute protein contains an N-terminal domain, PAZ domain, Mid domain and PIWI domain. **(B)** A shematic depiction of the model for siRNA-directed mRNA cleavage. The 3'overhang of siRNA is anchored in the PAZ domain of Argonaute protein where the 5'end sits between the cleft of Mid and PAZ domain. The catalytic center in the PIWI domain (shown as scissors) cleaves the mRNA at the position that corresponding to 9^{th} and 10^{th} nucleotides of siRNA. Adapted by permission from Elsevier, *Science* © 2004 (Paroo et al., 2007).

1.5.2 Activation step

Even though duplex siRNA is loaded onto RISC, RISC is still inactive because the siRNA duplex must be unwinded into single strand to anneal with the target mRNA. Thus, the central step of RISC activation is the unwinding of duplex siRNA. For each siRNA duplex, only one strand, which is called guide strand, will associate with Argonaute protein and eventually guide RISC cleaves its target mRNA. The other strand, which is called passenger strand, is discarded. Strand selection is determined by the thermodynamic asymmetry of the siRNA duplex. Specifically, R2D2 senses the thermodynamically more stable end of the siRNA duplex, whereas Dicer-2 interacts with the less stable end (Tomari et al., 2004b).

The central step of RISC activation is the unwinding of duplex siRNA. Two models have been proposed for mechanisms of duplex siRNA unwinding: One is called "helicase model" and the other is named "slicer model" (Figure 1-4) (Paroo et al., 2007).

Heilcase model for siRNA unwinding

An ATP-dependent siRNA unwinding activity correlated with the RISC activity following gel filtration chromatography (Nykanen et al., 2001). Therefore, a RNA helicase was proposed to unwind duplex siRNA and promote RISC activation. Since then, multiple helicases have been discovered to be involved in the siRNA pathway (Meister et al., 2005; Robb and Rana, 2007; Tomari et al., 2004a), but so far none of these have been shown to biochemically unwind duplex siRNA and facilitate RISC activation *in vitro*. Thus, the "helicase model" has not been validated to date.

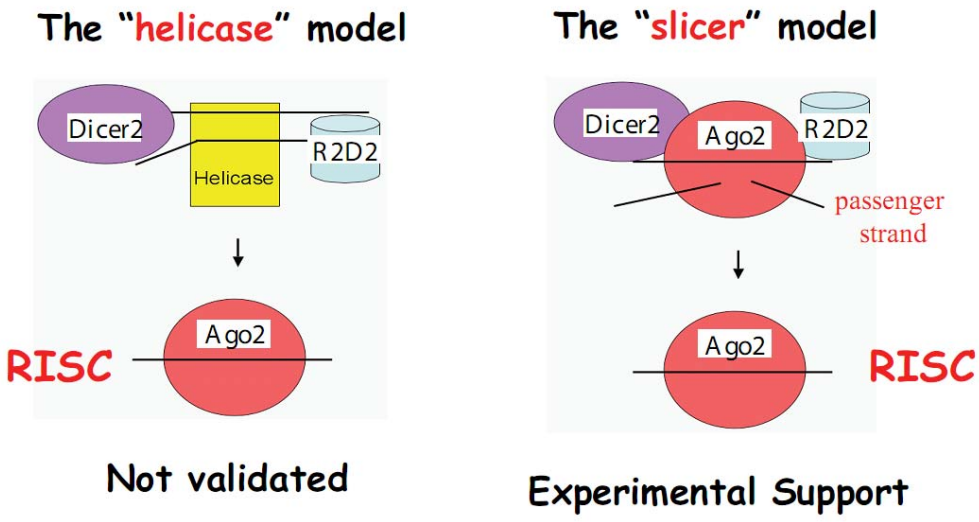


Figure 1-4 Mechanisms for siRNA unwinding and RISC activation

In the "helicase model", an unknown helicase unwinds duplex siRNA prior to RISC loading step, generating single-stranded siRNA. The guide strand itself is then loaded on to RISC. This model has not been validated to date. In the "slicer model", after loading with duplex siRNA, Ago2 cleaves in the middle of passenger strand. The passenger strand cleavage products dissociate from RISC, leaving guide strand behind with Ago2 to form active RISC. Several experiments support this model.

Slicer model for siRNA unwinding

In the "slicer model" of RISC activation, Ago2 cleaves passenger strand of duplex siRNA into 9- and 12-nt fragments. Passenger strand cleavage products will then dissociate from guide strand, leaving guide strand behind with Ago2 to form an active RISC (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). To support this model, phosphorothioate and 2'-O-methyl modifications on siRNA, which blocked passenger stand cleavage, attenuated RISC activation (Matranga et al., 2005; Rand et al., 2005).

1.5.3 Effector step

Once RISC associates with only guide strand of duplex siRNA, it is active and can anneal with the target mRNA. Active RISC is a multiple-turnover enzyme, wherein single-stranded guide RNA directs Ago2 to catalyze sequence-specific cleavage of complementary mRNA. The specific interaction of RISC with its target is mostly determined by the 5' seed region of the siRNA (Ameres et al., 2007).

Concluding Remarks

The early work of Andrew Fire and Craig Mello provided clear evidence that dsRNA is the trigger of gene silencing and opened up the RNAi field. Since then, cell-free systems were employed by researchers to recapitulate steps of RNA silencing pathway *in vitro*, which allowed the identification of key components of the pathway. These outstanding studies established the framework for subsequence studies to understand the detailed regulatory mechanism of the RNAi pathway.

However, so far nearly all the *in vitro* studies were carried out in cell extract or with immunoprecipitated endogenous proteins. An in vitro reconstitution system with recombinant proteins alone is urgently needed to better elucidate the detailed molecular mechanism of RNAi. The minimal reconstitution system can also be used to identify novel components in the RNAi pathway and study post-translational regulations of RNAi, therefore, connecting RNAi to other cellular signaling pathways. As such, these biomedical studies could have a major and lasting impact on the biological understanding and therapeutic application of RNAi.

Chapter 2 : Identification and characterization of C3PO

Abstract

The catalytic engine of RNAi is the RNA-induced silencing complex (RISC), wherein the endoribonuclease Argonaute and single-stranded siRNA direct target mRNA cleavage. Here we have reconstituted long dsRNA- and duplex siRNA-initiated RISC activities using recombinant *Drosophila* Dicer-2, R2D2 and Ago2 proteins. We employed this core reconstitution system to purify an RNAi regulator-component 3 promoter of RISC (C3PO), a complex of Translin and Trax. C3PO is a Mg^{2+}-dependent endoribonuclease that promotes RISC activation by removing siRNA passenger strand cleavage products. These studies establish an *in vitro* RNAi reconstitution system and identify C3PO as a key activator of the core RNAi machinery.

Introduction

RNA interference (RNAi) is post-transcriptional gene silencing initiated by the RNase III Dicer that processes double-stranded (ds)RNA into 21- to 22-nucleotide (nt) small interfering RNA (siRNA) (Carthew and Sontheimer, 2009; Paroo et al., 2007; Siomi and Siomi, 2009). Nascent siRNA duplex is assembled into the effector RNA-induced silencing complex (RISC), wherein single-stranded siRNA guides the endoribonuclease Argonaute (Ago) to catalyze sequence-specific cleavage of complementary mRNA (Carthew and Sontheimer, 2009; Paroo et al., 2007; Siomi and Siomi, 2009). A minimal RISC can be reconstituted with recombinant Ago2 and single-

stranded siRNA, but not duplex siRNA, suggesting that additional factors are required for loading nascent siRNA onto Ago2 (Liu et al., 2004). In *Drosophila*, Dicer-2 (Dcr-2) and R2D2 coordinately recruit duplex siRNA to Ago2 to promote RISC assembly (Liu et al., 2003; Liu et al., 2006; Pham et al., 2004). Moreover, Dcr-2/R2D2 complex senses thermodynamic asymmetry of siRNA and facilitates the guide strand selection (Tomari et al., 2004b). It remains unclear as to what constitute holo-RISC, how RISC is assembled, and how RISC is regulated.

2.1 Reconstitution of the *Drosophila* RNAi pathway

In order to identify all components that are necessary and sufficient for the formation of a functional RISC complex, we initially employed a classical biochemical fractionation and reconstitution approach. Unfortunately, salt treatment or chromatographic fractionation irreversibly damaged the ability of *Drosophila* embryo or S2 extract to form a functional RISC (Figure 2-1). Therefore, we took an alternative candidate approach to reconstitute the core RISC activity using recombinant Dcr-2, R2D2 and Ago2 proteins, all of which are essential for *Drosophila* RISC assembly (Liu et al., 2006; Okamura et al., 2004; Pham et al., 2004).

Besides PAZ and PIWI domains, *Drosophila* Ago2 carries a long stretch of amino-terminal poly glutamine (Q) repeats that are absent in most Ago proteins (Figure 2-2A). We generated an active truncated His-Flag-tagged Ago2 that removes most polyQ repeats (Figure 2-2B) and fully restores duplex siRNA-initiated RISC activity in *ago2* mutant lysate (Figure 2-2C) (Okamura et al., 2004). Purified recombinant Dcr-

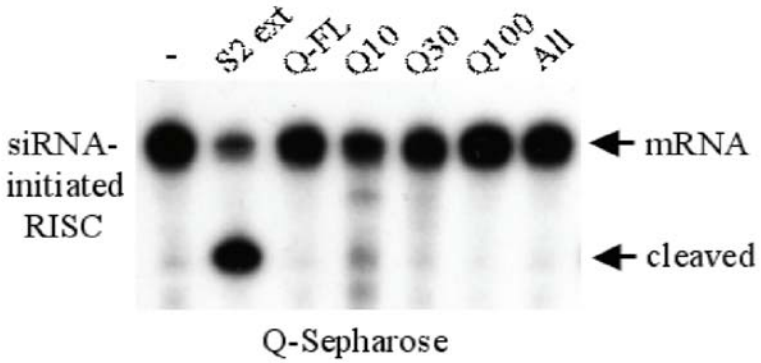


Figure 2-1 RISC activity is irreversibly damaged by chromatographic fractionation

Drosophila S2 cell extract, which has a robust RISC cleavage activity on its own, was fractionated onto a Q-sepharose column into four fractions: flow through (QFL), 10% (Q10), 30% (Q30), and 100% (Q100) salt elution. Duplex siRNA-initiated RISC assay were performed in buffer, S2 extract, different fractions or the combination of all fractions.

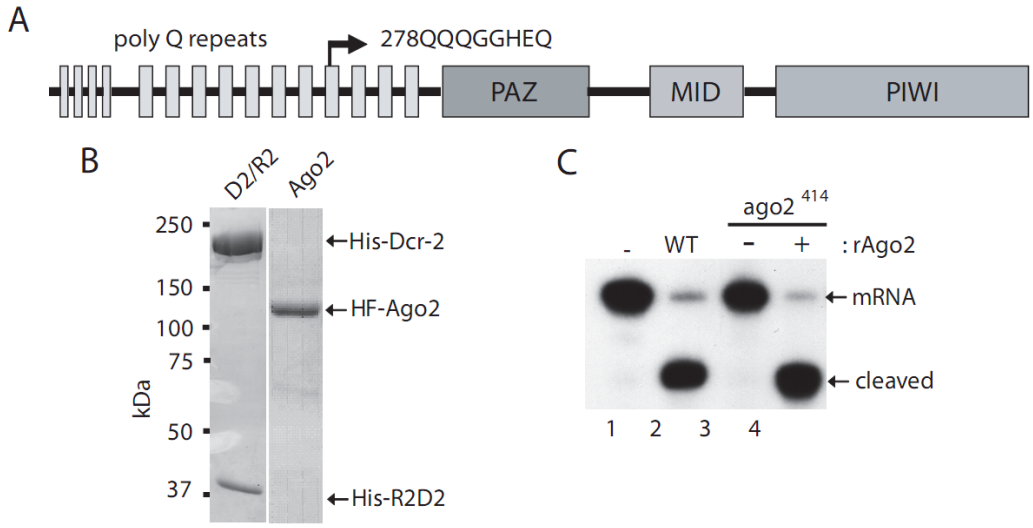


Figure 2-2 Generation of an active truncated Ago2 recombinant protein

(A) A schematic diagram showing the domain structure of *Drosophila* Ago2 protein, including four short and eleven long polyQ repeats at the N-terminus. The arrow indicates the start position of the truncated form of Ago2. **(B)** A Coomassie-stained SDS-PAGE gel showing purified His-tagged Dcr-2/R2D2 (D2/R2) complex and His-Flag(HF)-tagged truncated Ago2 recombinant proteins. **(C)** The siRNA-initiated RISC assays were performed in buffer (lane 1), wild-type (WT) ovary extract (lane 2), or *ago2*^{414} mutant ovary extract in the absence (lane 3) or presence (lane 4) of recombinant (r)Ago2.

2/R2D2 and Ago2 proteins could successfully reconstitute long dsRNA- and duplex siRNA-initiated RISC activities (Figure 2-3), suggesting that Dcr-2/R2D2 and Ago2 form the *Drosophila* core RISC machinery. Moreover, the RISC activity was abolished in the reconstitution system when using catalytic mutant Ago2, indicating that Ago2 was responsible for mRNA cleavage in this system (Figure 2-3, lane 6).

2.2 Purification of C3PO as an activator of RISC

However, recombinant Dcr-2/R2D2 and Ago2 generated lower RISC activities than did S2 extract (Figure 2-3, compare lane 6 to lane 2), suggesting that additional factors are required to achieve maximal RISC activity. Therefore, we used this core reconstitution system to search for new RISC-enhancing factors. Although mild heat treatment (HI, 37ºC for 30 minutes) abolished the RISC activity in S2 extract (Figure 2-4), addition of $S2^{HI}$ extract greatly enhanced the RISC activity of recombinant Dcr-2/R2D2/Ago2 (Figure 2-5), indicating the existence of an RNAi activator. We named this factor component 3 promoter of RISC (C3PO) because besides Dcr-2 and R2D2, this would be the third component that promotes RISC activity.

We purified C3PO from S2 extract following a seven-step chromatographic procedure (Figure 2-6). At the final step, two proteins, ~27 kDa and ~37 kDa, showed close correlation with the RISC-enhancing activity. They were identified by mass spectrometry as the evolutionarily conserved Translin/TB-RBP and Translin-associated factor X (Trax). Translin is a single-stranded DNA/RNA-binding protein that co-purifies

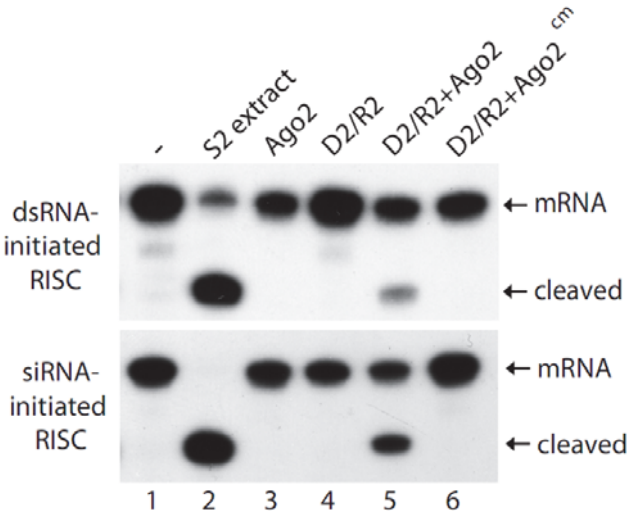


Figure 2-3 Reconstitution of dsRNA- and duplex siRNA-initiated RISC activity

The dsRNA- and duplex siRNA-initiated RISC assays were performed in buffer, S2 extract, recombinant Ago2, Dcr-2/R2D2 (D2/R2) complex, and D2/R2 plus wild-type or catalytic-mutant (cm/D965A) Ago2.

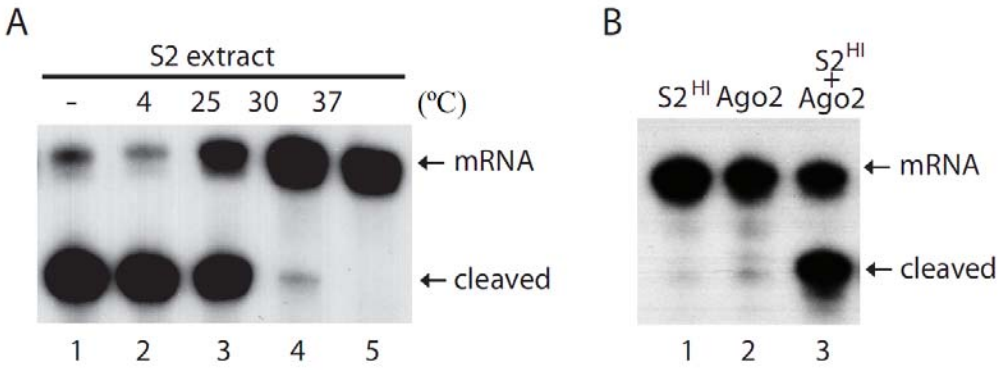


Figure 2-4 Mild heat treatment abolishes RISC activity by inactivating Ago2

(A) The siRNA-initiated RISC assays were performed with untreated S2 extract (lane 1) or S2 extracts that were pre-incubated at different temperatures (lanes 2-5) for 30 minutes. **(B)** The siRNA-initiated RISC assays were performed with heat-inactivated (37ºC, 30 min) S2 extract (lane 1, $S2^{HI}$), recombinant Ago2 (lane 2), or $S2^{HI}$ extract supplemented with recombinant Ago2 (lane 3).

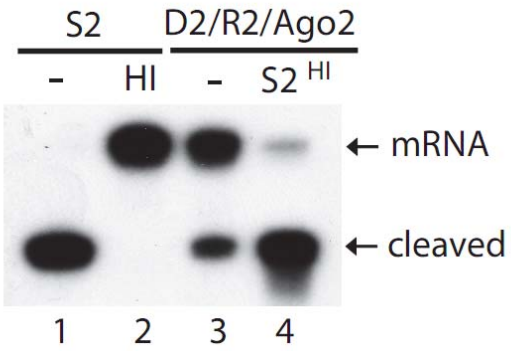
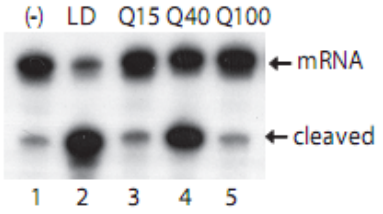


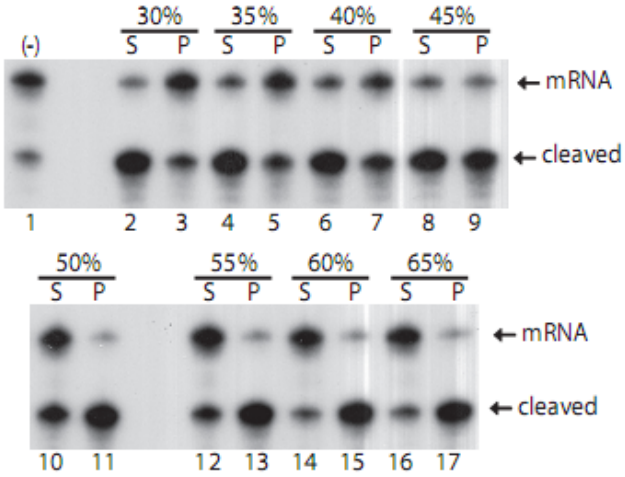
Figure 2-5 Heat-inactivated S2 extract contains RISC enhancing activity

The siRNA-initiated RISC assays were performed with untreated or heat-inactivated (HI) S2 extract, or recombinant Dcr-2/R2D2/Ago2 in the absence or presence of $S2^{HI}$ extract.

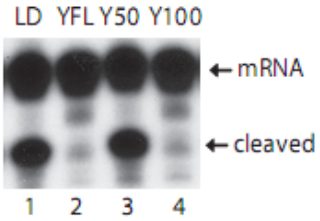
1. Q-Sepharose



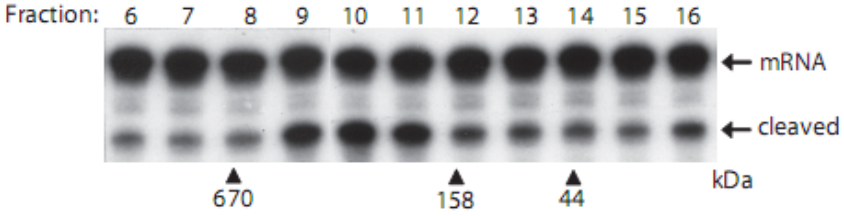
2. Ammonium sulfate



3. Phenyl

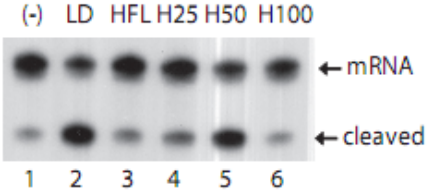


4. Superose 6

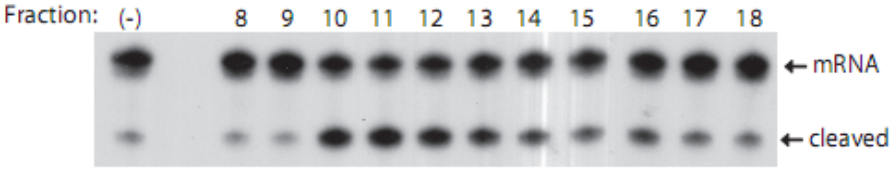


5. SP-Sepharose

6. Heparin



7. Hydroxyapatite



8. Mono Q

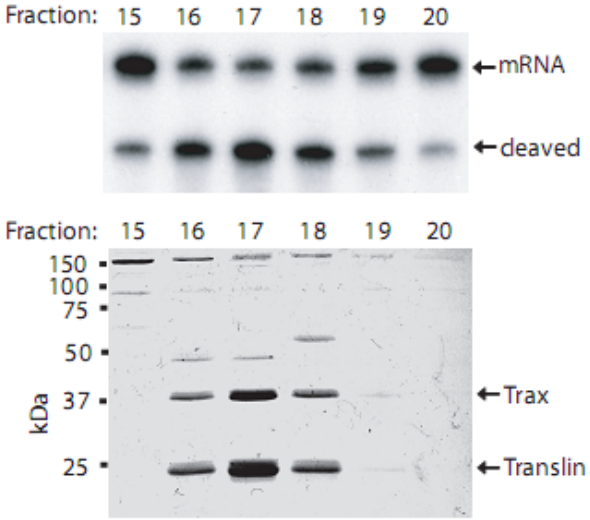


Figure 2-6 Purification of the RISC-enhancing activity

Purification of C3PO through a seven-step chromatographic procedure (described in methods and materials). Each fraction was assayed with recombinant Dcr-2/R2D2/Ago2 for the RISC-enhancing activity. Following the final Mono Q step, individual fractions were assayed with recombinant Dcr-2/R2D2/Ago2 for the RISC-enhancing activity (top) or resolved by SDS-polyacrylamide gel (PAGE) followed by Colloidal-staining (bottom). FL: flow-through; S: supernatant; P: pellet; Y: phenyl.

with siRNA after UV crosslinking (Claussen et al., 2006; Wang et al., 2004), whereas Trax has sequence similarity to and interacts with Translin (Suseendranathan et al., 2007).

