creasing the coefficient of variation of normalized link strength. This indicates that large food webs follow qualitatively different rules than smaller webs (*16*) and suggests that extreme link strengths should be rarer in larger food webs. Further, we found a power law for the scaling of food-web stability with species number and connectance and identified two topological rules governing food-web stability: For a given number of species and links, food-web stability is enhanced when (i) species at high trophic levels feed on multiple prey species and (ii) species at intermediate trophic levels are fed upon by multiple predator species. This pattern, with generalist apical predators preying upon intermediate specialist predators, is often encountered in empirical food webs (*7*, *11*, *14*, *15*) and is consistent with reported effects of allometric degree distributions (*15*) and of top predators connecting otherwise separate energy channels (*14*). In comparison with previous results, our study offers more predictive specificity based on a wider ensemble of models, which enhances confidence in the universality of the reported rules. Perhaps most importantly, the GM approach used here has much potential for addressing a large class of related questions.

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Materials and Methods
Figs. S1 and S2
Table S1
References

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C3PO, an Endoribonuclease That Promotes RNAi by Facilitating RISC Activation

Ying Liu,[1] Xuecheng Ye,[1] Feng Jiang,[1] Chunyang Liang,[1] Dongmei Chen,[2] Junmin Peng,[2] Lisa N. Kinch,[1,3] Nick V. Grishin,[1,3] Qinghua Liu[1]*

The catalytic engine of RNA interference (RNAi) is the RNA-induced silencing complex (RISC), wherein the endoribonuclease Argonaute and single-stranded small interfering RNA (siRNA) direct target mRNA cleavage. We reconstituted long double-stranded RNA– and duplex siRNA–initiated RISC activities with the use of recombinant *Drosophila* Dicer-2, R2D2, and Ago2 proteins. We used this core reconstitution system to purify an RNAi regulator that we term C3PO (component 3 promoter of RISC), 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.

RNA interference (RNAi) is posttranscriptional gene silencing initiated by Dicer, a ribonuclease (RNase) III that processes double-stranded RNA (dsRNA) into 21- to 22-nucleotide (nt) small interfering RNA (siRNA) (*1*–*3*). 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 (*1*–*3*). A minimal RISC can be reconstituted with recombinant Ago2 and single-stranded siRNA, but not duplex siRNA (*4*), which suggests that additional factors are required for loading nascent siRNA onto Ago2. In *Drosophila*, Dicer-2 (Dcr-2) and R2D2 coordinately recruit duplex siRNA to Ago2 to promote RISC assembly (*5*–*7*). Moreover, the Dcr-2–R2D2 complex senses thermodynamic asymmetry of siRNA and facilitates the guide strand selection (*8*). It remains unclear as to what constitutes holo-RISC, how RISC is assembled, and how RISC is regulated. These outstanding questions can be effectively addressed using a classic biochemical fractionation and reconstitution approach.

We took a candidate approach to reconstituting the core RISC activity with the use of recombinant Dcr-2, R2D2, and Ago2 proteins, all of which are essential for *Drosophila* RISC assembly (*6*, *7*, *9*). Besides PAZ and PIWI domains, *Drosophila* Ago2 carries a long stretch of N-terminal polyglutamine (Q) repeats that are absent in most Ago proteins. We generated an active truncated His-Flag–tagged Ago2 that removes most polyQ repeats and fully restores duplex siRNA–initiated RISC activity in *ago2* mutant lysate (fig. S1) (*9*). Furthermore, purified recombinant Dcr-2–R2D2 and Ago2 proteins could successfully reconstitute long dsRNA– and duplex siRNA–initiated RISC activities (Fig. 1A). The RISC activity was abolished when using catalytic mutant Ago2 (Fig. 1A), indicating that Ago2 was responsible for mRNA cleavage in this reconstituted system.

However, recombinant Dicer-2–R2D2 and Ago2 generated lower RISC activities than did S2 extract (Fig. 1A), which suggests that additional factors are required to achieve maximal RISC activity. Therefore, we used this core reconstitution system to search for new RISC-enhancing factors. We found that mild heat treatment (HI, 37°C for 30 min) abolished the RISC activity in S2 extract (fig. S2) and that addition of $S2^{HI}$ extract greatly enhanced the RISC activity of recombinant Dicer-2–R2D2 and Ago2 (Fig. 1B), which suggested the existence of an RNAi activator. We named this factor C3PO (component 3 promoter of RISC) because this is the third component besides Dcr-2 and R2D2 that promotes RISC activity.

We used a seven-step chromatographic procedure to purify C3PO from S2 extract. At the final step, two proteins, ~27 kD and ~37 kD, showed close correlation with the RISC-enhancing activity (Fig. 1C). They were identified by mass spectrometry as the evolutionarily conserved Translin, also known as testis-brain RNA binding protein (TB-RBP), and Translin-associated factor X (Trax). Translin is a single-stranded DNA and RNA binding protein that copurifies with siRNA after cross-linking by ultraviolet light (*10*, *11*), whereas Trax has sequence similarity to and interacts with Translin (*12*). Consistently, recombinant C3PO complex, but not Translin, greatly enhanced the RISC activity of recombinant Dicer-2–R2D2 and Ago2 (Fig. 1D and fig. S3A). Maximal RISC activity was obtained only when Dcr-2–R2D2, C3PO, and Ago2 were present (fig. S3B).

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 (Fig. 2A) (*11*), which suggests that Trax is unstable

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TRANSLIN和TRAX结合成C3PO复合体才能提高RISC效应而单独的TRANSLIN并不能明显作用。

without Translin. By contrast, the core RNAi components (i.e., Dcr-2, R2D2, and Ago2) remained at wild-type levels in *trsn* mutant lysate (Fig. 2A). Whereas siRNA-generating activity was slightly higher in the mutant lysate (fig. S4), duplex siRNA–initiated RISC activity was at ~25% of wild-type level in *trsn* extract (Fig. 2B), and this defect was rescued by adding recombinant C3PO (Fig. 2C). Thus, C3PO is required for optimal RISC activity in vitro.

To determine whether C3PO is required for 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 (*9*). After injection with *ftz* siRNA, more than 80% of wild-type embryos displayed a severe segmentation phenotype, whereas a substantial portion of *trsn* mutant embryos showed mild or no phenotype (Fig. 2, D and E). A similar phenomenon was observed with *ftz* dsRNA injection (Fig. 2F). These experiments indicate that C3PO is required for efficient RNAi in vivo.

To distinguish whether C3PO enhances RISC assembly or activity, we compared the amount of RISC activity generated by recombinant Dcr-2–R2D2 and Ago2 with C3PO added before or after RISC assembly (fig. S3C). In both cases, C3PO could enhance the core RISC activity; however, the RISC-enhancing effect was greatly diminished when C3PO was added late to preassembled RISC (fig. S3D). Therefore, we conclude that C3PO primarily promotes RISC activation but also enhances RISC-mediated target cleavage. Consistent with the latter, C3PO modestly enhanced single-stranded siRNA–initiated RISC activity (fig. S3E).

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 (*6*, *9*, *13*), 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 (*6*, *13*). Neither RLC nor RISC could form in *dcr-2* mutant extract, whereas only RISC was absent in *ago2* mutant extract (Fig. 3A). By contrast, all three siRNP complexes could form in *trsn* mutant extract, but the amount of RISC was much less than that in wild-type extract (Fig. 3A). These results suggest that C3PO facilitates the transition from RLC to active RISC.

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 by means of the siRNA-unwinding assay (*14*). In the reconstitution system, recombinant C3PO enhanced the siRNA-unwinding activity of Dcr-2–R2D2 and Ago2 (fig. S5A). Conversely, the efficiency of siRNA unwinding was lower in *trsn* mutant extract than in wild-type control extract (fig. S5B). Both results indicate that C3PO promotes siRNA unwinding and RISC activation.