2.3 C3PO is required for efficient RNAi *in vitro* and *in vivo*

We generated recombinant C3PO complex, or Translin subunit alone from *E.coli* (Figure 2-7A). Consistently, recombinant C3PO complex, but not Translin alone, greatly enhanced the RISC activity of recombinant Dcr-2/R2D2/Ago2 (Figure 2-7B). Maximal RISC activity was obtained only when Dcr-2/R2D2, C3PO and Ago2 were present (Figure 2-7C, lane 8).

Conversely, genetic depletion of C3PO diminished RISC activity in *Drosophila* ovary extract. Western blotting revealed that Translin and Trax were both missing in *translin* (*trsn*) mutant fly lysate, suggesting that Trax is unstable without Translin (Figure 2-8A). By contrast, the core RNAi components, i.e. Dcr-2, R2D2 and Ago2, remained at wild type levels in *trsn* mutant (Figure 2-8A). While siRNA-generating activity was slightly higher in the mutant lysate (Figure 2-9), duplex siRNA-initiated RISC activity was at ~25% of wild type level in *trsn* extract (Figure 2-8B), and this defect was rescued by adding recombinant C3PO (Figure 2-8C). Thus, C3PO is required for optimal RISC activity *in vitro*.

To determine if C3PO is required for efficient RNAi *in vivo*, we injected wild type and *trsn* mutant embryos with *fushi tarazu* (*ftz*) siRNA or dsRNA that causes segmentation defects by silencing *ftz* expression (Okamura et al., 2004). Following injection with *ftz* siRNA, more than 80% of wild type embryos displayed a severe

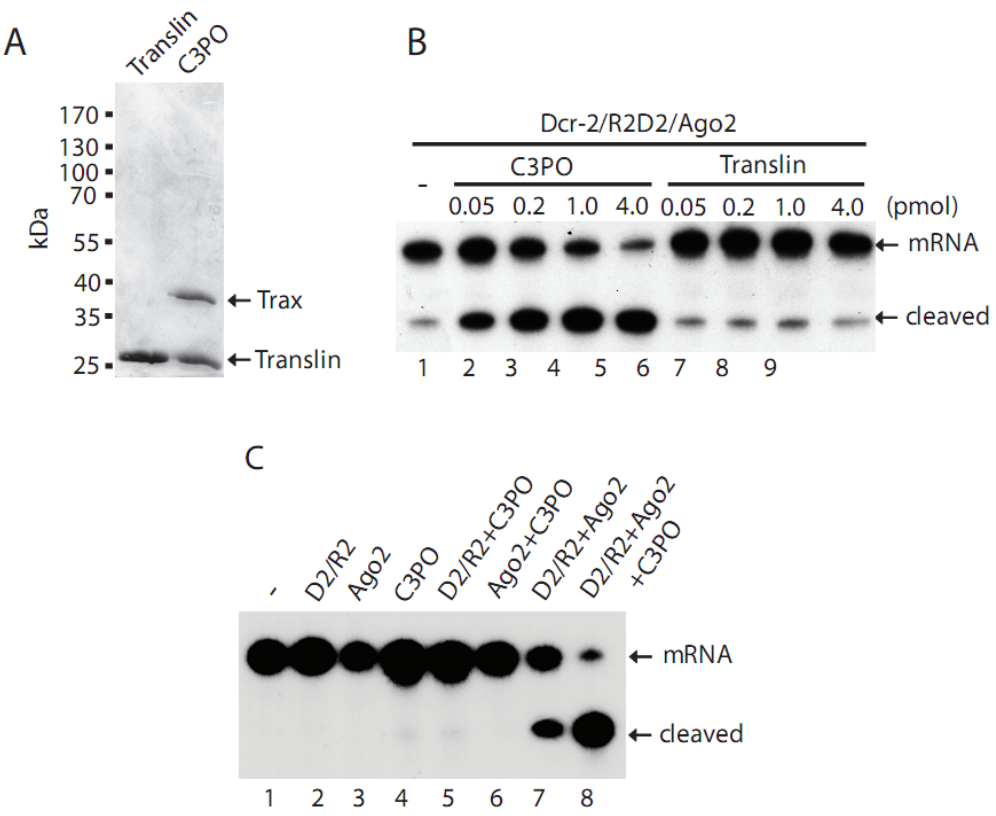


Figure 2-7 Recombinant C3PO enhances RISC activity *in vitro*

(A) A Coomassie-stained SDS-PAGE gel showing purified recombinant Translin and C3PO complex. **(B)** The siRNA-initiated RISC assays were performed using recombinant Dcr-2/R2D2/Ago2 alone or with increasing amount of recombinant C3PO or Translin. **(C)** The siRNA-initiated RISC assays were performed in buffer (lane 1), or using different combinations of recombinant Dcr-2/R2D2, C3PO, and Ago2 proteins (lanes 2-8).

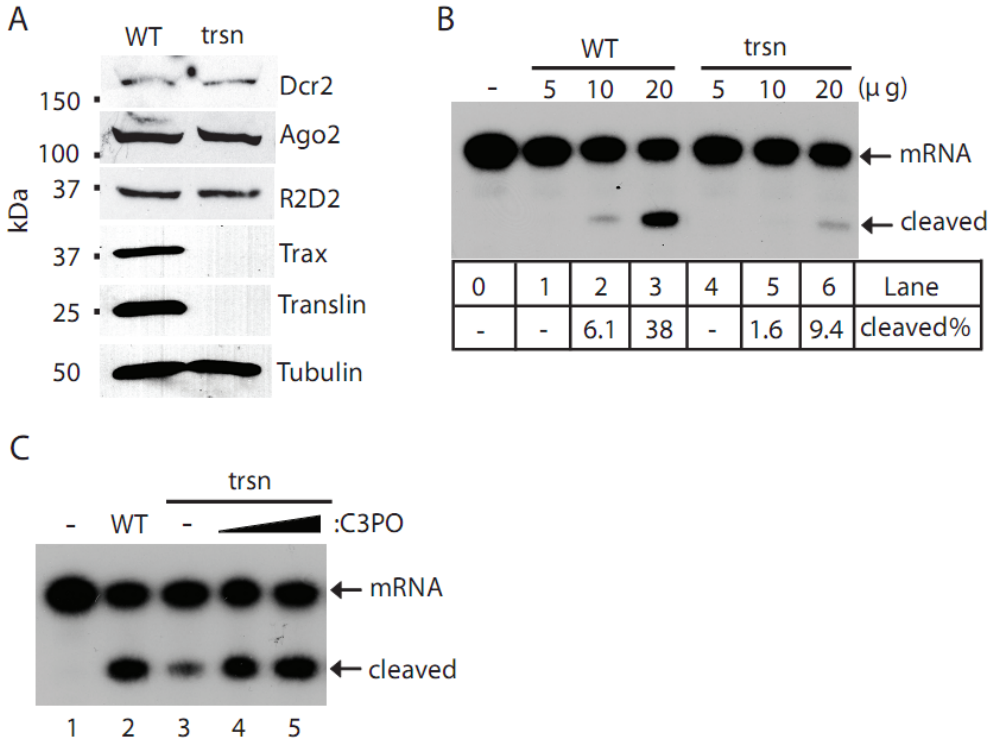


Figure 2-8 Genetic depletion of C3PO diminishes RISC activity *in vitro*

(A) Western blots comparing the levels of Dcr-2, R2D2, Ago2, Translin, Trax, and β-tubulin between wild-type (WT) and *trsn* mutant lysates. **(B)** The siRNA-initiated RISC assays were performed in buffer, and WT or *trsn* mutant ovary extracts. The RISC activity was measured by the percentage of cleaved mRNA. **(C)** The siRNA-initiated RISC assays were performed in buffer, 20μg WT extract, or 20μg *trsn* mutant extract without or with recombinant C3PO.

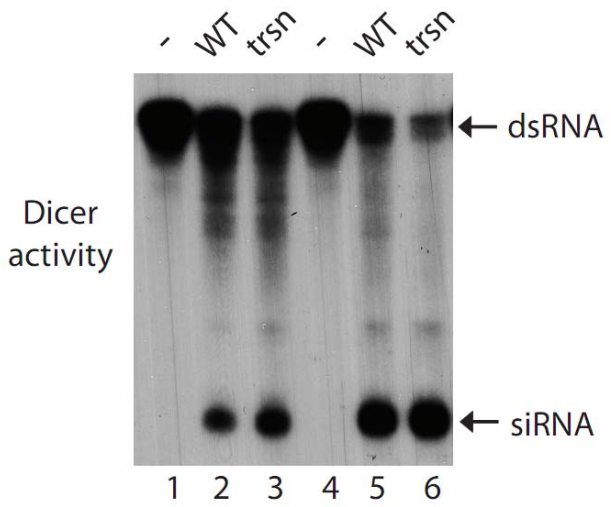


Figure 2-9 Comparison of siRNA generating activity in WT and *trsn* mutant

The Dicer assays were performed in buffer (lanes 1 and 4), or using 10 μg (lanes 2-3) or 20 μg (lanes 5-6) of WT and *trsn* mutant ovary extracts.

segmentation phenotype, whereas a significant portion of *trsn* mutant embryos showed mild or no phenotype (Figure 2-10A and Figure 2-10B). A similar phenomenon was observed with *ftz* dsRNA injection (Figure 2-10C). These experiments indicate that C3PO is required for efficient RNAi *in vivo*.

2.4 C3PO facilitates RISC assembly

To distinguish if C3PO enhances RISC assembly or activity, we compared the amount of RISC activity generated by recombinant Dcr-2/R2D2/Ago2 with C3PO added before or after RISC assembly (Figure 2-11A). In both cases, C3PO could enhance the core RISC activity, however, the RISC-enhancing effect was greatly diminished when C3PO was added late to pre-assembled RISC (Figure 2-11B). Therefore, we conclude that C3PO primarily promotes RISC activation, but it also enhances the RISC-mediated target cleavage. Consistent with the latter, C3PO modestly enhanced single-stranded siRNA-initiated RISC activity (Figure 2-11C).

To further dissect the role of C3PO in RISC activation, we examined the stepwise process of RISC assembly by native siRNA gel-shift assay. As previously described (Okamura et al., 2004; Pham et al., 2004; Tomari et al., 2004a), three siRNA-protein (siRNP) complexes, B, RISC loading complex (RLC) and RISC, were formed in wild-type ovary extract. RLC contains Dcr-2/R2D2 and siRNA, whose formation precedes and is required for RISC assembly (Pham et al., 2004; Tomari et al., 2004a). While neither RLC nor RISC could form in *dcr-2* mutant extract, only RISC was absent in *ago2* mutant extract (Figure 2-12). By contrast, all three siRNP complexes could form

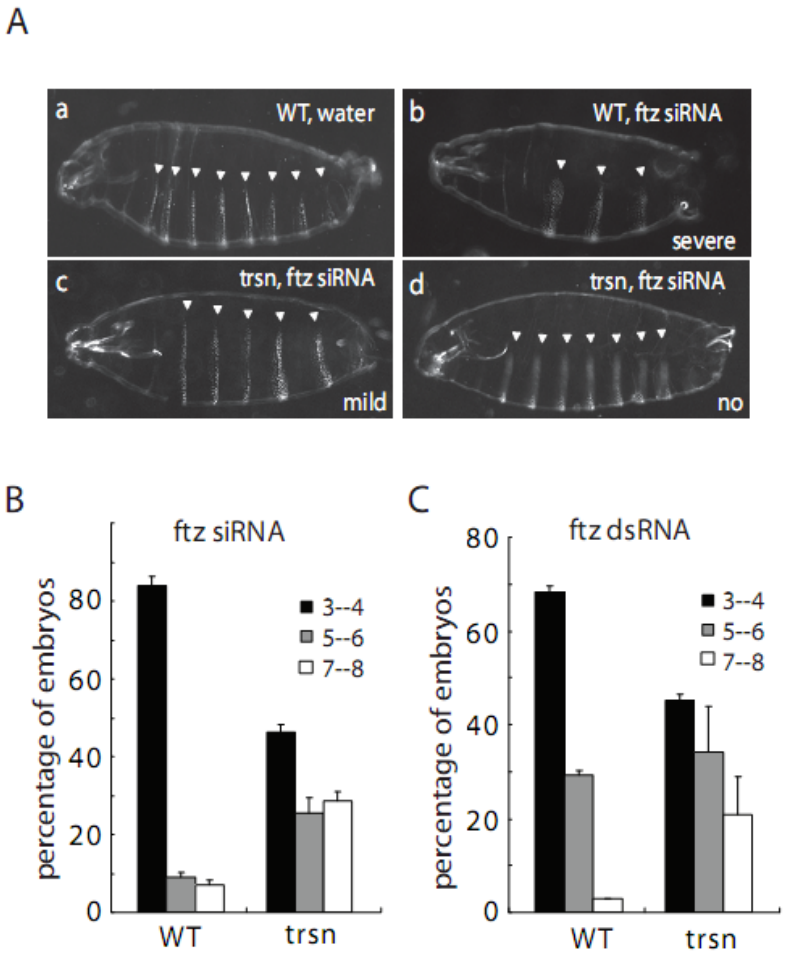


Figure 2-10 C3PO is required for efficient RNAi *in vivo*

(A) Images showing segmentation phenotypes of WT and *trsn* mutant embryos: (a) WT embryo injected with water (8 abdominal cuticle belts); (b) WT embryo injected with *ftz*-siRNA (severe, 3-4 belts); (c) *trsn* embryo injected with *ftz*-siRNA (mild, 5-6 belts); (d) *trsn* embryo injected with *ftz*-siRNA (no phenotype, 7-8 belts). **(B-C)** Graphs showing distribution of WT or *trsn* mutant embryos with severe, mild, or no phenotype following injection of *ftz*-siRNA **(B,** n>100**)** or *ftz*-dsRNA **(C,** n>150**)**.

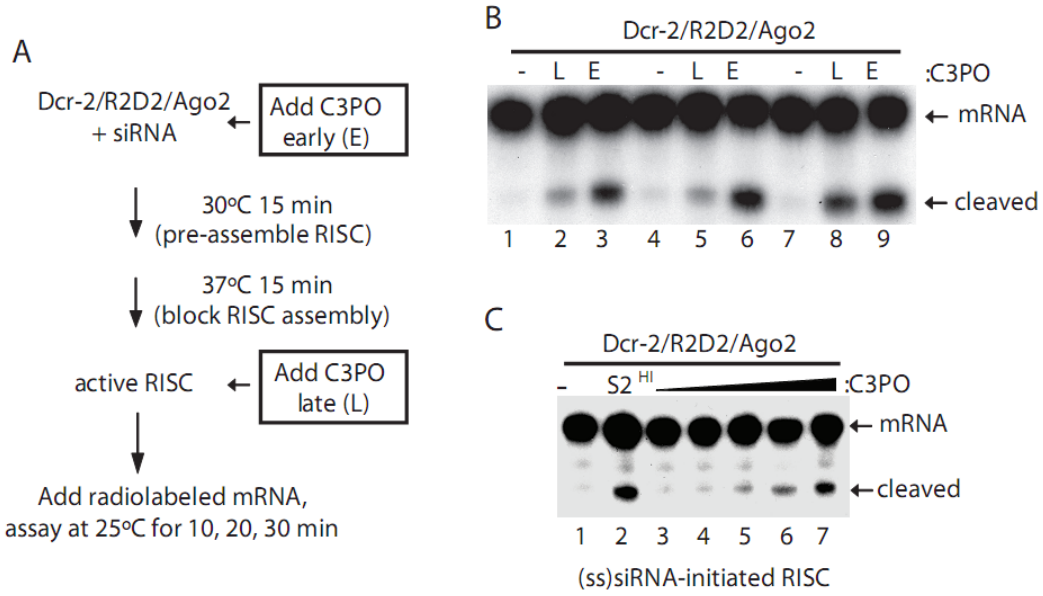


Figure 2-11 C3PO facilitates RISC activation

(A) A flow chart of the experimental procedure to distinguish if C3PO promotes assembly and/or activity of RISC. **(B)** The siRNA-initiated RISC assays were performed as described in (A) using recombinant Dcr-2/R2D2/Ago2 alone, or with C3PO added late (L) to pre-assembled RISC, or added early (E) prior to RISC assembly. After adding radiolabeled mRNA substrate, reactions were incubated at room temperature for 10 (lanes 1-3), 20 (lanes 4-6), and 30 (lanes 7-9) minutes. **(C)** The single-stranded (ss)siRNA-initiated RISC assays were performed with recombinant Dcr-2/R2D2/Ago2 proteins alone (lane 1), or in combination with heat-inactivated S2 ($S2^{HI}$) extract (lane 2) or with a series of dilutions of recombinant C3PO (lanes 3-7).

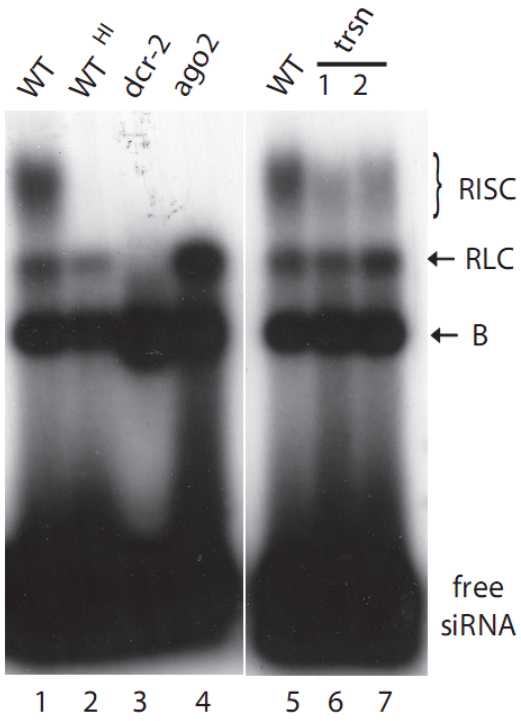


Figure 2-12 C3PO facilitates the transition from RLC to active RISC

Native siRNA gel-shift assays were performed using radiolabeled let-7 siRNA with untreated or heat-inactivated (HI) WT, *dcr-2*, *ago2* mutant ovary extract (lanes 1-4), or 40µg of WT and two preps of *trsn* mutant ovary extract (lanes 5-7). B: an unknown complex; RLC: RISC loading complex.

in *trsn* mutant extract, but the amount of RISC was significantly less than that in wild-type extract (Figure 2-12). These results support the hypothesis that C3PO facilitates the transition from RLC to active RISC.

2.5 C3PO degrades passenger strand cleavage products

The central step of RISC activation is the unwinding of duplex siRNA and loading of the guide strand onto Ago2. Thus, we measured the efficiency of RISC assembly using the siRNA-unwinding assay (Nykanen et al., 2001). In the reconstitution system, recombinant C3PO enhanced the siRNA-unwinding activity of Dcr-2/R2D2/Ago2 (Figure 2-13A). Conversely, the efficiency of siRNA unwinding was lower in *trsn* mutant extract than wild type control (Figure 2-13B). Both results indicate that C3PO promotes siRNA unwinding and RISC activation.

Two different models have been proposed for mechanisms of duplex siRNA unwinding (Figure 1-4) (Paroo et al., 2007). In the "helicase model", an ATP-dependent RNA helicase unwinds duplex siRNA and promotes RISC activation (Nykanen et al., 2001). However, this model has not been validated to date. In the "slicer model", Ago2 cleaves the passenger strand of duplex siRNA into 9- and 12-nt fragments. Passenger strand cleavage products will then dissociate from guide strand, leaving guide strand behind with Ago2 to form an active RISC (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005).

To study the relative contribution of different RISC activation mechanisms, we supplemented heat-inactivated S2 ($S2^{HI}$) extract, which displayed no siRNA-unwinding

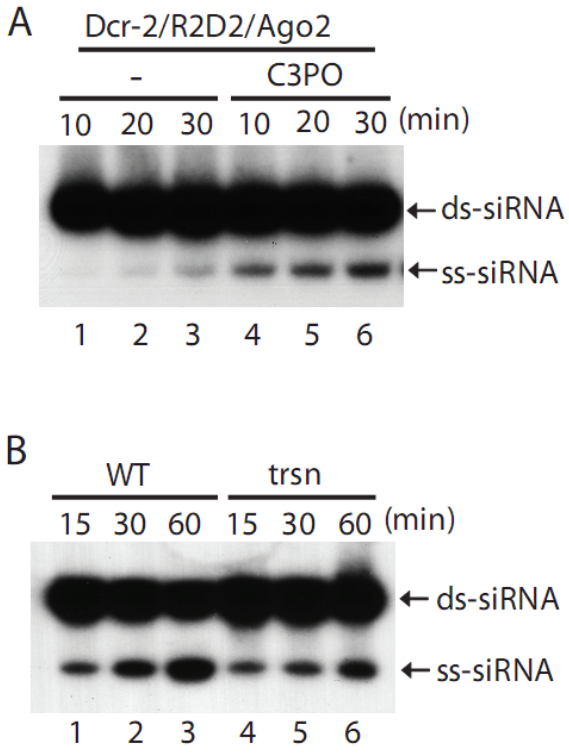


Figure 2-13 C3PO promotes the unwinding of duplex siRNA

(A) The siRNA-unwinding assays were performed with an asymmetric siRNA of which the guide strand was radiolabeled using recombinant Dcr-2/R2D2/Ago2 proteins in the absence (lanes 1-3) or presence (lanes 4-6) of recombinant C3PO complex. **(B)** The siRNA-unwinding assays were performed by incubating radiolabeled siRNA with 40 µg of WT (lanes 1-3) or *trsn* mutant (lanes 4-6) ovary extract. (Courtesy of Dr. Qinghua Liu)

activity due to Ago2 inactivation (Figure 2-14A and Figure 2-4B), with recombinant wild-type or catalytic mutant Ago2. Only wild-type, but not mutant, Ago2 could effectively rescue siRNA unwinding in $S2^{HI}$ extract (Figure 2-14B). This result, together with previous studies (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005), strongly supports that the catalytic activity of Ago2 is indispensable for siRNA unwinding and RISC activation. Therefore, the "slicer model" is the primary mechanism for siRISC activation.