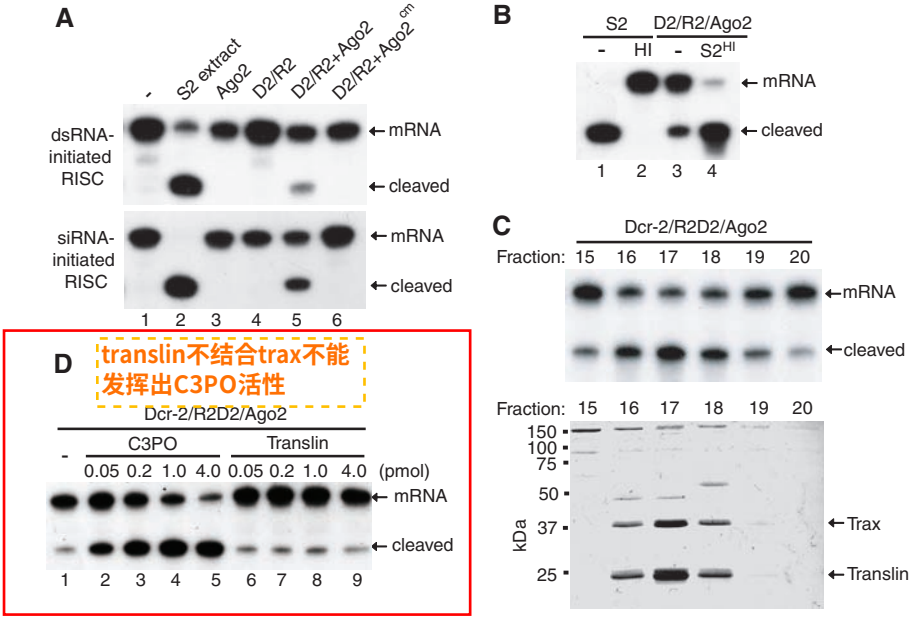

Fig. 1. Purification of C3PO as a RISC activator. (**A**) The dsRNA- and siRNA-initiated RISC assays were performed in buffer, S2 extract, recombinant Ago2, Dcr-2–R2D2 (D2/R2) complex, and D2/R2 plus wild-type or catalytic mutant (cm; $Asp^{965} \rightarrow Ala$) Ago2. All siRNA-initiated RISC assays used duplex siRNA except as noted. (**B**) The siRNA-initiated RISC assays were performed with untreated or heat-inactivated (HI) S2 extract or with recombinant Dcr-2–R2D2 and Ago2 in the absence or presence of $S2^{HI}$ extract. (**C**) Purification of C3PO through a seven-step chromatographic procedure. After the final Mono Q step, individual fractions were assayed with recombinant Dcr-2–R2D2 and Ago2 for the RISC-enhancing activity (top) or resolved by SDS–polyacrylamide gel electrophoresis followed by colloidal staining (bottom). (**D**) The siRNA-initiated RISC assays were performed using recombinant Dcr-2–R2D2 and Ago2 alone or with increasing amounts of recombinant C3PO or Translin.