Surprisingly, by passenger strand cleavage assay (Matranga et al., 2005; Rand et al., 2005), we observed that both 9-nt and 12-nt fragments displayed longer half-life in *trsn* mutant than wild-type extract (Figure 2-15). Moreover, addition of C3PO complex, but not Translin, resulted in rapid degradation of the 9-nt fragment in the reconstituted system (Figure 2-16). Together, these findings support the model that C3PO promotes RISC activation by removing siRNA passenger strand cleavage products.

2.6 C3PO is a novel Mg^{2+}-dependent endoribonuclease

Recombinant C3PO displayed potent ribonuclease (RNase) activity towards single-stranded (ss)siRNA, but showed little or no activity towards double-stranded siRNA or ssDNA (Figure 2-17). The RNase activity of C3PO is Mg^{2+}-dependent and could be blocked by EDTA, but not by EGTA (Figure 2-18A). Further support for this endonuclease activity can from the observation that C3PO could degrade circular as well as linear RNA (Figure 2-18B). Moreover, the endogenous C3PO complex closely correlated with the RISC-enhancing activity as well as an ssRNase activity following

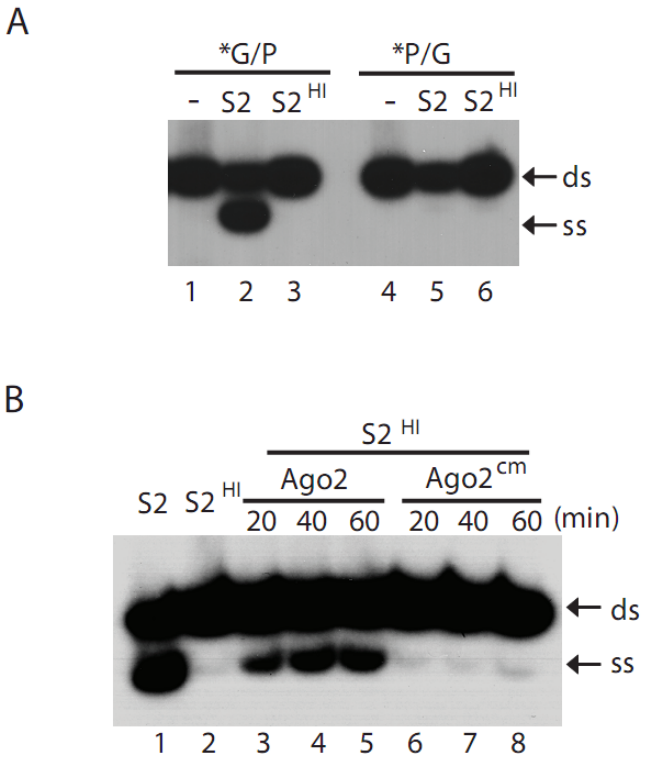


Figure 2-14 The "slicer model" plays a dominant role in RISC activation

(A) The siRNA-unwinding assays were performed using radiolabeled *G/P (lanes 1-3) or *P/G (lanes 4-6) siRNA in buffer, untreated S2 extract, or heat-inactivated S2 ($S2^{HI}$) extract. **(B)** The siRNA-unwinding assays were performed in untreated (lane 1) or heat-inactivated (HI) (lane 2) S2 extract, or $S2^{HI}$ extract supplemented with recombinant wild-type (lanes 3-5) or catalytic mutant (cm/D965A) (lanes 6-8) Ago2. (Courtesy of Dr. Qinghua Liu)

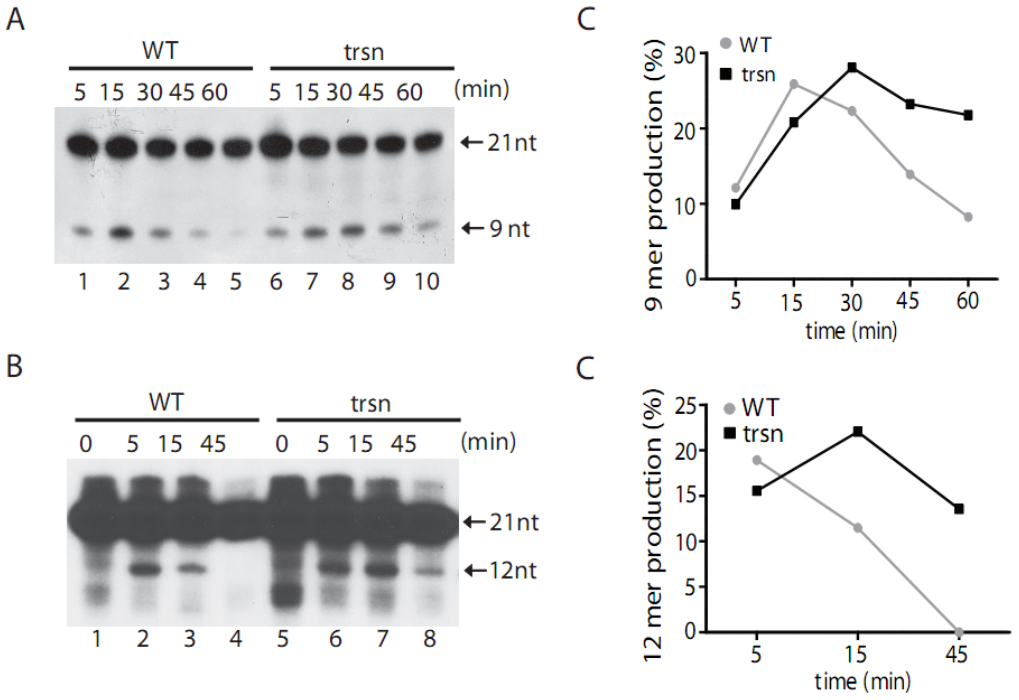


Figure 2-15 C3PO promotes RISC activation by removing the passenger strand cleavage producs

(A-B) Passenger strand cleavage assays were performed with 40µg of WT or *trsn* mutant extract. The 9-nt or12-nt cleavage products were detected separately using siRNA whose passenger strand was radiolabeled at the 5' (A) or 3' (B) end. **(C-D)** The data in (A-B) was converted into graphs illustrating different stability of the 9-mer and 12-mer in WT and *trsn* extracts. (Courtesy of Dr. Qinghua Liu)

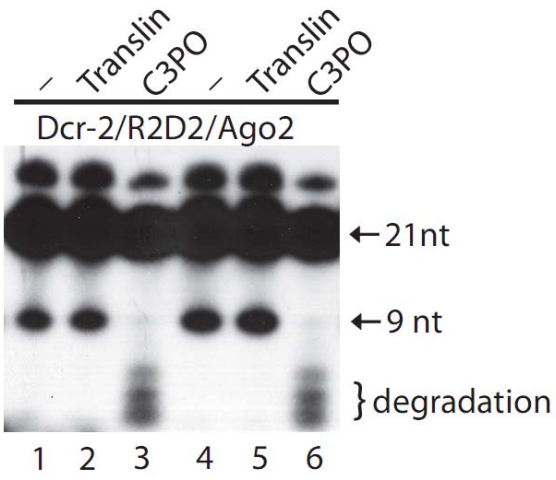


Figure 2-16 C3PO degrades passenger strand cleavage products in the reconstitution system

The passenger strand cleavage assays were performed using recombinant Dcr-2/R2D2/Ago2 alone (lanes 1, 4), or in combination with Translin (lanes 2, 5) or C3PO complex (lanes 3, 6) in the presence of 0.1μM (lanes 1-3) or 0.2μM (lanes 4-6) 2'-O-methyl Trap oligo. (Courtesy of Dr. Qinghua Liu)

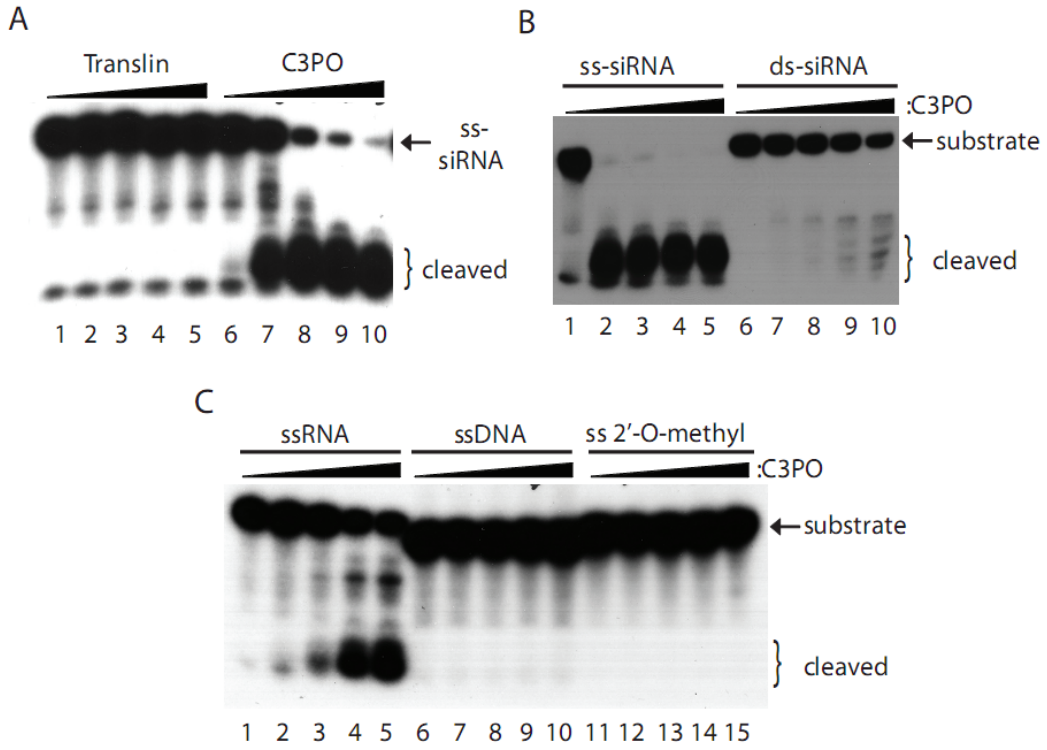


Figure 2-17 C3PO displays RNase activity toward ss-siRNA

(A) 5'-radiolabeled single-stranded (ss)-siRNA was incubated with increasing amounts of recombinant Translin or C3PO complex. **(B)** The RNase assays were performed with recombinant C3PO complex in the presence of radiolabeled single-stranded (ss) (lanes 1-5) or double-stranded (ds) siRNA (lanes 6-10). **(C)** The RNase activity of recombinant C3PO complex was compared with radiolabeled ssRNA, ssDNA or ss 2'-O-methylated RNA oligo as substrates. (Courtesy of Dr. Xuecheng Ye)

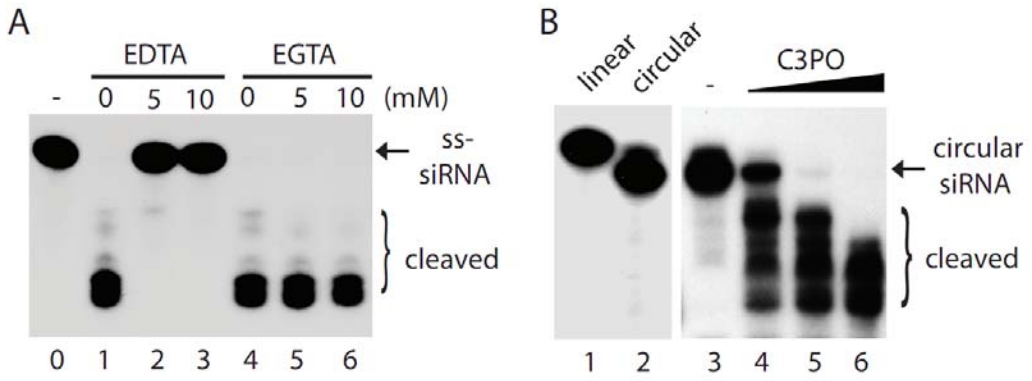


Figure 2-18 C3PO is a Mg^{2+}-dependent endoribonuclease

(A) The RNase assays were performed with recombinant C3PO in the absence or presence of EDTA or EGTA. **(B)** The RNase assays were conducted by incubating circular ss-siRNA with increasing amounts of recombinant C3PO. (Courtesy of Dr. Xuecheng Ye)

sequential chromatography (Figure 2-19) .

C3PO is a novel class of endoribonuclease because neither subunit shows similarity to any known RNase by bioinformatics and structural analyses (Sugiura et al., 2004). To identify the catalytic sites of C3PO, we performed a multi-sequence alignment of Translin/Trax (Table 1) and observed three acidic residues (E123, E126, D204) that were invariant in Trax, but missing in all Translin (Figure 2-20). Furthermore, modeling the structure of *Drosophila* Trax after crystal structure of human Translin (Sugiura et al., 2004) revealed that these residues existed in close spatial proximity, suggesting that they may coordinate Mg^{2+} ion for catalysis (Figure 2-20).

To test this hypothesis, we individually mutated E123, E126 and D204 residues of Trax to alanine. Recombinant mutant C3PO complexes were generated by co-expressing His-tagged wild-type Translin and untagged mutant Trax (Figure 2-21A). These point mutations did not affect protein folding or complex formation because wild-type and mutant C3PO displayed same column behaviors and bound single-stranded siRNA equally well in a non-cleaving condition (Figure 2-21B). Consistently, mutating each putative catalytic residue abolished the RNase activity and the RISC-enhancing activity of C3PO (Figure 2-22). By contrast, mutation of other highly conserved D/E residues in Translin or Trax did not significantly affect the RNase or RISC-enhancing activity of C3PO (Figure 2-23). These studies suggest that the intrinsic RNase activity of C3PO is required for its RISC-enhancing activity.

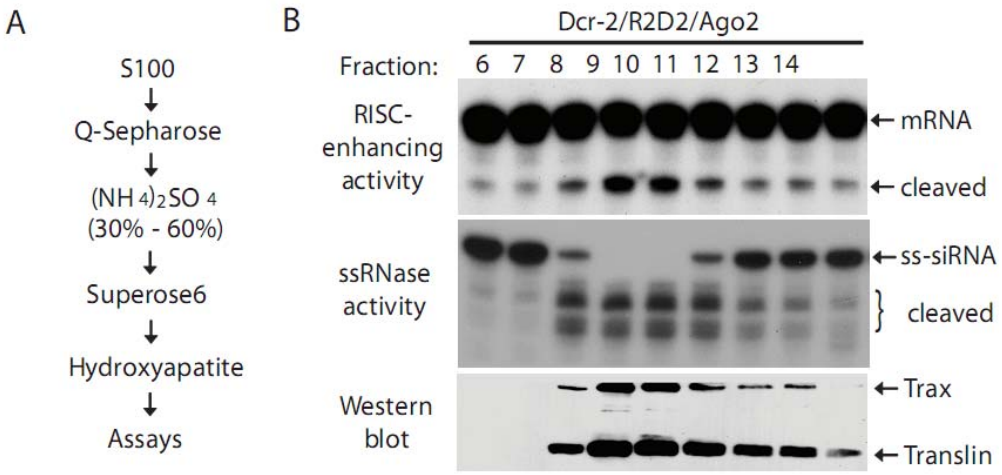


Figure 2-19 Endogenous C3PO correlates with both RISC-enhancing activity and ssRNase activity

(A) A flow chart of sequential chromatography of S2 extract. **(B)** Following sequential chromatography, fractions were assayed for the RISC-enhancing activity (top), RNase activity (middle), or Western blotting to detect Translin and Trax (bottom). (Courtesy of Dr. Xuecheng Ye)

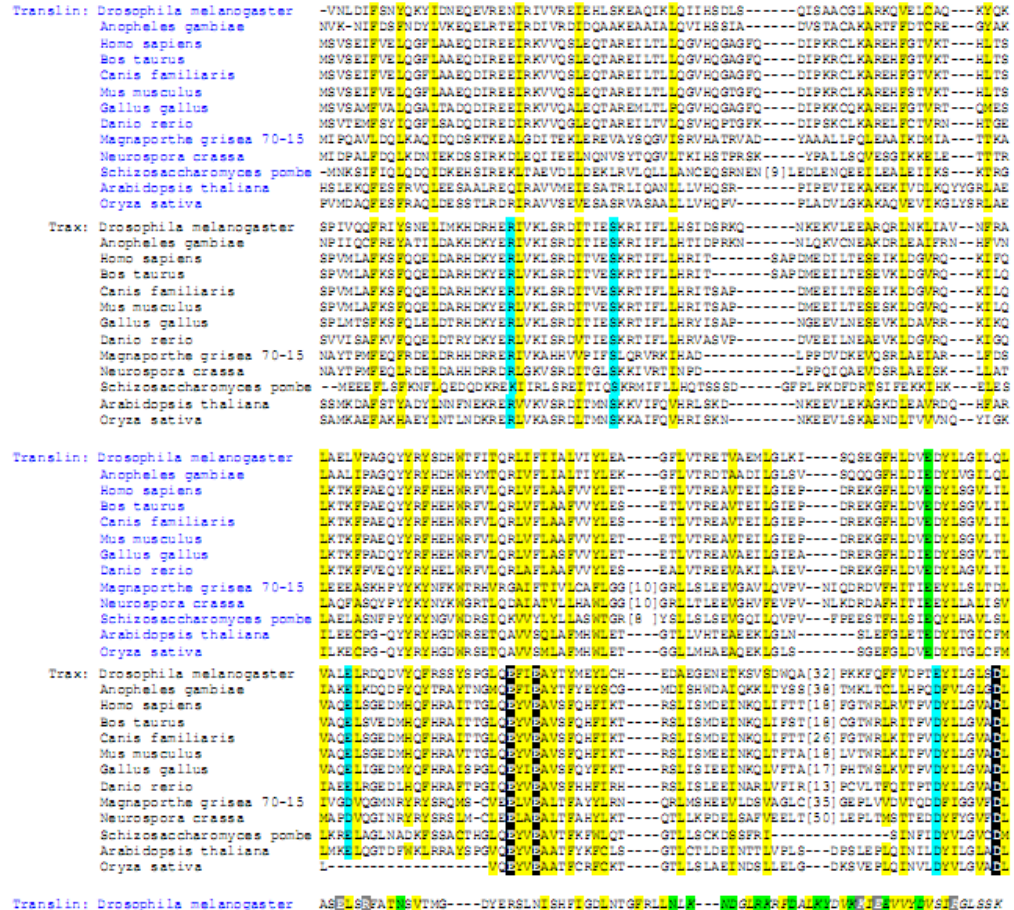

Translin: Drosophila melanogaster
Anopheles gambiae
Homo sapiens
Bos taurus
Canis familiaris
Mus musculus
Gallus gallus
Danio rerio
Magnaporthe grisea 70-15
Neurospora crassa
Schizosaccharomyces pombe
Arabidopsis thaliana
Oryza sativa
Trax: Drosophila melanogaster
Anopheles gambiae
Homo sapiens
Bos taurus
Canis familiaris
Mus musculus
Gallus gallus
Danio rerio
Magnaporthe grisea 70-15
Neurospora crassa
Schizosaccharomyces pombe
Arabidopsis thaliana
Oryza sativa

Table 1 A multi-sequence alignment of Translin and Trax

Translin and Trax sequences are colored in blue or black, respectively. Residue positions are highlighted according to conservation: hydrophobic positions in both Translin and Trax (yellow), charged residues in both Translin and Trax (gray), polar residues in Translin (green), polar residues in Trax (blue). Most importantly, the putative catalytic D/E residues that are invariant in all Trax, but missing in all Translin, are marked in black. (Courtesy of Dr. Lisa Kinch)

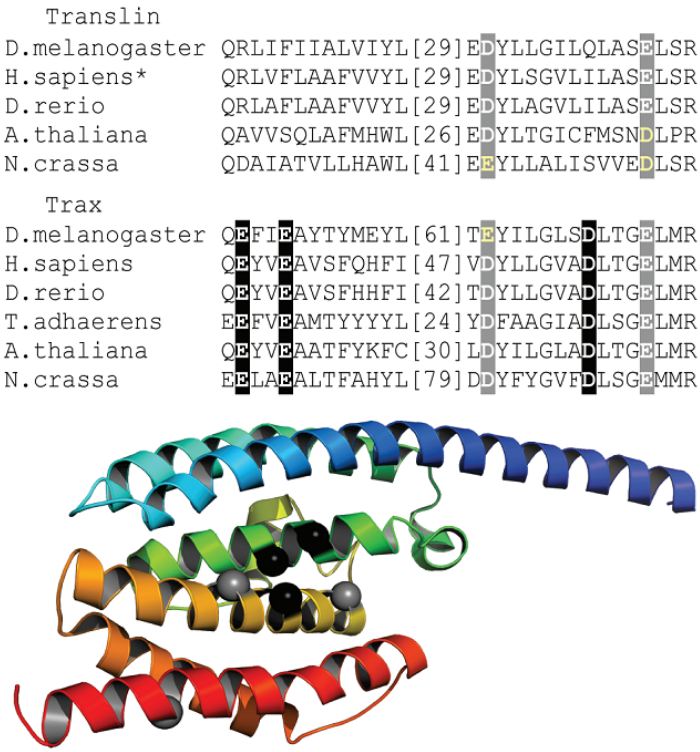


Figure 2-20 Identification of the putative catalytic center of C3PO

A partial Translin/Trax multi-sequence alignment (top) and a modeled structure of *Drosophila* Trax based on crystal structure of human Translin from 1j1j (bottom). The comprehensive alignment of Translin/Trax is shown in **Error! Reference source not found.**. The putative catalytic D/E residues are colored in black. (Courtesy of Dr. Lisa Kinch)

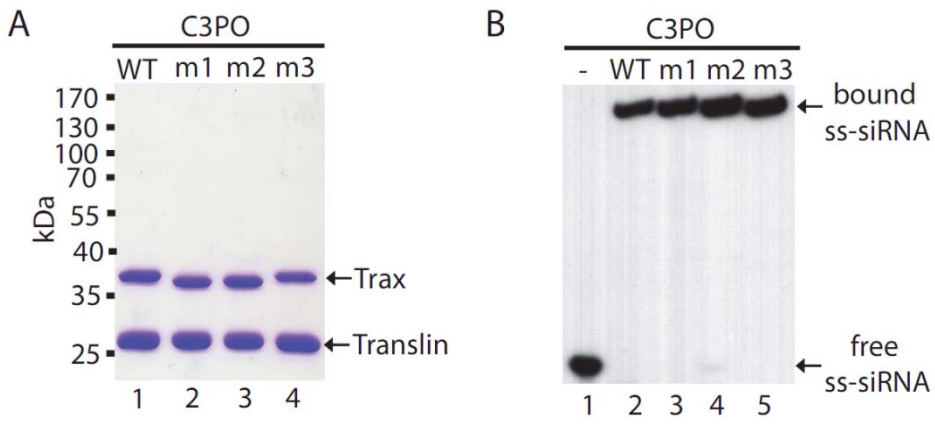


Figure 2-21 Generation of catalytic mutant C3PO

(A) A Coomassie-stained SDS-PAGE gel showing purified recombinant wild-type (WT) and three catalytic mutant (m1=E123, m2=E126, m3=D204) C3PO complexes. **(B)** Native gel-shift assays were performed by incubating radiolabeled sslet-7 siRNA in buffer (lane 1), or with WT (lane 2) or mutant C3PO (lanes 3-5) in the presence of EDTA to block catalysis.

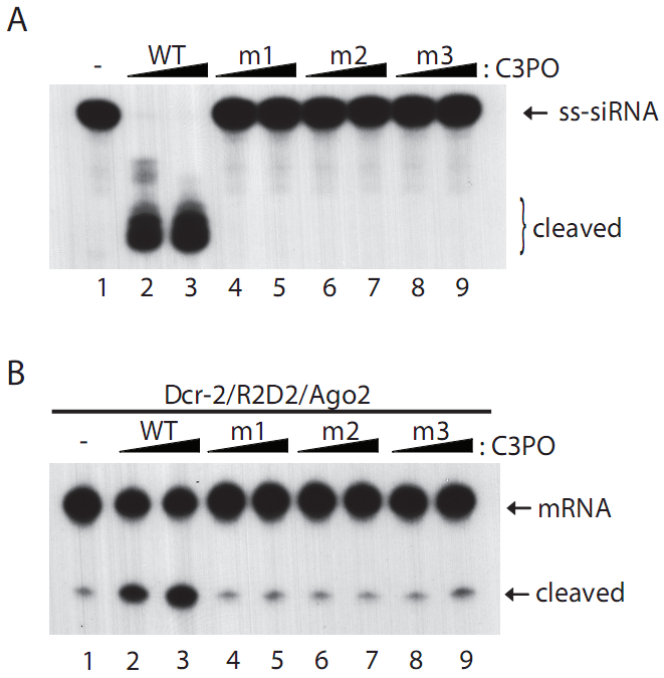


Figure 2-22 The RNase activity of C3PO is required for RISC activation

(A) The RNase activity was compared between WT and three catalytic mutant (m1=E123, m2=E126, m3=D204) C3PO complexes. **(B)** The RISC-enhancing activity was compared between WT and catalytic mutant C3PO by assaying together with recombinant Dcr-2/R2D2/Ago2.

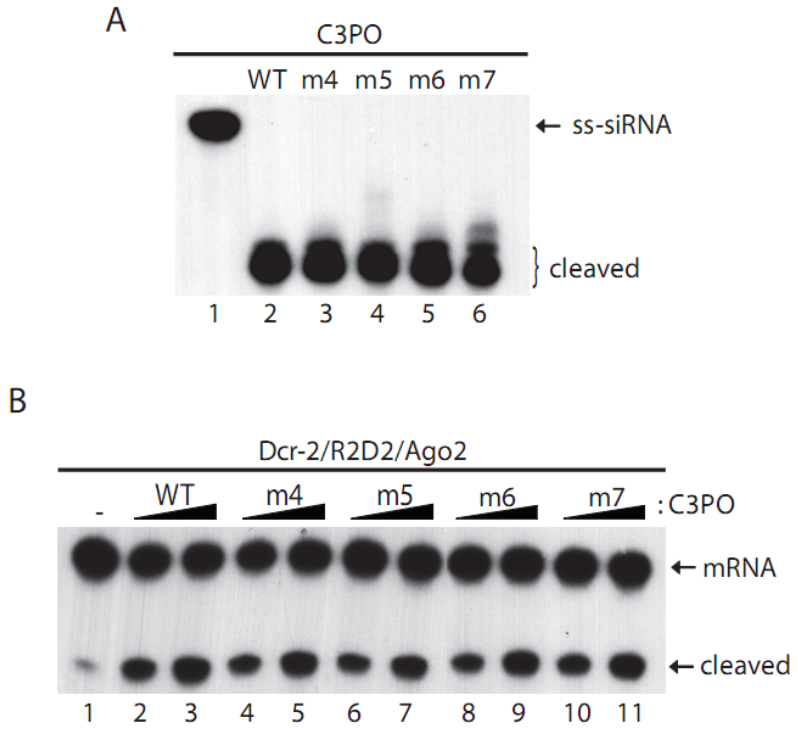


Figure 2-23 Mutagenesis studies of other highly conserved D/E residues in C3PO

(A) The RNase assays were performed with WT (lane 2) and four mutant C3PO complexes (lanes 3-6) in which other highly conserved D/E residues of Trax (m4=E197A, m5=E49A) or Translin (m6=E208A, m7=D202A) were mutated. **(B)** The RISC-enhancing assays were performed using recombinant Dcr-2/R2D2/Ago2 proteins together with WT (lanes 2-3) and four mutant C3PO complexes (lanes 4-11).

2.7 C3PO plays a conserved role in human RISC actiavation

In a recent study from our lab, Dr. Xuecheng Ye reconstituted duplex siRNA-initiated human RISC activity with the use of recombinant human Ago2 and C3PO proteins. Human C3PO could dramatically enhance the RISC activity of Ago2 (Figure 2-24A), suggesting an evolutionarily conserved role of C3PO in RISC activation.

Importantly, I have shown that hC3PO was also required for nicked duplex siRNA-initiated RISC activity, but could not activate hAgo2-RISC when using a nicked duplex siRNA carrying 2'-O-methylated passenger fragments that are resistant to C3PO cleavage (Figure 2-24B). Therefore, the removal of passenger fragments from Ago2 is not a passive process, but requires active degradation by C3PO. These studies strongly suggest that hC3PO facilitates human RISC activation by specifically degrading the Ago2-nicked passenger strand.

Discussion

We establish for the first time an *in vitro* reconstitution system for dsRNA and duplex siRNA-initiated RISC activities, demonstrating that Dcr-2/R2D2/Ago2 comprise the catalytic core of *Drosophila* RNAi. This reconstitution system also enables us to purify C3PO, a multimeric complex of Translin and Trax, as a key activator of the core RNAi machinery. Our biochemical studies show that the "slicer" mechanism plays a dominant role in *Drosophila* RISC assembly and that C3PO promotes efficient RISC activation by removing siRNA passenger strand cleavage products (Figure 2-25). The exonuclease QIP functions in a similar manner to promote RISC activation in

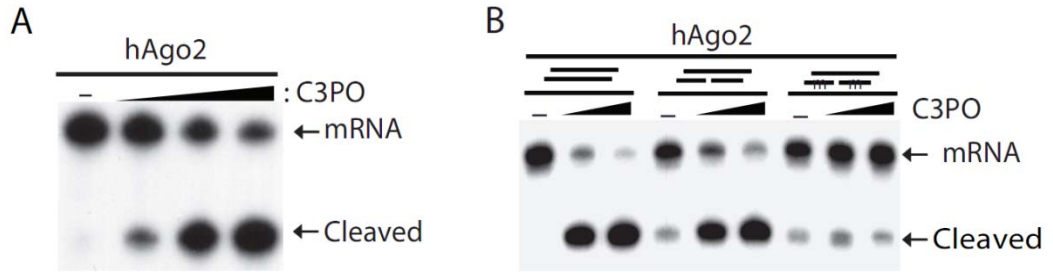


Figure 2-24 C3PO promotes human RISC activation

(A) duplex (ds)siRNA-initiated RISC assays were performed with the use of recombinant human Ago2 alone, or in the presence of increasing amount of human C3PO. (**B**) Comparison of the RISC activities of recombinant hAgo2 with or without C3PO using a perfect duplex siRNA, a nicked duplex siRNA, and a nicked duplex siRNA carrying 2'-O-methylated passenger fragments as triggers. —m— indicates 2'-O-methylated RNA.

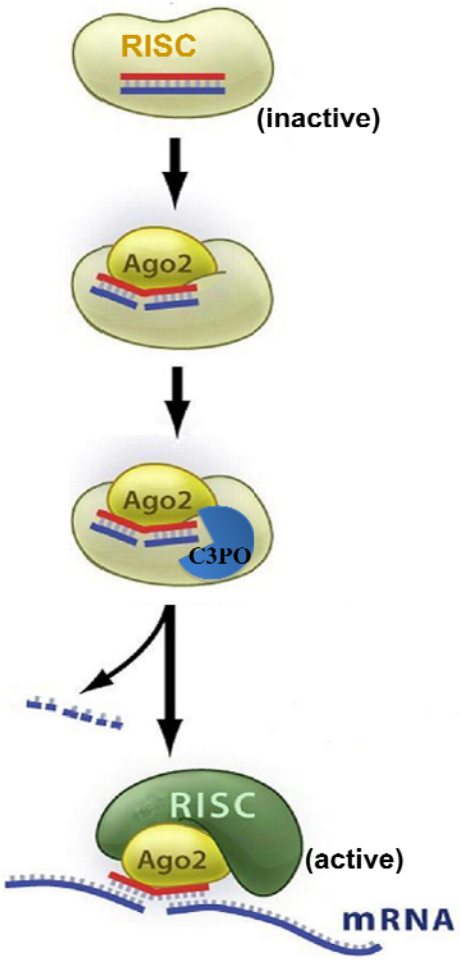


Figure 2-25 Working model for C3PO

A schematic graph showing the working model for C3PO. After duplex siRNA is loaded onto RISC, Ago2 cleaves in the middle of passenger strand, generating 9-mer and 12-mer cleavage products. C3PO then degrades the passenger strand cleavage products and facilitates RISC activation. Adapted and modified by permission from Elsevier, *Cell* © 2005.

Neurospora crassa (Maiti et al., 2007). C3PO is a Mg^{2+}-dependent endoribonuclease of which Trax is the catalytic subunit. The RNase activity of C3PO is required for RISC activation and may be stimulated by Ago2-mediated nick in duplex siRNA and/or fraying of the ends of cleavage fragments. Additionally, we established that C3PO plays an evolutionarily conserved role in *Drosophila* and human RISC activation.

Previous studies suggest that the interaction between Ago and RNA is a highly dynamic process associated with large conformational changes in both components (Parker; Wang et al., 2008a; Wang et al., 2009; Wang et al., 2008b): 1) duplex siRNA is loaded onto Ago2; 2) Ago2-nicked passenger strand has to be removed to activate RISC; 3) target mRNA then comes in to form a duplex with the guide strand; 4) the sliced mRNA products need to dissociate in order for RISC to cleave another target. Therefore, the nicked duplex siRNA is not all deeply buried within Ago2 at all time, and parts of it are accessible to other regulatory factors. We think it is unlikely that the entire nicked duplex siRNA is transferred from Ago2 to C3PO, where the passenger fragments are degraded and the guide strand is transported back to Ago2. We prefer a model that the guide strand remains bound to Ago2, while the passenger fragments are transferred to the active site of C3PO to be degraded. It is possible that the dissociation and degradation of passenger fragments are closely coupled and jointly promoted by C3PO and Ago2. It will be exciting and challenging for future studies, including structural determination of C3PO-RNA complexes, to unravel the precise mechanism of this dynamic process.

Chapter 3 : Purification and Characterization of La

Abstract

Activation of the RNA-induced silencing complex (RISC) involves the recruitment of duplex siRNA (guide strand/passenger strand) to Argonaute2 (Ago2), Ago2-mediated nick of passenger strand, and degradation of passenger fragments by C3PO nuclease. Active RISC is a multiple-turnover enzyme, wherein single-stranded guide RNA directs Ago2 to catalyze sequence-specific cleavage of complementary mRNA. How this effector step of RNA interference (RNAi) is regulated is currently unknown. Here, we used human minimal RISC system to purify autoantigen La, also known as Sjögren's syndrome antigen B (SS-B), as a novel activator of the RISC-mediated mRNA cleavage activity. We demonstrated that La was required for efficient RNAi, antiviral defense, and transposon silencing in *Drosophila* and mammalian cells. Our reconstitution and kinetic studies reveal that La promotes the multiple-turnover RISC catalysis in an ATP-independent manner. Consistent with this function, La exhibits an RNA unwinding activity and facilitates the release of cleaved mRNA from Ago2. Taken together, our findings of La and C3PO reveal a general concept that regulatory factors are required to remove Ago2-cleaved products to assemble or restore active RISC.

Introduction

RNA interference (RNAi), in all of its manifestations, impacts many aspects of eukaryotic gene expression and is involved in numerous biological and disease processes.

Multiple classes of 20- to 32-nucleotide (nt) small regulatory RNAs, including small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-interacting RNA (piRNAs), have been identified and characterized in a wide variety of eukaryotic organisms (Carthew and Sontheimer, 2009; He and Hannon, 2004; Kim, 2005; Liu and Paroo; Siomi and Siomi, 2009). The primary function of siRNAs in plants and animals is to serve as an ancient defense mechanism against invading nucleic acids, such as viruses and transgenes (Ding and Voinnet, 2007; Li et al., 2002). Mounting studies have revealed abundant sources of endogenous (endo)-siRNAs that are derived from transposable elements, complementary annealed transcripts, and long fold-back transcripts (Okamura and Lai, 2008). In *Schizosaccharomyces pombe*, centromeric repeats-derived endo-siRNAs are involved in the recruitment of histone modifiers to establish and maintain centromeric heterochromatin and transcriptional silencing (Hall et al., 2002; Reinhart and Bartel, 2002; Volpe et al., 2002). In *Drosophila*, the endo-siRNAs are important for transposon silencing in the somatic cells (Czech et al., 2008; Ghildiyal et al., 2008). Hundreds to thousands of miRNAs function in diverse developmental, physiological, or pathological processes by acting as molecular switches or fine tuner of genome expression (Bartel, 2004; Carthew and Sontheimer, 2009; He and Hannon, 2004). In animals, the piRNA pathway plays essential roles in transposon silencing and maintenance of genomic integrity in the germ line (Saito and Siomi).

The canonical form of RNAi is an evolutionarily conserved, double-stranded RNA (dsRNA)-induced post-transcriptional gene silencing mechanism (Fire et al., 1998). In principle, the RNAi pathway consists of initiation, activation, and effector steps

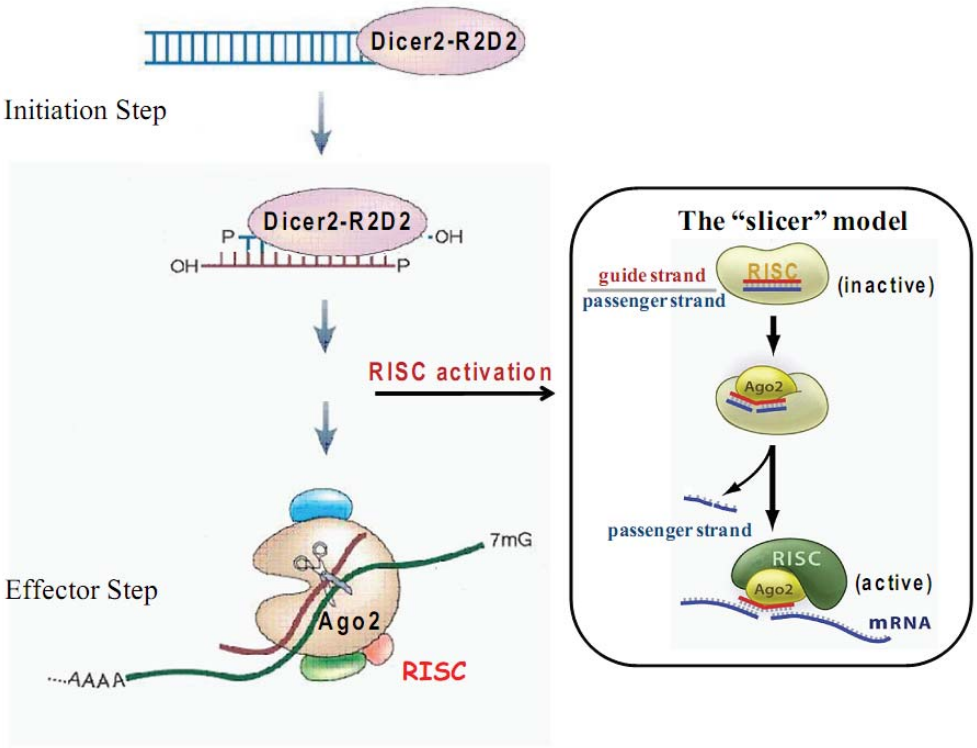


Figure 3-1 The canonical RNAi pathway

The canonical RNAi pathway comprises initiation, activation and effector steps. In the initiation step, Dicer-2-R2D2 complex processes long dsRNA into duplex siRNA and facilitates the loading of siRNA onto RISC. The central step for RISC activation is the unwinding of duplex siRNA. In the "slicer model" of RISC activation, Ago2 cleaves in the middle of passenger strand and results in the dissociation of passenger strand cleavage products from guide strand, leaving guide strand behind with Ago2 to form active RISC. In the effector step, Ago2, the catalytic enzyme of RISC catalyzes sequence-specific cleavage of the target RNA (Preall and Sontheimer, 2005). Adapted by permission from Elsevier, *Cell* © 2005.

(Figure 3-1). In the initiation step, the ribonuclease (RNase) III Dicer processes long dsRNA into ~21-nucleotide (nt) small interfering RNA (siRNA) (Bernstein et al., 2001; Elbashir et al., 2001b; Lee et al., 2004; Liu et al., 2003). In the activation step, nascent siRNA duplex is assembled into the effector RNA-induced silencing complex (RISC) (Hammond et al., 2000). In the effector step, active RISC employs single-stranded (ss) siRNA to direct sequence-specific cleavage of complementary mRNA (Haley and Zamore, 2004; Liu et al., 2004; Martinez et al., 2002; Rivas et al., 2005; Song et al., 2004).

Recent biochemical studies suggest a three-step process for *Drosophila* RISC activation (Liu and Paroo). First, Dicer-2 (Dcr-2) and its dsRNA-binding partner R2D2 coordinately recruit duplex siRNA (guide strand/passenger strand) to Argonaute 2 (Ago2) (Liu et al., 2003; Liu et al., 2006; Pham et al., 2004; Tomari et al., 2004b). Second, Ago2, an endonuclease, nicks the passenger strand into 9-nt and 12-nt fragments (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). Finally, C3PO, another endonuclease, activates RISC by degrading the passenger fragments (Liu et al., 2009). In *Drosophila*, recombinant Dicer-2-R2D2 and Ago2 reconstitute a basal level of duplex siRNA-initiated RISC activity that is greatly enhanced by C3PO (Liu et al., 2009). However, recombinant human Ago2 and C3PO can reconstitute duplex siRNA-initiated RISC activity in the absence of Dicer-TRBP complex, suggesting a Dicer-independent mechanism for human RISC activation (Ye et al.). In both fly and human systems, heat shock proteins, such as Hsp90/Hsc70, have also been reported to facilitate the loading of duplex siRNA onto Ago2 (Iki et al.; Iwasaki et al.; Miyoshi et al.). In *Neurospora*, QIP,

a putative exonuclease, plays a similar role as C3PO to degrade QDE-2 (homolog of Ago2)–nicked duplex siRNA to activate RISC (Maiti et al., 2007). Collectively, these studies support a general model for siRNA-initiated RISC activation in diverse eukaryotes.

Relatively little is known about the regulatory mechanisms of the RNAi effector step. The siRNA-programmed RISC is a multiple-turnover enzyme with classical Michaelis-Menton kinetics (Haley and Zamore, 2004; Rivas et al., 2005). In *Drosophila* embryo extract, the rate of multiple rounds of RISC catalysis appears to be limited by ATP-dependent release of cleaved mRNA from Ago2 (Haley and Zamore, 2004). Here, we used human minimal RISC system to purify autoantigen La, also known as Sjögren's syndrome antigen B (SS-B) (Mattioli and Reichlin, 1974), as a novel regulator of the RISC-mediated mRNA cleavage activity.

Sjögren's syndrome is a chronic autoimmune disorder in which immune cells attack and destroy the exocrine glands that produce tears and saliva. Sjögren's syndrome can occur alone (primary Sjögren's syndrome), or in association with other autoimmune disorder such as systemic Lupus erythematosus (secondary Sjögren's syndrome) (Srinivasan and Slomovic, 2007). One hallmark of Sjögren's syndrome is the presence of antibodies against self-antigens, most commonly known of which are SS-A/Ro and SS-B/La (Mattioli and Reichlin, 1974). The evolutionarily conserved La protein plays important roles in many processes of RNA metabolism from yeast to human (Wolin and Cedervall, 2002).