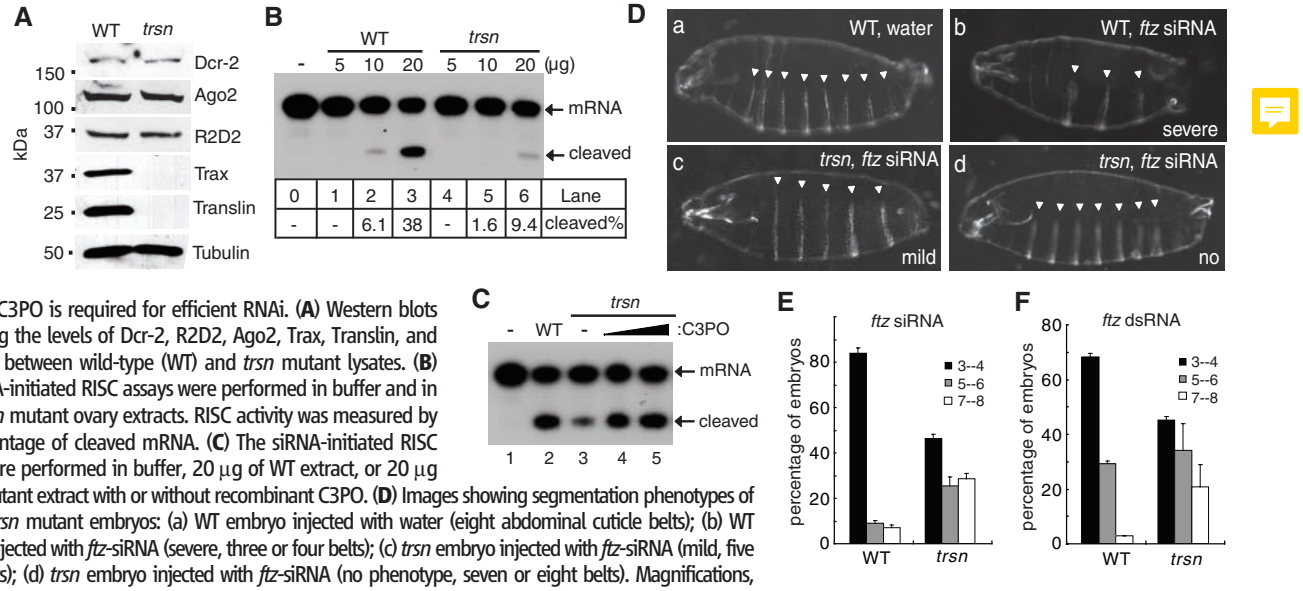

Fig. 2. C3PO is required for efficient RNAi. (**A**) Western blots comparing the levels of Dcr-2, R2D2, Ago2, Trax, Translin, and β-tubulin between wild-type (WT) and *trsn* mutant lysates. (**B**) The siRNA-initiated RISC assays were performed in buffer and in WT or *trsn* mutant ovary extracts. RISC activity was measured by the percentage of cleaved mRNA. (**C**) The siRNA-initiated RISC assays were performed in buffer, 20 μg of WT extract, or 20 μg of *trsn* mutant extract with or without recombinant C3PO. (**D**) Images showing segmentation phenotypes of WT and *trsn* mutant embryos: (a) WT embryo injected with water (eight abdominal cuticle belts); (b) WT embryo injected with *ftz*-siRNA (severe, three or four belts); (c) *trsn* embryo injected with *ftz*-siRNA (mild, five or six belts); (d) *trsn* embryo injected with *ftz*-siRNA (no phenotype, seven or eight belts). Magnifications, 100×. (**E** and **F**) Graphs showing distribution of WT or *trsn* mutant embryos with severe, mild, or no phenotype after injection of *ftz* siRNA [(E), $n > 100$] or *ftz* dsRNA [(F), $n > 150$].

To study the relative contribution of different RISC activation mechanisms, we supplemented $S2^{HI}$ extract, which displayed no siRNA-unwinding activity due to Ago2 inactivation (figs. S2 and S6B), with recombinant wild-type or catalytic mutant Ago2. Only wild-type, but not mutant, Ago2 could effectively rescue siRNA unwinding in $S2^{HI}$ extract (fig. S6C). This result, together with previous studies (*15*–*17*), strongly supports the idea that the catalytic activity of Ago2 is indispensable for siRNA unwinding and RISC activation.

In the "slicer" model, Ago2 cleaves the passenger strand of siRNA into 9- and 12-nt fragments that simply melt away because of low binding energy, leaving the guide strand behind to form an active RISC with Ago2. By passenger strand cleavage assay (*15*, *17*), we observed that both 9- and 12-nt fragments displayed a longer half-life in *trsn* mutant extract than in wild-type extract (Fig. 3, B to E, and fig. S7). Moreover, addition of C3PO complex, but not Translin, resulted in rapid degradation of the 9-nt fragment in the reconstituted system (fig. S8A). Together, these findings suggest that C3PO promotes RISC activation by removing siRNA passenger strand cleavage products.

In further support of this idea, recombinant C3PO displayed potent RNase activity toward single-stranded siRNA but showed little or no activity toward double-stranded siRNA or single-stranded DNA (Fig. 4A and fig. S8, B and C). The RNase activity of C3PO is Mg^{2+}-dependent and could be blocked by EDTA, but not by EGTA (Fig. 4B). In addition, C3PO acts as an endonuclease, because it could degrade circular as well as linear RNA (Fig. 4C). Moreover, the endogenous C3PO complex closely correlated with the RISC-enhancing activity as well as a single-stranded RNase activity after sequential chromatography (Fig. 4D and fig. S8D).

Neither subunit of C3PO shows similarity to any known RNase by bioinformatics and structural analyses (*18*). To identify the catalytic sites of C3PO, we performed a multisequence alignment of Translin and Trax and observed three acidic residues (Glu^{123}, Glu^{126}, and Asp^{204}) that were invariant in Trax but missing in all Translin (Fig. 5A and fig. S9). Furthermore, modeling the structure of *Drosophila* Trax after the crystal structure of human Translin (*18*) revealed that these residues existed in close spatial proximity, which suggests that they may coordinate Mg^{2+} for catalysis (Fig. 5A).

To test this hypothesis, we individually mutated the Glu^{123}, Glu^{126}, and Asp^{204} residues of Trax to alanine. Recombinant mutant C3PO complexes were generated by coexpressing His-tagged wild-type Translin and untagged mutant Trax (fig. S10A). These point mutations did not affect protein folding or complex formation, because wild-type and mutant C3PO displayed the same column behaviors and bound single-stranded siRNA equally well in a noncleaving condition (fig. S10B). Consistently, mutating each putative catalytic residue abolished the RNase activity and the RISC-enhancing activity of C3PO (Fig. 5, B and C, and fig. S10C). By contrast, mutation of other highly conserved Asp or Glu residues in Translin or Trax did not significantly affect the RNase or RISC-enhancing activity of C3PO (fig. S10, D and E). These studies suggest that the intrinsic RNase activity of C3PO is required for its RISC-enhancing activity.