In this study, we report three principal findings: 1) Autoantigen La is required for efficient RNAi, antiviral defense and transposon silencing in *Drosophila* and mammalian cells. 2) La promotes the multiple-turnover of RISC catalysis. 3) La exhibits an ATP-independent RNA unwinding activity even though it bears no resemblance to any known helicases. These findings significantly enhance our current understanding of the regulatory mechanisms of the RNAi effector step.

3.1 Purification of La as an activator of the RISC activity

We took an unbiased biochemical fractionation and reconstitution approach to study the regulatory mechanisms of the RNAi effector step. Specifically, we used recombinant Ago2 and ss-siRNA to assemble minimal RISC *in vitro* and searched for activators or inhibitors of the RISC activity by supplementing chromatographic fractions of S2 and HeLa cell extracts. Recombinant human (h)Ago2 (Liu et al., 2004; Rivas et al., 2005), but not *Drosophila* (d)Ago2, generated robust ss-siRNA-initiated RISC activity *in vitro* (Figure 3-2). Therefore, we used human minimal RISC system to search for new regulators of the RNAi effector step. Addition of the cytoplasmic (S100) extract of HeLa cells, which had no detectable ss-siRNA-initiated RISC activity, greatly enhanced the hAgo2-RISC-mediated mRNA cleavage activity (Figure 3-3A), suggesting the existence of a new activator of RISC activity. This RISC activator was subsequently purified to homogeneity through a four-step chromatographic procedure. At the final step, a single ~50 kDa protein appeared on the silver-stained SDS-polyacrylamide gel and correlated closely with the RISC-enhancing activity (Figure 3-3B). This protein was identified as autoantigen La/SS-B by mass spectrometric analysis. Human La, also known as

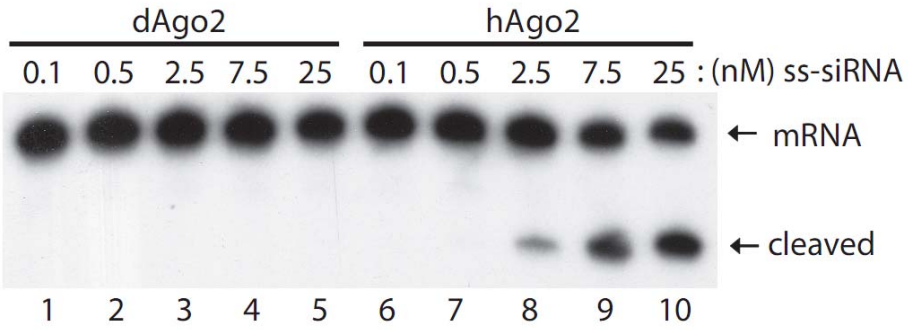
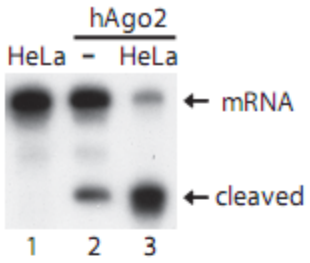


Figure 3-2 Reconstitution of human minimal RISC

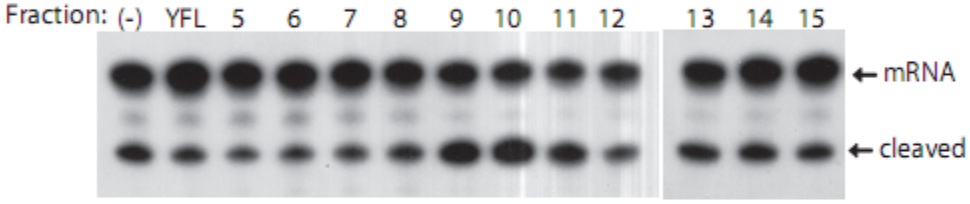
Recombinant dAgo2 (lanes 1-5) and hAgo2 (lanes 6-10) proteins were compared for single-stranded (ss)-siRNA-initiated RISC activity.

A



B

Phenyl Gradient:



SP Gradient:

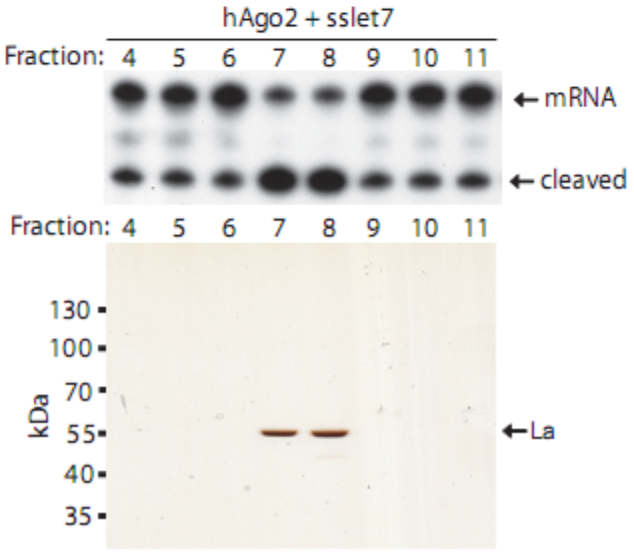


Figure 3-3 Purification of La as an activator of the RISC activity

(A) The ss-siRNA-initiated RISC assays were performed with HeLa extract or recombinant hAgo2, or both. **(B)** Purification of La through a four-step chromatographic procedure. Here shows the activities after phenyl gradient and final Mono S column. Following the final Mono S step, individual fractions were assayed with recombinant hAgo2 for the RISC-enhancing activity (top) or resolved by SDS-polyacrylamide gel (PAGE) followed by silver-staining (bottom). YFL: phenyl flow-through.

Sjogren's syndrome antigen B (SS-B), was identified as a common autoantigen in patients suffering rheumatic diseases, such as Sjogren's syndrome and systemic lupus erythematosus (Mattioli and Reichlin, 1974). The evolutionarily conserved La protein plays important roles in many processes of RNA metabolism from yeast to human (Bayfield et al.).

3.2 Recombinant La promotes fly and human RISC activity *in vitro*

To reconstitute this RISC-enhancing activity, we generated recombinant human and *Drosophila* La proteins using an insect cell expression system (Figure 3-4A). Consistent with the purification result, addition of recombinant human La significantly increased the hAgo2 minimal RISC activity (Figure 3-4B). Similarly, addition of *Drosophila* La proteins greatly enhanced the duplex siRNA-initiated RISC activity of recombinant Dcr-2-R2D2 and dAgo2 proteins (Figure 3-5). To distinguish if dLa enhanced the RISC assembly or activity, we sought to examine the activity of La on the pre-assembled *Drosophila* RISC. Because dAgo2 exhibited little ss-siRNA-initiated RISC activity (Figure 3-2), we assembled *Drosophila* RISC by incubating recombinant Dicer-2-R2D2 and dAgo2 proteins with duplex siRNA followed by mild heat treatment to inactivate free dAgo2 and block further RISC assembly (Figure 3-6A) (Liu et al., 2009). Robust RISC-enhancing activity was observed no matter when dLa was added before or after the assembly of RISC (Figure 3-6B), suggesting that La primarily promotes the RISC-mediated mRNA cleavage activity. Thus, our biochemical fractionation and reconstitution studies identify autoantigen La as a key regulator of the effector step of the *Drosophila* and human RNAi pathways.

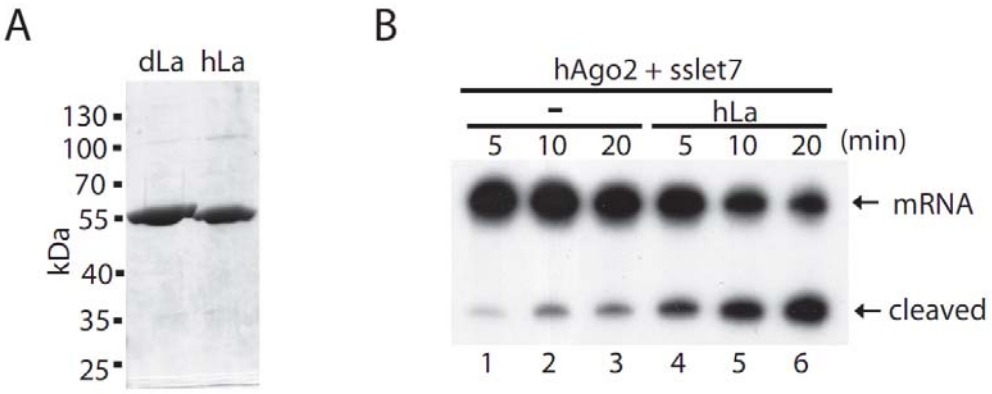


Figure 3-4 hLa enhances human minimal RISC activity

(A) Generation of recombinant *Drosophila* and human La. Drosophila and human La proteins were generated from insect cells using a baculovirus expression system. **(B)** The ss-siRNA-initiated RISC assays were performed using recombinant hAgo2 in the absence or presence of hLa.

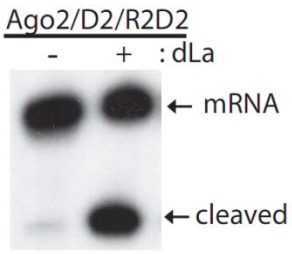


Figure 3-5 dLa enhances *Drosophila* RISC activity

The duplex siRNA-initiated RISC assays were performed using recombinant dAgo2/D2/R2D2 in the absence or presence of dLa.

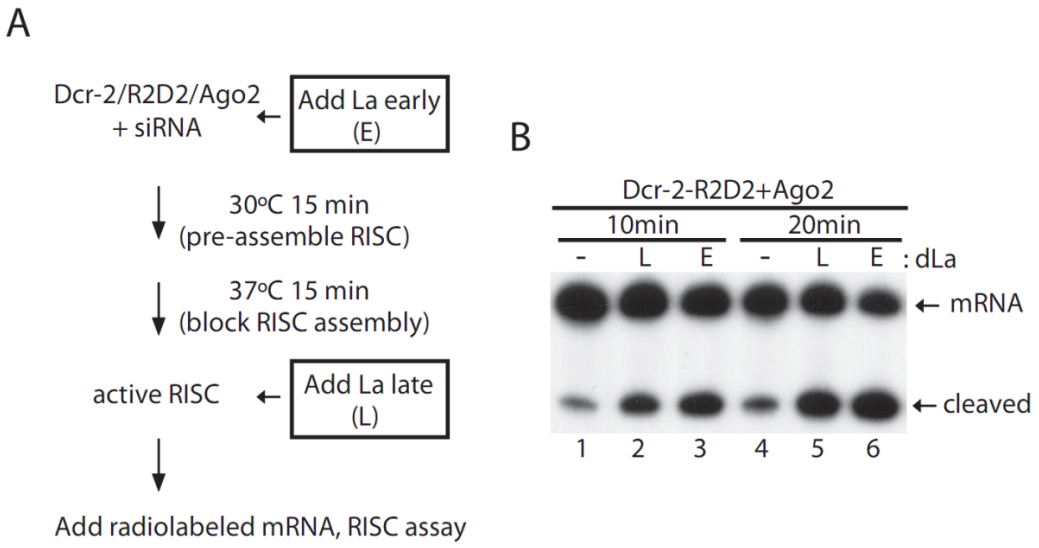


Figure 3-6 La enhances Drosophila RISC activity after RISC assembly step

(A) A flow chart of the experimental procedure used in (B). **(B)** The duplex siRNA-initiated RISC assays were conducted using recombinant Dcr-2-R2D2 and Ago2, or with La added before (E) or after (L) RISC assembly.

3.3 La physically associates with Ago2

We performed co-immunoprecipitation (IP) to examine the association between La and Ago2, the catalytic subunit of RISC, in mammalian cells. Reciprocal co-IP experiments detected the stable association of ectopically expressed Myc-Ago2 and Flag-La proteins in HeLa cells (Figure 3-7A and Figure 3-7B). Additionally, the physical interaction between La and Ago2 was confirmed by co-IP experiments using purified recombinant proteins. Recombinant human La efficiently brought down hAgo2 protein in the absence or presence of siRNA (Figure 3-7C), suggesting that La could interact with Ago2 before or after the loading of siRNA.

3.4 La is required for efficient RNAi *in vivo*

To determine if La is required for efficient RNAi in vivo, we used siRNA to knock down the expression of La in HeLa cells (Figure 3-8A) followed by co-transfection of a psiCheck2 construct, which expressed both *Firefly* (FL) and *Renilla* (RL) luciferase reporters, together with a control or *RL*-siRNA. The *RL*-siRNA could effectively silence the expression of *RL*-luciferase, but not that of *FL*-luciferase, which served as an internal control. As expected, siRNA-mediated depletion of Ago2 almost fully restored the expression of *RL*-luciferase (Figure 3-8B). Consistent with in vitro biochemical studies, depletion of La significantly compromised the efficiency of RNAi silencing of *RL*-luciferase in HeLa cells (Figure 3-8B). The same result was obtained when using two siRNAs that targeted different regions of La, suggesting that this phenotype is less likely due to an off-target effect (Figure 3-8B). Similar RNAi defects were also observed when

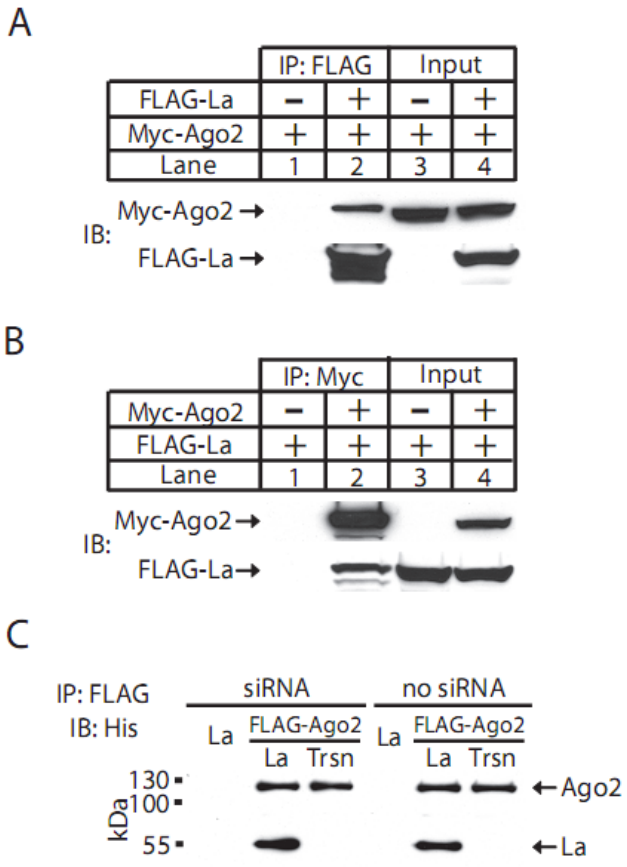


Figure 3-7 La interacts Ago2 *in vivo* and *in vitro*

(A-B) Following transfection of 293T cells with Myc-hAgo2 and/or Flag-hLa constructs, co-IP experiments were performed with anti-Flag (A) or anti-Myc (B) antibodies followed by Western blotting with the corresponding antibodies. **(C)** Co-IP experiments were performed using purified His-Flag-hAgo2 and His-La or His-Translin recombinant proteins after pre-incubation with or without ss-siRNA. (Courtesy of Huiling Tan)

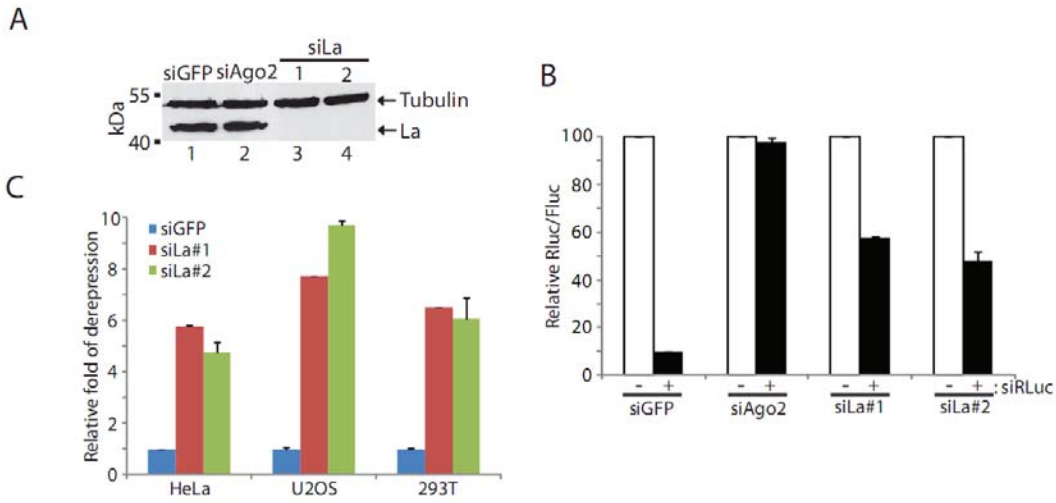


Figure 3-8 La is required for efficient RNAi in mammalian cells

(A) siRNA oligos against GFP, hAgo2 or two different siRNAs against hLa were transfected into mammalian cells. Cells were harvest three days after transfection. The protein level of La was measured in different lysates by immunoblotting using anti-La antibody. Tubulin level was served as a loading control. **(B)** After siRNA-mediated knockdown in HeLa cells for three days, a control or *RL*-siRNA were co-transfected with the psiCheck2 reporter followed by dual luciferase assays within twenty-four hours. The efficiency of RNAi silencing was measured by the relative *Renilla* / *Firefly* luciferase activity. **(C)** A graph illustrating the fold of de-repression of the *Renilla* luciferase after similar experiments in (B) were carried out in HeLa, U2OS and 293T cells.

La was depleted in U2OS and HEK293T cells. In all three cell lines, the lack of La expression typically resulted in a 5 to 10-fold de-repression of the *RL*-luciferease reporter (Figure 3-8C). Consistently, dsRNA-mediated knockdown of La also compromised the efficiency of RNAi silencing in *Drosophila* S2 cells (Figure 3-9).

To test if La was also critical for RNAi silencing of an endogenous gene, we employed a Tet_{on}-inducible U2OS cell line, in which tetracycline induction of a short hairpin RNA (shRNA) could effectively silence the expression of the endogenous Mcl-1 gene. The siRNA-mediated knockdown of Dicer, the enzyme required for processing shRNA into siRNA restored Mcl-1 expression in the presence of tetracycline (Figure 3-10). Similarly, depletion of La by siRNA transfection also significantly attenuated RNAi silencing of the endogenous Mcl-1 gene (Figure 3-10). Taken together, these genetic studies indicate that La promotes efficient RNAi in *Drosophila* and mammalian cells.

microRNA (miRNA) is a class of genome-encoded small regulatory RNAs that control diverse developmental, physiological, and pathological processes (Carthew and Sontheimer, 2009; He and Hannon, 2004; Liu and Paroo; Siomi and Siomi, 2009). In animal cells, the majority of miRNAs mediate post-transcriptional gene silencing through translational repression and/or mRNA decay (Djuranovic et al.; Fabian et al.). Additionally, miRNA-programmed RISC (miRISC) can also cleave near perfectly matched target mRNA in a similar manner as siRISC *in vitro* and *in vivo* (Haley and Zamore, 2004; Hutvagner and Zamore, 2002; Yekta et al., 2004). To test if La could also

regulate the action of miRISC, we co-transfected HeLa cells with the psiCheck2 reporter and a previously described synthetic miR-Luc duplex. The presence of miR-Luc

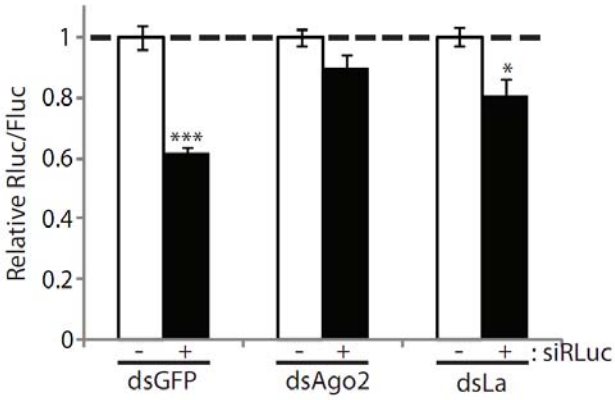


Figure 3-9 La promotes RNAi silencing in *Drosophila*

La promotes RNAi silencing in *Drosophila*. The expression of Ago2, La, or both were depleted in S2 cells by dsRNA treatment for three days. A control or *RL*-siRNA were co-transfected with the psiCheck2 reporter construct followed by dual luciferase assays within twenty-four hours. The efficiency of RNAi silencing was measured by the relative *Renilla* / *Firefly* luciferase activity. P-value was calculated with two tailed distribution. *, $P < 0.05$; ***, $P < 0.001$. (Courtesy of Huiling Tan)

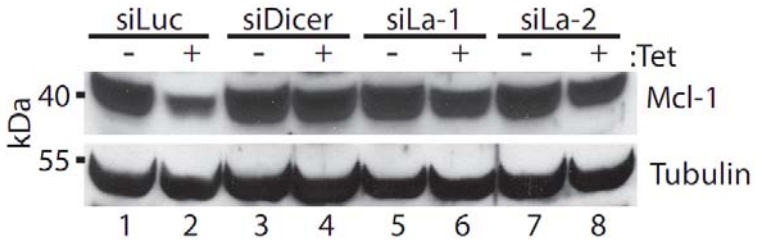


Figure 3-10 La is critical for efficient RNAi silencing of endogenous genes

After siRNA-mediated knockdown in the Tet_{on}/shMc-l U2OS cells for two days, tetracycline were added to the growth media for two days to induce the expression of shRNA to silence the endogenous Mcl-1 gene. The protein levels of Mcl-1 and α-Tubulin were compared among various treated cells.

normally repressed *RL*-luciferase activity by approximately fifty-fold, however, depletion of Ago2 or La significantly attenuated this silencing activity (Figure 3-11A). A similar phenomenon was observed when co-transfecting duplex miR-30a with a psiCheck2 construct carrying two perfect miR-30a target sites in the 3' untranslated region (UTR) of *RL*-luciferase (Figure 3-11B). These studies suggest that autoantigen La may be also important for the miRNA-mediated silencing of target genes.

3.5 La promotes anti-viral defense and transposon silencing in *Drosophila*

The RNAi pathway serves as an important antiviral defense mechanism in plants and animals (Ding and Voinnet, 2007; Li et al., 2002). To study the involvement of La in the antiviral response, we used dsRNA treatment to specifically knockdown the expression of Ago2, La, or both in *Drosophila* S2 cells (Figure 3-12), and transfected these cells with a self-replicating *flock house* virus (FHV) mutant (FR1-ΔB2) construct (Li et al., 2004). The lack of viral B2, a suppressor of RNAi, allowed S2 cells to effectively repress viral expression through RNAi silencing (Li et al., 2004). Knocking down either Ago2 or La resulted in ~1.5-fold accumulation of FHV RNA, whereas depletion of both proteins caused a ~2.5-fold induction of viral RNA expression (Figure 3-13). Thus, La is involved in the RNAi-mediated antiviral defense in *Drosophila* cells.

In *Drosophila* and mammals, the endogenous (endo)-siRNAs are frequently derived from complementary annealed transcripts and long fold-back transcripts (Okamura and Lai, 2008). The long hairpin (hp) transcripts-derived endo-siRNAs can be

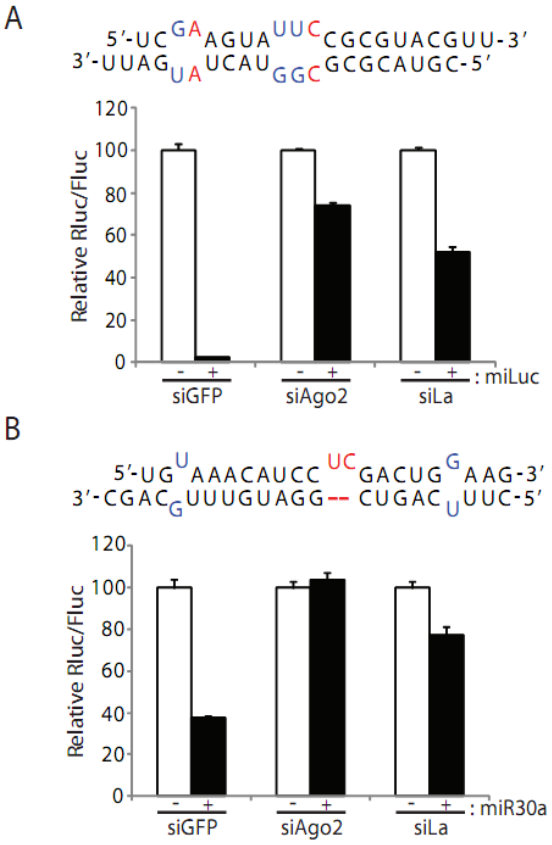


Figure 3-11 La is important for the miRNA-mediated silencing of target genes

(A) A luciferase miRNA duplex (Yoda et al.) (sequence shown on top) was co-transfected with the psiCheck2 reporter containing a perfect target site followed by dual luciferase assays within twenty-four hours. The efficiency of RNAi silencing was measured by the relative *Renilla* / *Firefly* luciferase activity. **(B)** A miR30a duplex (sequence shown on top) was co-transfected with the psiCheck2 reporter construct containing two perfect miR30a target sites in the 3'UTR of *RL*-luciferase followed by dual luciferase assays within twenty-four hours to measure the relative *Renilla*/*Firefly* luciferase activity. (Courtesy of Dr. Hui Tian)

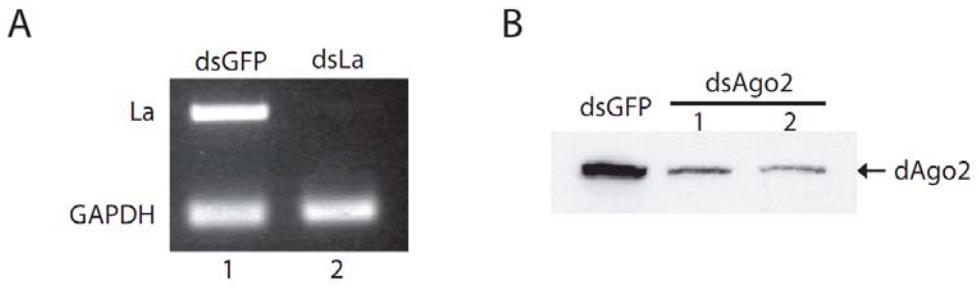


Figure 3-12 Depletion of La and Ago2 in *Drosophila* S2 cells

dsRNAs against GFP, La or Ago2 were generated by in *vitro* transcription and added into the growth medium of *Drosophila* S2 cells for three days. **(A)** Total RNA was extracted from cells using Trizol reagent. RT-PCR was then carried out to compare the endogenous mRNA level of La under different treatment. GAPDH was served as a control. **(B)** Immunoblotting was performed using anti-dAgo2 antibody to detect endogenous Ago2 levels.

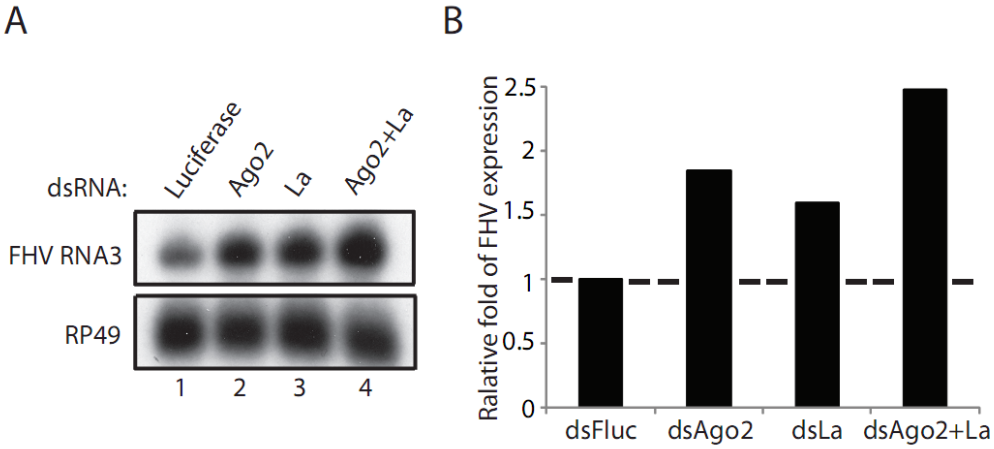


Figure 3-13 La promotes anti-viral defense in *Drosophila*

(A) The expression of Ago2, La, or both were depleted in S2R(+) cells by dsRNA treatment for three days. Cells were transfected with a self-replicating *flock house* virus (FHV) mutant (FR1-ΔB2) construct (Li et al., 2004). Total RNAs were extracted two days after Cu^{2+}induction and Northern blotting was performed with a probe specific to viral RNA or RP49. **(B)** A graph illustrating the quantification of viral RNA levels normalized to RP49 transcript in (A).

loaded onto Ago2 to cleave complementary target mRNA (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008). In S2 cells, ectopic expression of plasmid encodes hp-CG18854, but not hp-CG4068, repressed the expression by ~70% of a luciferase reporter carrying target sites for a hp-CG18854-derived siRNA (Figure 3-14A) (Okamura et al., 2008). Depletion of Ago2 or La proteins by dsRNA treatment (Figure 3-12) significantly attenuated the endo-siRNA-mediated repression of the luciferase reporter (Figure 3-14A). Conversely, when hp-CG4068 was ectopically expressed, loss of Ago2 or La de-repressed the luciferase reporter carrying target sites for hp-CG4068-derived siRNA (Figure 3-14B). These results indicate that La also plays a critical role in the *Drosophila* endo-siRNA pathway.

Another abundant source of endo-siRNAs is the transposable elements, whose activities must be silenced to protect genomic integrity and organism survival (Czech et al., 2008; Ghildiyal et al., 2008; Saito and Siomi; Tam et al., 2008). This class of endo-siRNAs contributes critically to transposon silencing in the somatic cells of *Drosophila* (Czech et al., 2008; Ghildiyal et al., 2008). To examine the role of La in transposon silencing, we knocked down the expression of La, Ago2, or both in S2 cells followed by quantitative RT-PCR to compare the expression level of retrotransposon ZAM. While knocking down La or Ago2 respectively caused ~2- or 5-fold accumulation of ZAM transcript, depletion of both proteins resulted in up to 20-fold induction of the transposon expression (Figure 3-15A). This synergistic effect further suggests that Ago2 and La work cooperatively at the RISC effector step. Furthermore, we examined the expression levels of a panel of transposons and observed significant accumulation of the transcripts

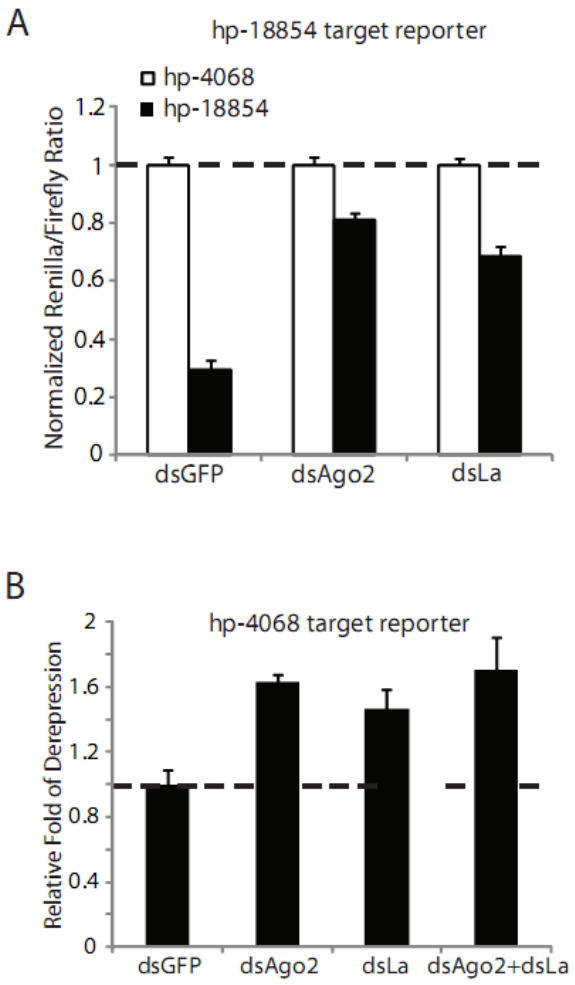


Figure 3-14 La enhances endo-siRNA-mediated silencing

Following dsRNA treatments of S2 cells for two days, the hp-4068 or hp-18854 construct was co-transfected with a luciferase reporter carrying the target sites for a hp-CG18854-derived endo-siRNA, and luciferase activities were monitored after 24 hours. (Courtesy of Huiling Tan)

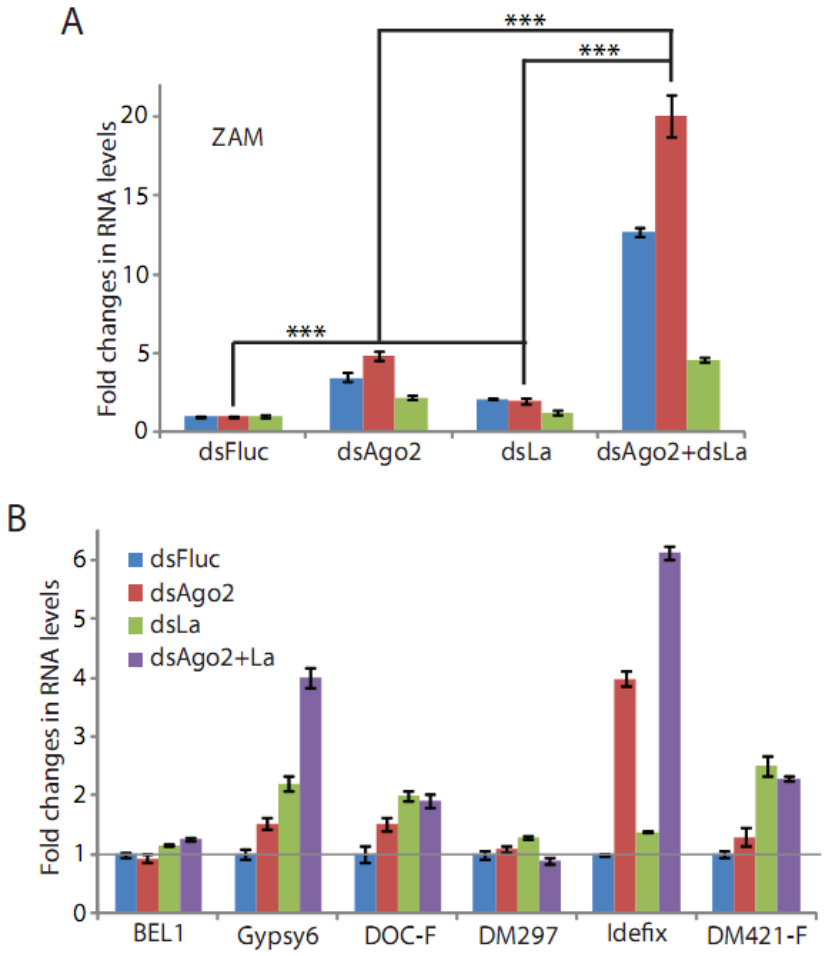


Figure 3-15 La promotes transposon silencing in *Drosophila*

(A) A graph showing relative fold of changes in the ZAM transcript levels in S2 cells following various dsRNA treatments for eight days. The ZAM RNA level was measured by quantitative RT-PCR and normalized to that of RP49 in three independent experiments. **(B)** In a similar experiment as described in (A), the RNA levels of six different transposons were compared among various dsRNA-treated S2 cells. (Courtesy of Huiling Tan)

of *Idefix*, *DOC*, *gypsy6* and *412*, but not those of *DM297* and *BEL1*, in La and/or Ago2-depleted cells (Figure 3-15B). These results suggest that La and Ago2 function cooperatively in the endo-siRNA-mediated transposon silencing.

3.6 La promotes the multiple-turnover of RISC catalysis

In principle, the RISC-mediated mRNA cleavage activity can potentially be regulated at three steps: target recognition, mRNA cleavage, and product release. Thus, we performed a single- vs. multiple-turnover reactions to further dissect at which step La enhances the RISC activity. Regulation of target recognition and RISC catalysis by La, but not the product release, should affect the rate of both single- and multiple-turnover RISC reactions. In a single-turnover condition, in which RISC was in excess over target mRNA, we observed identical rates of RISC-mediated cleavage in the absence or presence of La (Figure 3-16A). In a multiple-turnover condition, in which target mRNA was in large excess, a burst of cleaved products was detected early in the reaction (single-turnover phase) followed by a significantly slower rate of target cleavage (multiple-turnover phase) in the absence of La (Figure 3-16B). Addition of La greatly enhanced the efficiency of RISC-mediated mRNA cleavage in the multiple-turnover phase (Figure 3-16B). Moreover, the slower steady-state rate of catalysis observed in the absence of La is not due to inactivation of RISC, such as by releasing the guide strand from Ago2, during incubation. Addition of La could still enhance the RISC activity even after a 20-minute pre-incubation of hAgo2-RISC with target mRNA (Figure 3-16C). Taken together, these kinetic studies strongly suggest that La promotes multiple-turnover of RISC catalysis by facilitating the release of cleaved products.

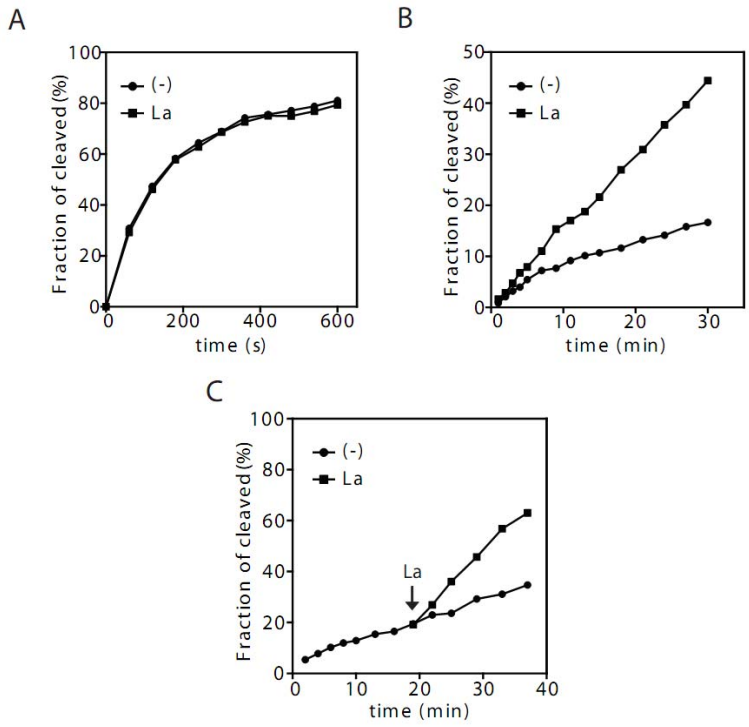


Figure 3-16 La promotes the multiple-turnover of RISC catalysis

(A) The ss-siRNA-initiated RISC assays were performed using recombinant hAgo2 in the absence or presence of hLa in a single-turnover condition (The estimated concentration of RISC was ~4nM and target mRNA was 0.2nM). **(B)** The ss-siRNA-initiated RISC assays were performed using recombinant hAgo2 in the absence or presence of hLa in a multiple-turnover condition (The estimated concentration of RISC was ~4nM and target mRNA was 60nM). **(C)** The ss-siRNA-initiated RISC assays were performed using recombinant hAgo2 in a multiple-turnover condition as described in (B). Recombinant La protein or buffer control was added nineteen minutes after the start of the reaction. Reactions were allowed to continue at room temperature for another twenty minutes.

The product release from RISC can be facilitated by weakening the interaction between the 3' end of guide strand and target mRNA (Haley and Zamore, 2004). Thus, we compared the steady-state velocities of RISC reactions in the absence and presence of La with the use of ss-let-7 siRNA carrying zero or 4-nt 3' mismatches with the target mRNA (Figure 3-17A). In support of our hypothesis, introducing mismatches at the 3' end of guide strand significantly mitigated La's RISC-enhancing effect (Figure 3-17B).

A recent study suggested that ATP was required for the multiple rounds of catalysis by RISC in *Drosophila* embryo extract (Haley and Zamore, 2004). However, we did not observe any appreciable effect of ATP on the RISC-enhancing activity of highly purified endogenous human La (Figure 3-18A). Similar robust RISC-enhancing activity was observed with the use of recombinant hLa proteins even after ATP depletion (Figure 3-18B). Although La was previously reported to possess ATPase activity (Bachmann et al., 1990), bioinformatics analysis did not detect any ATP-binding motif in *Drosophila* or human La, nor could recombinant hLa bind the ATP-Sepharose beads (Figure 3-18C). Thus, we conclude that La promotes the multiple-turnover of RISC catalysis in an ATP-independent manner.

3.7 La exhibits RNA unwinding activity and facilitates mRNA release from RISC

It was previously reported that mammalian La proteins exhibited unwinding activity toward the DNA-RNA or RNA-RNA hybrid with 5' or 3' overhangs (Bachmann et al., 1990; Huhn et al., 1997). Consistently, both recombinant and endogenous human

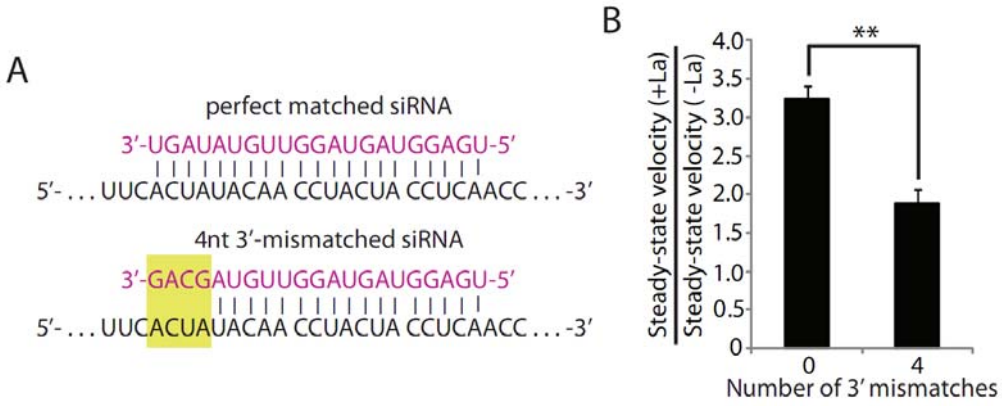


Figure 3-17 La facilitates the release of RISC cleaved products

(A) Alignment of the ss-siRNA bearing 0 or 4-nt 3' mismatches with complementary target mRNA. **(B)** A graph comparing the ratio of the steady-state velocities of minimal RISC with and without La for the two ss-siRNAs described in (A). The P-value was calculated by paired t-test.

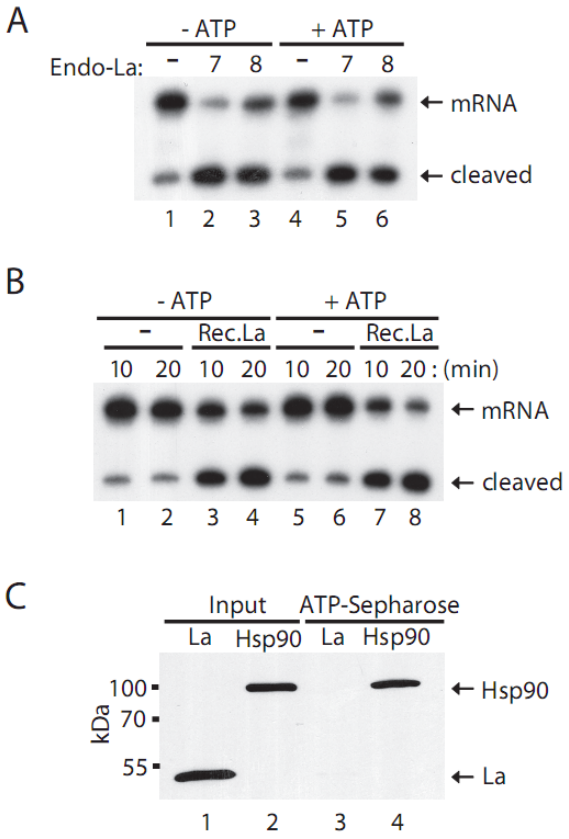


Figure 3-18 La enhances RISC activity independent of ATP

(A) The minimal hAgo2-RISC activity was assayed with highly purified endogenous La (fractions 7 and 8 in **Error! Reference source not found.**B) in the absence or presence of ATP. **(B)** La enhances RISC activity independent of ATP. The minimal hAgo2-RISC activity was assayed with highly purified recombinant La in the absence or presence of ATP. For –ATP reactions, ATP depletion was performed by incubating recombinant proteins with 1 unit of hexokinase and 20mM (final concentration) glucose at room temperature for 30 min. **(C)** ATP-Sepharose beads were used to pull-down highly purified recombinant La or Hsp90 proteins.

La proteins, but not the HIV TAR-RNA binding protein (TRBP) that functions in complex with Dicer in human RNAi pathway (Chendrimada et al., 2005; Gatignol et al., 1991; Haase et al., 2005), could unwind the duplex between radiolabelled guide strand and complementary target mRNA, and accumulate radiolabelled guide strand on the gel (Figure 3-19A). By contrast, recombinant hLa was incapable of unwinding duplex siRNA with 2-nt 3' overhang (Figure 3-19B), suggesting that long overhangs might be required for La's unwinding activity of dsRNA.

To determine if La promoted mRNA release from RISC, we loaded hAgo2-RISC with radiolabelled target mRNA and then incubated the immobilized RISC/target complex with recombinant La or TRBP proteins. Incubation with La, but not TRBP, could facilitate the release of target mRNA and cleavage products from immobilized RISC into the supernatant (Figure 3-20). This result was consistent with that La, but not TRBP, exhibited the RISC-enhancing activity (Figure 3-21). Since La could also release uncleaved mRNA from RISC, the action of La might not require Ago2 to cleave target mRNA before hand. In principle, La may increase the fidelity of mRNA cleavage and specificity of RNAi by facilitating the exchange of off-target mRNA from Ago2.

Discussion

The RNAi pathway consists of initiation, activation, and effector steps. How the effector step of RNAi is regulated is currently unknown. Here, we took a classic biochemical approach to identify autoantigen La as a novel regulator of the RNAi

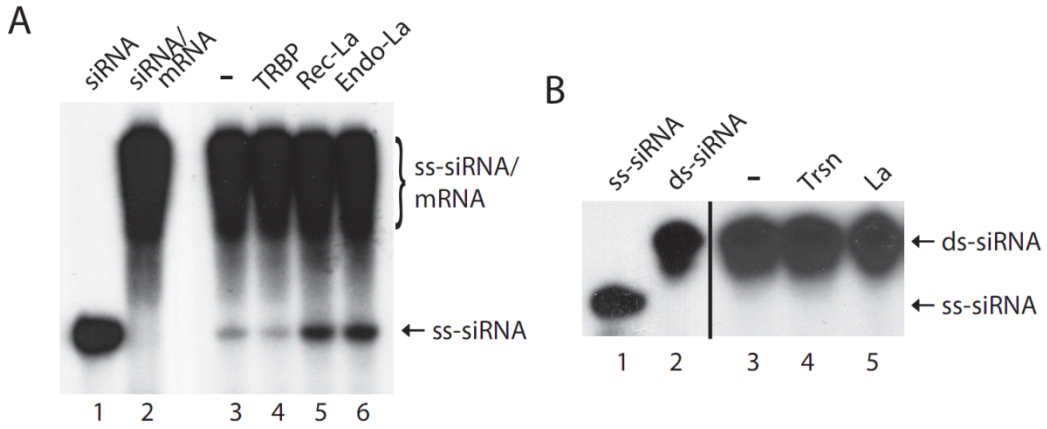


Figure 3-19 La unwinds ss-siRNA/mRNA duplex but not duplex siRNA

(A) The unwinding assays were performed by incubating radiolabelled ss-siRNA/mRNA duplex with buffer, recombinant TRBP, recombinant (Rec-La) or endogenous La (Endo-La) protein at 37 ºC for 15min. **(B)** The unwinding assays were performed by incubating radiolabelled duplex siRNA with buffer, recombinant Translin (Trsn) or La protein at 37 ºC for 30min.

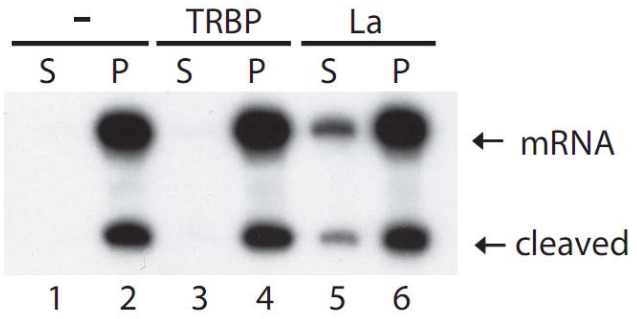


Figure 3-20 La facilitates the release of mRNA and cleavage products from active RISC

Immobilized hAgo2-RISC/target mRNA complex was incubated with buffer (lanes 1-2), TRBP (lanes 3-4) or La (lanes 5-6) to induce the release of mRNA from RISC. Supernatant (S) and beads (P) were recovered after a 5 min incubation.

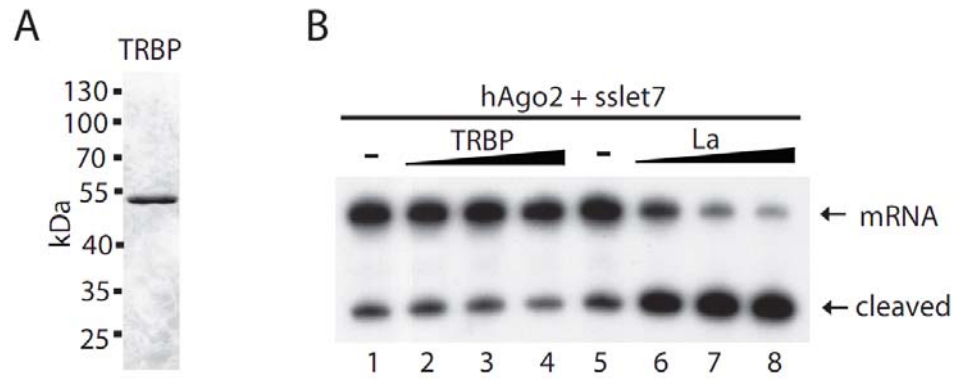


Figure 3-21 TRBP does not enhance minimal RISC activity

(A) Recombinant TRBP was purified from insect cells using a baculovirus expression system. **(B)** The minimal hAgo2-RISC activity was assayed with recombinant recombinant TRBP (lane 2-4) or hLa (lane 6-8).

effector step, i.e. La could interact with Ago2 and enhance the fly and human RISC-mediated mRNA cleavage activity. Specifically, La promoted the multiple-turnover of RISC catalysis by facilitating the release of cleaved mRNA from Ago2 (Figure 3-22). Moreover, our studies demonstrated that La played a critical role in RNAi, antiviral response, and transposon silencing in *Drosophila* and mammalian cells.

A previous study suggested that ATP was required for the multiple-turnover of RISC catalysis in *Drosophila* embryo extract (Haley and Zamore, 2004). However, we observed robust RISC-enhancing activity for highly purified endogenous or recombinant La protein in the absence or presence of ATP. It is quite possible that another ATP-dependent factor may function independently or in tandem with La to promote the multiple-turnover of RISC catalysis in *Drosophila* embryos.

We find it intriguing that the La protein could unwind ss-siRNA/target mRNA duplex although it shares no similarity to any known helicases, which typically require ATP hydrolysis to unwind DNA or RNA duplexes. While La was previously reported to possess ATPase activity (Bachmann et al., 1990), we could not observe the binding of recombinant La to ATP-Sepharose, nor detect any known ATP-binding motif in the La protein by bioinformatics analysis. We suspect that the previous experiments used partially purified La from mammalian tissues and the weak ATPase activity might be attributed to other contaminating proteins (Bachmann et al., 1990). There are a few examples of ATP-independent DNA unwindases. For example, Adenovirus single-stranded DNA binding protein (DBP) forms protein filament and the multimerization is the driving force for ATP-independent DNA unwinding (Dekker et al., 1998). In the

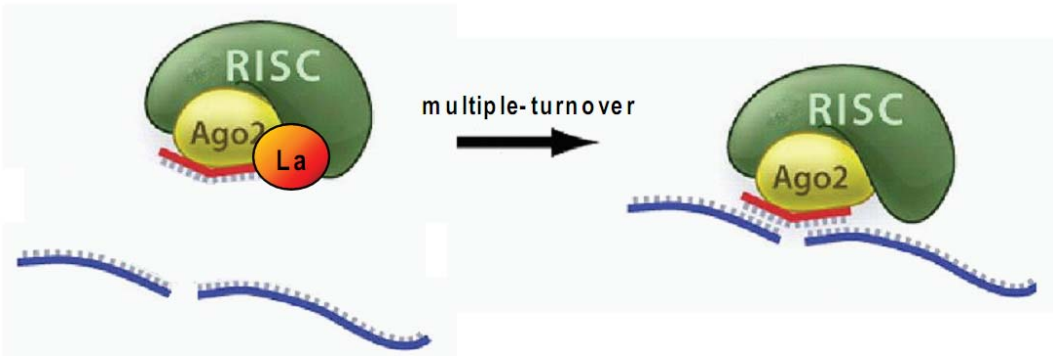


Figure 3-22 Working model for La

A schematic graph showing the working model for La. La interacts with Ago2 and promotes the multiple-turnover of RISC catalysis by facilitating the release of cleavage products. Adapted and modified by permission from Elsevier, *Cell* © 2005.

absence of ATP, binding of UL9-ICP8 protein complex can promote the unwinding of the AT-rich segment, permitting access of the replication machinery to the origin of replication of a herpes simplex virus type 1 (HSV-1) (He and Lehman, 2001). Thus, it will be of great interest to study the mechanism by which La unwinds RNA duplex.

The interaction among Ago2, siRNA, and target mRNA is a highly dynamic process in which significant conformational changes occur in both protein and RNA components (Parker; Wang et al., 2009). During the assembly of RISC, Ago2 receives duplex siRNA and nicks the passenger strand, and then C3PO activates RISC by degrading Ago2-nicked passenger strand (Liu et al., 2009; Ye et al.). During the RISC-mediated mRNA cleavage, target mRNA comes in to form a duplex with the guide RNA and Ago2 slices the target mRNA, and then La promotes the product release from RISC to initiate another round of catalysis. While these are two very similar processes, C3PO primarily functions in RISC activation because it prefers to degrade small RNA rather than long mRNA (Ye et al.)(data not shown). In contrast, La specifically promotes the RISC-mediated mRNA cleavage activity. Consistent with this function, the La protein could unwind the ss-siRNA/target mRNA duplex, but not a siRNA duplex with 2-nt 3' overhang, suggesting that long overhangs may be a prerequisite for La's unwinding activity. Thus, the substrate specificity of C3PO and La dictates that they regulate two different steps of RISC functions.

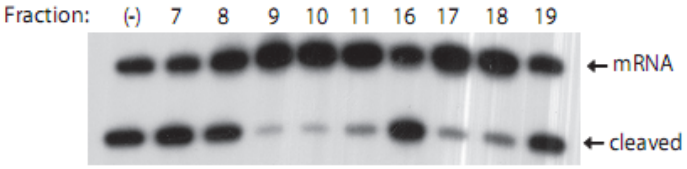
The world of RNAi is rapidly expanding, with many classes of small regulatory RNAs, including siRNA, miRNA and piRNA, playing fundamental roles in diverse biological and disease processes (Carthew and Sontheimer, 2009; He and Hannon,

2004; Kim, 2005; Liu and Paroo; Siomi and Siomi, 2009). It will be interesting to investigate the functions of La in the miRNA and piRNA pathways in the future studies. We anticipate that La may represent the first of many regulators that modulate the multiple-turnover catalysis, target recognition, or catalytic activity of the siRNA, miRNA, and piRNA effector complexes. Finally, La is an autoantigen commonly associated with the Lupus and Sjogren's syndromes (Wolin and Cedervall, 2002), these current and future studies of La may provide fresh insights into the pathogenesis of debilitating autoimmune diseases.

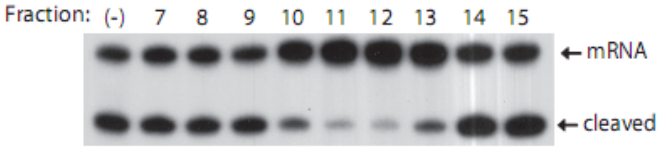
Chapter 4 Purification of *Drosophila* Yoda

The robust *in vitro* reconstitution system is a powerful platform for in-depth studies of the assembly, function and regulation of RISC. We took advantage of this system and purified a novel inhibitor of RISC which inhibits RISC cleavage activity at the effector step. After a five-step chromatographic fractionation, a ~25 kDa protein, which closely correlated with the RISC-inhibitory activity, was identified by mass spectrometric analysis (Figure 4-1). It is an unknown protein with relatively small size; therefore we decided to name it Yoda. We are currently trying to generate the recombinant Yoda protein from insect cells and reconstitute the inhibitory activity *in vitro*.

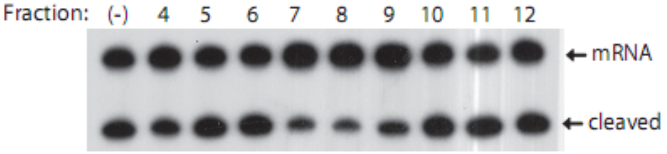
1. SP-Sepharose



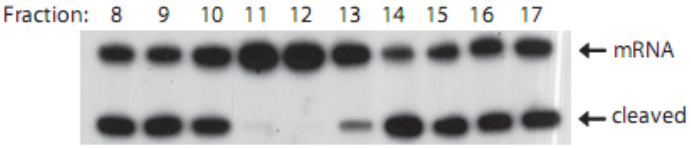
2. Phenyl



3. Q-Sepharose



4. Heparin



5. Mono S

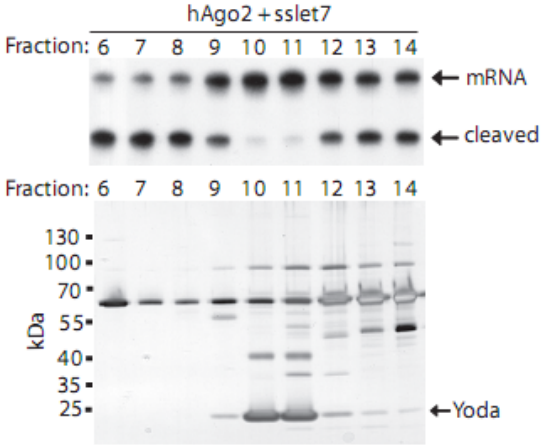


Figure 4-1 Purification of Yoda as an inhibitor of the RISC activity

Purification of Yoda through a five-step chromatographic procedure. Individual fractions were assayed with recombinant hAgo2 for the RISC activity. Two inhibitory activity peaks were revealed after the first SP column. I followed the activity in the first peak (fractions 9-11). Following the final Mono S step, individual fractions were assayed with recombinant hAgo2 for the RISC-inhibitory activity (top) or resolved by SDS-polyacrylamide gel (PAGE) followed by silver-staining (bottom).

Concluding Remarks

Our findings of La and C3PO reveal a general concept that regulatory factors are required to remove Ago2-cleaved products to assemble or restore active RISC. The Ago family of proteins may be evolutionarily designed to be important but lousy enzymes that require an entourage of co-factors to help them function efficiently. First, Dicer-2-R2D2 complex and heat shock factors help recruit nascent siRNA duplex to Ago2 to promote RISC assembly (Iki et al.; Iwasaki et al.; Liu et al., 2003; Liu et al., 2006; Miyoshi et al.; Pham et al., 2004; Tomari et al., 2004b). Second, the nucleases C3PO and QIP are needed to degrade Ago2-nicked duplex siRNA to efficiently activate RISC (Liu et al., 2009; Maiti et al., 2007; Ye et al.). Finally, after Ago2 slices the target mRNA, La facilitates the unloading of the cleaved products from Ago2 to promote the multiple-turnover of RISC catalysis. It is possible that eukaryotic cells are mindful of the negative consequences when the RNAi pathways are overly active and thus, putting in place of these regulators to carefully modulate the activities of Ago proteins. Conceptually, mutations of these key regulators of RISC may lead to malfunctions of the RNAi pathways that could potentially contribute to human diseases, such as cancer.

The robust reconstitution system establishes a powerful platform for in-depth studies of the assembly, function, regulation of RISC, the catalytic engine of RNAi. Similar to the discovery of C3PO and La, I employed it to identify Yoda as a novel inhibitor of RISC. The reconstitution system can also be used in the future to study post-translational regulations of RNAi, therefore, connecting RNAi to other cellular signaling

pathways. As such, these biomedical studies could have a major and lasting impact on the biological understanding and therapeutic applications of RNAi.

Materials and Methods

General reagents and antibodies

General RNA reagents were purchased from Ambion and Promega, isotopes were from MP Biomedicals, and siRNAs were synthesized by Dharmacon and IDT. The Ago2 antiserum was raised against a C-terminal fragment, whereas both Dcr-2 and R2D2 antibodies have been described previously (Liu et al., 2003). The Translin and Trax antibodies were a generous gift from Dr. Rafael Koch and Dr. Beat Suter. The β-tubulin antibody was purchased from Abm. The w^{1118} fly strain was used as a wild type control. The $dcr\text{-}2^{R416X}$ and $ago2^{414}$ mutant were generous gifts from Dr. Richard Carthew and Dr. Mikiko Siomi and Haru Siomi (Lee et al., 2004; Okamura et al., 2004). The *trsn* mutant (y^1w^{67c23}; P{EPgy2}translinEY06981) was obtained from the Bloomington fly stock center (Suseendranathan et al., 2007). The human La antibody was purchased from Santa Cruz Biotechnology. Tet_{on}-inducible U2OS cell line was a generous gift from Dr. Xiaodong Wang. FR1-ΔB2 construct was a generous gift from Dr. Shou-Wei Ding. Constructs for endo-siRNA sensor assay were generous gifts from Dr. Eric Lai.

Preparation of recombinant Ago2 and Dcr-2/R2D2 proteins

The cDNA of truncated Ago2 protein was amplified and cloned into pFastBac-HTC vector (Invitrogen). To produce N-terminal His-Flag-tagged Ago2 construct, an annealed oligo pair encoding 3 × Flag peptide sequence was phosphorylated by T4

polynucleotide kinase (NEB) and ligated into pFastBac-His-Ago2 construct. The catalytic mutant Ago2 (D965A) was generated by Quikchange (Stratagene). Recombinant Ago2 proteins were expressed in insect cells using the Bac-to-Bac baculovirus expression system from Invitrogen. Sf9 insect cells were infected with different Ago2 viruses. After 60 hours, the infected cells were harvested, washed in PBS, and lysed by 40 strokes with a Douncer in buffer T (20 mM Tris-Hcl, pH8.0, 50 mM Nacl, 5 mM β-mercaptoethanol) containing 20 mM imidazole with freshly added protease inhibitors, including 1 mM Pefabloc SC, 5 μg/ml Leupeptine, and 0.7 μg/ml Pepstain (Roche). Followed by an 18,000g spin at 4 ºC for 30 minutes, the supernatant was then incubated with 0.5 ml Ni-NTA beads (Qiagen) at 4 ºC for overnight, loaded onto a column, washed sequentially with buffer T containing 20 mM imidazole and buffer T with 50 mM imidazole and, eluted in buffer T containing 250 mM imidazole. For Flag-affinity purification, the Nickel column elution was directly incubated with 100 μl of Anti-Flag M2 Agarose beads (Sigma) at 4 ºC for 6 hours. The Flag beads were washed five times with buffer T and then eluted in 200 μl buffer T containing 500 ng/μl 3 × Flag peptide (Sigma). Purified recombinant Ago2 was stored in aliquots in 20% glycerol at -80 ºC. Expression and purification of His-tagged Dcr-2/R2D2 complex were carried out essentially as described (Liu et al., 2003).

Preparation of recombinant Translin and C3PO complex.

Translin cDNA alone was cloned in pET-28 vector (Novagen), whereas both Translin and Trax cDNAs were cloned in pETDuet-1 vector (Novagen). *E. coli* BL21(DE3) cells that contain the Translin or C3PO expression construct were grown in 1

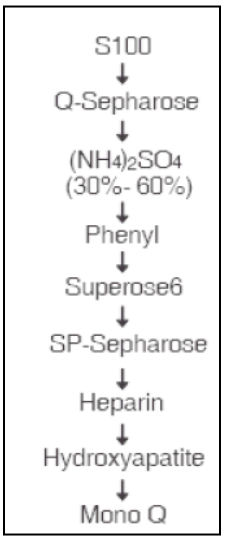
liter LB medium containing 100 μg/ml Ampicillin to OD_{600} of 0.6 at 37 ºC. Following induction by 1 mM IPTG for three hours, cells were harvested by centrifugation. The cell pellet was resuspended in buffer A (10 mM KOAc, 10 mM HEPES, pH7.4, 2 mM $Mg(OAc)_2$, 5 mM β-mecaptoethanol) with freshly added protease inhibitors and 20 mM imidazole, sonicated on ice and centrifuged at 18,000g for 30 min. The supernatant was then incubated with 1 ml Ni-NTA beads at 4 ºC for overnight, loaded onto a column, washed sequentially with buffer B (Buffer A with1 M Nacl) containing 20 mM imidazole, buffer A containing 20 mM imidazole and buffer A with 50 mM imidazole, and eluted in 250 mM imidazole buffer A. The recombinant proteins were further purified by a step elution on a Heparin-Sepharose column and followed by fractionation on a Superose 6 gel filtration column. Purified recombinant proteins were stored in aliquots at -80 ºC in 10% glycerol.

Purification of the RISC-enhancing activity (C3PO)

The *Drosophila* S2 cells (ATCC) were cultured in suspension in Drosophila-SFX media (Invitrogen), harvested at ~2 x 10^7 cells/ml, washed with PBS, and resuspended in three times pellet volume of buffer A supplemented freshly with protease inhibitors. After sitting on ice for 20 minutes, cells were broken with a Douncer with 40 strokes followed by an 18,000g spin at 4 ºC for 30 minutes. The supernatant were further centrifuged at 100,000g for one hour at 4 ºC to make the cytoplasmic (S100) extract.

All purification steps were carried out at 4 ºC. All columns were purchased from GE Healthcare except for the Hydrxyapatite column from BioRad. Approximately

100ml of S100 (~10-15 mg/ml) prepared from S2 cells were diluted two fold with buffer A and loaded in multiple runs onto 20 ml Q-Sepharose column. Following a step wash with 15%, 40%, and 100% Buffer B (Buffer A with 1M NaCl), the RISC-enhancing activity was eluted by ~0.4 M NaCl (40% buffer B) and precipitated by addition of Ammonium Sulfate to 30% saturation. After a 30-min, 18,000g spin, the supernatant was directly loaded in multiple runs onto a 5 ml Phenyl-Sepharose Hydrophobic column. The peak activity was step eluted at ~ 18% saturation ammonium sulfate, and following Ammonium sulfate precipitation at 60% saturation, the pellet was resuspended in 4 ml Buffer A, filtered, and loaded in two runs onto a 140 ml hand-packed Superose 6 gel filtration column. The peak activity flowed through a 5 ml SP-Sepharose column, and was loaded directly onto a 5 ml Heparin-Sepharose column. The peak activity was eluted at ~0.5 M NaCl after a step wash of 25%, 50%, and 100% buffer B, and directly loaded onto a 1 ml Hydroxyapatite column. The peak activity was eluted at ~13% by a 0-20% gradient wash with 1M phosphate buffer (pH7.2), and following overnight dialysis, was fractionated on a 100 µl Mono Q column on a smart HPLC machine. The peak activity was eluted at ~ 0.35 M NaCl concentration by a 20%-55% gradient wash with Buffer B.



RNAi in embryo

Microinjections of *fushi taraze* (*ftz*) siRNA or dsRNA were performed as previously described (Kennerdell and Carthew, 1998; Okamura et al., 2004). The same

region of the ftz dsRNA was PCR amplified and in vitro transcribed using RiboMax kit (Promega). The ftz siRNA were purchased from Dharmacon. dsRNA (1.2mg/ml in H2O) or siRNA (0.2 mg/ml in H2O) were injected into syncytial blastoderm embryos. The survived embryos were scored for the number of ventral denticle belts after 24-36 hours at room temperature. In principle, silencing of ftz expression results in the disappearance of odd number (A1, A3, A5, A7) belts. However, we scored a partial belt as one if it was more than half, or zero if less than half of full-length. Because A8 belt was not always clear under the dissecting microscope, we had embryos with only three belts and grouped embryos with seven belts into the "no phenotype" category. For cuticle preparation and imaging, embryos were dechorionated with 50% bleach, and rinsed with water. Dechorionated embryos were rotated for 20 minutes in fix solution (700µl of heptane, 560µl of 1X PBS, and 140µl of 37% formaldehyde). Fixed embryos were then devittelenized by methanol. Embryos were incubated at 60 ºC overnight in glycerol/acetic acid (1:4), placed on a microscope slide and covered with Hoyer's/lactic acid (1:1) medium. The slides were incubated at 60 ºC for more than six hours with a plastic scintillation vial filled with water on top before imaging.

Native siRNA gel-shift assay

The native siRNA gel-shift assays were performed as previously described using horizontal Agarose gel electrophoresis (Miyoshi et al., 2005). In brief, the RISC reactions (10 µl) were performed by incubating radiolabeled siRNA duplex with various extracts in the absence of mRNA substrate. Following a 30-min incubation at room temperature, the reactions were transferred on ice and 0.8 µl glycerol was added, different

siRNA-protein (siRNP) complexes were resolved on a pre-chilled horizontal Agarose gel at 4 ºC in the running buffer (0.5 × TBE, 1.5 mM $MgCl_2$) at 120V for 3 hours. Afterwards, the Agarose gels were dried under vacuum onto a Zeta-Probe membrane (BioRad) and directly exposed to X-ray film.

The duplex siRNA-unwinding assay

The guide or passenger strand of an asymmetric siRNA was 5'-^{32}P-radiolabeled with T4 polynucleotide kinase (PNK), purified on a 16% denaturing PAGE gel, and annealed with 1.5 fold excess of cold phosphorylated complementary strand in the annealing buffer (10 mM Tris-HCl, pH8.0, 5 mM $MgCl_2$). The siRNA-unwinding assays (10 μl) were carried out in the same buffer as the RISC assay essentially as described (Nykanen et al., 2001), stopped by addition of 200 μl 0.3 M NaOAc, phenol/chloroform extracted, ethanol precipitated with glycogen as a carrier, dissolved in water and native dye, resolved on a 16% native polyacrylamide gel and was directly exposed to X-ray film.

The passenger strand cleavage assay

Radiolabeled duplex siRNA was prepared as described above. The passenger strand cleavage assays were performed as described in the same RISC reaction condition except for the addition of the 2'-*O*-methyl oligonucleotide complementary to the passenger strand to stabilize the cleavage products (Rand et al., 2005). The 9-mer and 12-mer cleavage products were detected in separate assays using 5'-radiolabeled (by PNK with γ-^{32}P ATP) or 3'-radiolabeled (by T4 RNA ligase with ^{32}pCp) passenger strand, respectively. The reactions were stopped by addition of 200 μl 0.3 M NaOAc,

phenol/chloroform extracted, ethanol precipitated with glycogen as a carrier, resolved on a denaturing 16% polyacrylamide gel, and was directly exposed to X-ray film.

The RNase assay

Preparation of radiolabeled single-strand or duplex siRNA is described as above. The RNase assays were performed as described in the same RISC reaction condition except for the reaction time is 5 minutes. The reactions were stopped by addition of 200 µl 0.3 M NaOAc, phenol/chloroform extracted, ethanol precipitated with glycogen as a carrier, resolved on a denaturing 16% polyacrylamide gel, and was directly exposed to X-ray film.

Sequence alignment and structure prediction

The multiple sequence alignment was created by PROMALS-3D (Pei et al., 2008) using Translin and trax sequences collected from HomoloGene Release 63 (Sayers et al., 2009), and inspected for conservations contained within Translin, Trax, or both sequences. A homology model of *Drosophila* Trax was built with HHPRED (Soding et al., 2005) using the most complete *H. sapiens* Translin structure (PDB:1j1j) as a template. In the Trax model, the N- and C-terminal extensions as well as a long insert (indicated as gray dots in the **Error! Reference source not found.**structure) were removed for clarity.

Preparation of recombinant *Drosophila* and human La proteins

The cDNAs of *Drosophila* and human La proteins were amplified and cloned into pFastBac-HTC vector (Invitrogen). Recombinant La proteins were expressed in

insect cells using the Bac-to-Bac baculovirus expression system from Invitrogen. Sf9 insect cells were infected with different La viruses. After 48 hours, the infected cells were harvested, washed in PBS, and lysed by 40 strokes with a Douncer in buffer T (20 mM Tris-Hcl, pH8.0, 50 mM Nacl, 5 mM β-mercaptoethanol) containing 20 mM imidazole with freshly added protease inhibitors, including 1 mM Pefabloc SC, 5 μg/ml Leupeptine, and 0.7 μg/ml Pepstain (Roche). Followed by an 18,000g spin at 4 ºC for 30 minutes, the supernatant was then incubated with Ni-NTA beads (Qiagen) at 4 ºC for overnight, loaded onto a column, washed sequentially with 1M Nacl buffer T containing 20mM imidazole, buffer T containing 20 mM imidazole and buffer T with 50 mM imidazole and, eluted in buffer T containing 250 mM imidazole. Purified recombinant proteins were stored in aliquots at -80 ºC in 10% glycerol.

Minimal RISC assay

The human Ago2 recombinant protein was incubated first with single-stranded let-7 at 37 ºC for 5min to form a minimal RISC. A 5'G-cap labeled 300 nt RNA containing a perfect let-7 target site was then added into reaction as target RNA. After incubating at 37 ºC for the indicated time, the reactions were stopped by addition of 200 μl 0.3 M NaOAc, phenol/chloroform extracted, ethanol precipitated with glycogen as a carrier, resolved on a denaturing 6% polyacrylamide gel, and was directly exposed to X-ray film.

Purification of the RISC-enhancing activity (La)

The pellet of HeLaS3 cells were purchased from National Cell Culture Center (NCCC). Cells were grown in log phase and harvested at the density of ~$0.5x10^6$ cells/ml. Cells were then collected via centrifugation at 2500g, followed by two washes in cold PBS, and resuspended in four times pellet volume of human lysis buffer (1M HEPES pH7.4, 2M KCl, 1M $MgCl_2$, 0.5M EDTA, 0.5M EGTA, 1M DTT) supplemented freshly with protease inhibitors. After sitting on ice for 30 minutes, cells were broken with a Douncer with 40 strokes followed by an 18,000g spin at 4 ºC for 30 minutes. The supernatant were further centrifuged at 100,000g for one hour at 4 ºC to make the cytoplasmic (S100) extract.

All purification steps were carried out at 4 ºC. All columns were purchased from GE Healthcare. Approximately 40ml of S100 (~4-6 mg/ml) prepared from HeLa cells were loaded in two runs onto 20 ml Q-Sepharose column. Following a step wash with 15%, 30%, and 100% Buffer B (10 mM KOAc, 10 mM HEPES, pH7.4, 2 mM $Mg(OAc)_2$, 1M NaCl, 2.5mM DTT), the RISC-enhancing activity was eluted by 1 M NaCl (100% buffer B) and precipitated by addition of Ammonium Sulfate to 30% saturation. After a 30-min, 18,000g spin, the supernatant was directly loaded in two runs onto a 1 ml Phenyl-Sepharose Hydrophobic column. The peak activity was gradient eluted at ~ 14% saturation ammonium sulfate, and following overnight dialysis, was fractionated on a 100 µl Mono S column on a micro FPLC machine. The peak activity was eluted at ~ 0.52 M NaCl concentration by a 30%-100% gradient wash with Buffer B.

RNA releasing assay

Recombinant His-FLAG-hAgo2 was incubated with anti-Flag M2 magnetic beads at 4 ºC for 1 hour. Beads were then extensively washed with Buffer A and loaded with ss-siRNA and its target at 37 ºC for 10min. After extensive wash, beads were then incubated with buffer alone, or with different recombinant proteins at 37 ºC for 5min. Supernatant was saved and the beads were washed again by Buffer A. 200 µl 0.3 M NaOAc was added to supernatant or beads. After phenol/chloroform extraction, ethanol precipitation with glycogen as a carrier, RNA was resolved on a native 6% polyacrylamide gel, dried under vacuum onto a filter paper, and exposed to X-ray film.

The duplex RNA unwinding assay

The single-stranded siRNA was 5'-^{32}P-radiolabeled with T4 polynucleotide kinase (PNK), purified on a 16% denaturing PAGE gel, and annealed with 1.5 fold excess of cold complementary target RNA which is used in the RISC assay in the annealing buffer (10 mM Tris-HCl, pH8.0, 5 mM $MgCl_2$). The duplex RNA unwinding assays (10 µl) were carried out in the same buffer as the RISC assay essentially as described (Nykanen et al., 2001), stopped by addition of 200 µl 0.3 M NaOAc, phenol/chloroform extracted, ethanol precipitated with glycogen as a carrier, dissolved in native dye, resolved on a 16% native polyacrylamide gel and was directly exposed to X-ray film.

In vivo co-immunoprecipitation and western blotting

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin

(Invitrogen) in a 5% CO2 atmosphere at 37^0C. FLAG-tagged hLa and/or Myc-tagged hAgo2 vectors were transfected with lipofectamin2000 (Invitrogen) following the recommended procedure. After 48 hours, cells pellet were resuspended in Hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl , 2 mM EDTA ,0.1% CHAPS) supplemented with fresh protease inhibitors. After sitting on ice for 30min, cells were lysed by passthrough a 22 gauge syringe for 40 times. Lysate containing the same amount of protein was incubated with 20µl of anti-Flag M2 magnetic beads (Sigma) or 20µl of protein A agarose beads mixed with 1µg of anti-Myc antibody overnight at 4 °C. After incubation, the beads were extensively washed for four times in wash buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% CHAPS). The precipitated proteins were eluted by boiling in 2x SDS sample buffer for 10 min, loaded onto SDS-PAGE and transferred onto Nitrocellulose membrane (Bio-Rad). After blocked with 5% nonfat milk, the membrane was probed with the designated first and second antibodies and developed with the enhanced chemiluminescence method (Perkin Elmer) and visualized by X-ray film.

In vitro co-immunoprecipitation and western blotting

Purified recombinant proteins were incubated in a 20µl reaction under RISC assay condition with or without siRNA at 30 °C for 30 min. The reaction mixtures were then incubated with 20 µl of anti-Flag M2 magnetic beads in 500 µl RISC buffer (100 mM KOAc, 10 mM HEPES at pH 7.4, 2 mM $Mg(OAc)_2$, and 5 mM DTT) at 4 °C overnight. After incubation, the beads were extensively washed four times for 5 min in wash buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% CHAPS). The precipitated proteins were eluted by boiling in 2x sample buffer and followed by Western blotting.

Luciferase reporter assay in mammalian cells

$2.5x10^4$ mammalian cells were plated into each well of a 24 well plate. Following the manufacturer's instruction, different siRNAs (eg. siGFP, siLa and siAgo2) were mixed together with lipofectamine RNAiMax (Invitrogen) in100 µl opti-MEM (Sigma). After incubating at room temperature for 15min, the transfection mixtures were added to cells. Three days after transfection, siRNAs were co-transfected again together with psiCHECK2 vector (Promega) and control siGFP, or siRNA against *Renilla* luciferase into cells using lipofectamine2000. 24 hours after transfection, cells were lysed and subjected to the dual luciferase assay (Promega). We perform triplicate transfections for each experiment.

RNA silencing of endogenous Mcl-1

Transgenic U2OS cell line which generates short hairpin RNAs to repress endogenous Mcl-1 under tetracycline treatment was a generous gift from Dr. Xiaodong Wang. $4x10^5$ cells were plated into each well of a 6 well plate. Following the manufacturer's instruction, different siRNAs (eg. siGFP, siLa and siAgo2) were mixed together with lipofectamine RNAiMax (Invitrogen) in 200 µl opti-MEM (Sigma). After incubating at room temperature for 15min, the transfection mixtures were added to cells. Three days later, same transfection was performed again. Doxycycline was added to the medium at the same time to induce the expression of short hairpin RNAs. 24 hours after induction, cells were harvested and washed once with cold PBS. Cell pellets were then resuspended in 30 µl of the lysis buffer (10 mM KOAc, 10 mM HEPES, pH7.4, 2 mM

$Mg(OAc)_2$, 2.5mM DTT, 1% TritonX-100) with freshly added protease inhibitors. After sitting on ice for 15min, lysates were spun at 13,000g for 5min at 4 °C and protein concentrations were measured in the supernatants. Same amount of proteins in each group of lysate were loaded onto SDS-PAGE and subjected to western blotting to detect the protein level of endogenous Mcl-1.

Viral RNA expression

pFR1-ΔB2 was a generous gift from Dr. Shou-Wei Ding. Detection of viral RNA expression was performed essentially as described before (Li et al., 2004). S2R(+) cells were adapted and cultured in serum-free SFX insect cell medium (Hyclone) at 25°C. $1x10^6$ cells were plated into a 12-well format and 300ng pFR1-ΔB2 plus 300ng different dsRNAs (eg. dsFluc, dsAgo2 and dsLa) were transfected using cellfectin reagent (Invitrogen) following manufacturer's instruction. Viral RNA transcription was induced by adding 0.5M $CuSO_4$ into medium. Two days after induction, cells were harvested and total RNA was extracted by Trizol reagent (Invitrogen). 6μg RNA samples were then analyzed by Northern blot (NorthernMax kit, Ambion). DNA fragments corresponding to the FHV or rp49 were labeled as probe using Rediprime II random prime labeling system (Amersham BioSciences). RNA quantification from Northern blot was carried out using phosphor-Imager.

Sensor assays for endo-siRNA

Constructs for the endo-siRNA sensor assays are generous gift from Dr. Eric Lai. Luciferase sensor assays were performed essentially as described before (Okamura et

al., 2008). $6x10^5$ S2 cells were plated in 24-well plate format. 200ng of different dsRNAs (eg. dsGFP, dsAgo2 and dsLa) were transfected into cells using cellfectin reagent. Two days after dsRNA transfection, cells were resuspended and re-plated in triplicate into 48-well plate. 25ng target, 12.5ng ub-Gal4, and 25ng UAS-DsRed-hpRNA plasmids were transfected into cells. Cells were then lysed and subjected to dual luciferase assay two days after the second transfection.

Quantitative real-time PCR

$3x10^6$ S2R(+) cells were soaked in 1ml serum-free SFX medium containing 15μg dsRNAs in 6-well plate. Cells were treated with dsRNAs for every two days. Five or eight days after initial dsRNA treatment, cells were harvested and total RNA was extracted using Trziol reagent. RNA was then treated with DNase (Invitrogen) and subjected to reverse transcription by SuperScript III Reverse Transcriptase and random primers (Invitrogen). Quantitative real-time PCR was carried out using SYBR GREEN PCR Master Mix (Applied Biosystems). Quantification of transposons was normalized to ribosomal protein rp49 and relative expression levels were calculated as described previously (Bookout and Mangelsdorf, 2003). Primers for rp49 and transponsons analysis are chosen from previous literature (Czech et al., 2008; Ghildiyal et al., 2008).

ATP-binding assay

1μg of highly purified recombinant His-La or His-Hsp90 proteins were incubated with ATP-sepharose beads at 4°C for 2 hours in buffer A containing 1% BSA. After extensive wash with buffer A containing 200mM NaCl, 30μl of 2X SDS loading

buffer was added to the beads. Protein samples were boiled and resolved on the SDS-PAGE gel, and followed by western blotting using anti-His antibody.

Purification of the RISC-inhibitory activity (Yoda)

The *Drosophila* S2 cells (ATCC) were cultured in suspension in Drosophila-SFX media (Invitrogen), harvested at ~2 x 10^7 cells/ml, washed with PBS, and resuspended in three times pellet volume of buffer A supplemented freshly with protease inhibitors. After sitting on ice for 20 minutes, cells were broken with a Douncer with 40 strokes followed by an 18,000g spin at 4 ºC for 30 minutes. The supernatant were further centrifuged at 100,000g for one hour at 4 ºC to make the cytoplasmic (S100) extract.

All purification steps were carried out at 4 ºC. All columns were purchased from GE Healthcare except for the Hydrxyapatite column from BioRad. Approximately 50ml of S100 (~10-15 mg/ml) prepared from S2 cells were diluted two fold with buffer A and loaded in multiple runs onto 5 ml SP-Sepharose column. Following a gradient wash, the RISC-inhibitory activity was eluted by ~0.4 M NaCl (40% buffer B) and precipitated by addition of Ammonium Sulfate to 30% saturation. After a 30-min, 18,000g spin, the supernatant was directly loaded in multiple runs onto a 1 ml Phenyl-Sepharose Hydrophobic column. The activity was eluted by a 10%-50% buffer C (buffer A with 30% ammonium sulfate) gradient. The peak activity was dialyzed over night in buffer A and loaded onto a 1 ml Heparin-Sepharose column. The peak activity was eluted by a gradient wash of 20%-55% buffer B. After dialyzed in buffer A for three hours, the peak

activity was fractionated on a 100 µl Mono S column on a smart HPLC machine. The peak activity was eluted by a 5%-40% gradient wash with buffer B.

Table 2 Primer and siRNA sequences

Primers for quatitative RT-PCR

Detects	Forward primer, Reverse primer
RP49	ATGACCATCCGCCCAGCATAC, CTGCATGAGCAGGACCTCCAG
ZAM	ACTTGACCTGGATACACTCACAAC, GAGTATTACGGCGACTAGGGATAC
BEL1	ATTATACAAACGCCCAATTGCCAAAA, TCCGATGAAGCTGCAGACAAATAAGA
Gypsy6	GACAAGGGCATAACCGATACTGTGGA, AATGATTCTGTTCCGGACTTCCGTCT
DOC	TACCTTAAACAAACAAACATGCCCACC, TTTGTATGGGTGGTCAGCTTTTCGT
DM297	AAAGGGCGCTCATACAAATC, TGTGCACATAAAATGGTTCG
Idefix	ATGGCAGTCCCACAACTCTC, TTGTTCCACTTGGTTGACGA
DM412	AAAGTACGGTCCAATGAAGACG, GTGGTGATGAGCTGTTGATGTT

Primers for generating dsRNA

gene name	Primer sequence
dAgo2 #1 Forward	GGTAATACGACTCACTATAGGACCTTGACCTGTCCAAAA
dAgo2 #1 Reverse	GGTAATACGACTCACTATAGGGTAGTTGCGACTGTGGAA
dAgo2 #2 Forward	GGTAATACGACTCACTATAGGAATTTTGACGCAATGCAT
dAgo2 #2 Reverse	GGTAATACGACTCACTATAGGGACAATCGTTCGCTTTGC
dLa Forward	GCTAATACGACTCACTATAGGGGGCCTACATTGAGTTCGCCAAGGGCG
dLa Reverse	GCTAATACGACTCACTATAGGGGCCGTGGCGCTTGCGATTGAAGCG

Dharmacon siRNA oligos

gene name	Target sequence
siLa #1	GGUCGUAGAUUUAAAGGAA
siLa #2	GGUUAGAAGAUAAAGGUCA

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