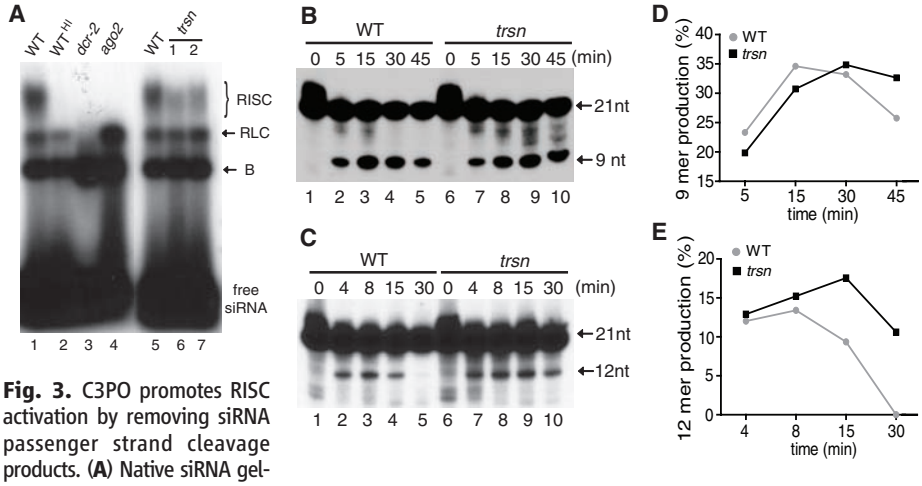

Fig. 3. C3PO promotes RISC activation by removing siRNA passenger strand cleavage products. (**A**) Native siRNA gel-shift assays were performed using radiolabeled *let-7* siRNA with untreated or heat-inactivated (HI) WT, *dcr-2*, or *ago2* mutant ovary extract (lanes 1 to 4), or 40 μg of WT and two preparations of *trsn* mutant ovary extract (lanes 5 to 7). (**B** and **C**) Passenger strand cleavage assays were performed with 40 μg of WT or *trsn* mutant extract. The 9- or 12-nt cleavage products were detected separately using siRNA whose passenger strand was radiolabeled at the 5′ (B) or 3′ (C) end. (**D** and **E**) The data in (B) and (C) were converted into graphs illustrating different stability of the 9- and 12-nt oligomers in WT and *trsn* extracts.

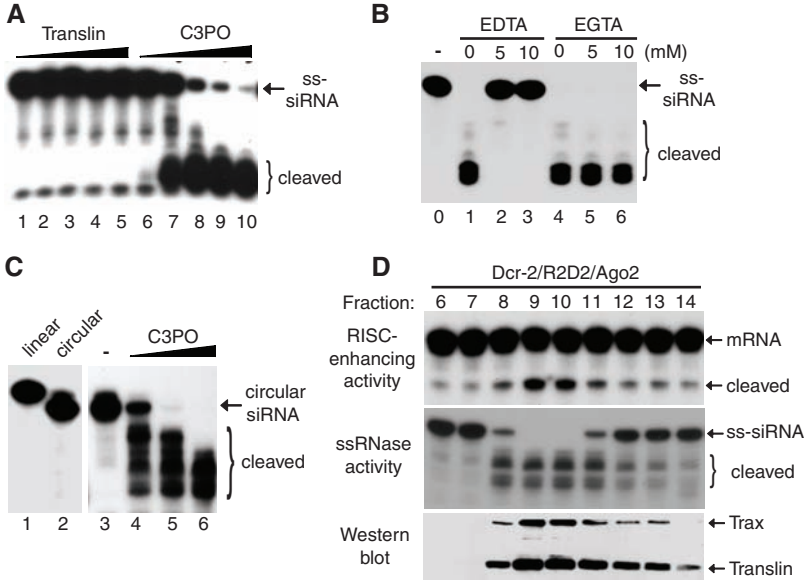

Fig. 4. C3PO is a Mg^{2+}-dependent endoribonuclease. (**A**) 5′-radiolabeled single-stranded (ss) siRNA was incubated with increasing amounts of recombinant Translin or C3PO complex. (**B**) RNase assays were performed with recombinant C3PO in the absence or presence of EDTA or EGTA. (**C**) RNase assays were conducted by incubating circular single-stranded siRNA with increasing amounts of recombinant C3PO. (**D**) After sequential chromatography, fractions were assayed for RISC-enhancing activity (top), RNase activity (middle), or Western blotting to detect Translin and Trax (bottom).

Our in vitro reconstitution system for dsRNA- and duplex siRNA–initiated RISC activities has revealed that Dcr-2–R2D2 and Ago2 constitute the catalytic core of *Drosophila* RNAi. This reconstitution system enables us to identify C3PO, a multimeric complex of Translin and Trax, as a key activator of the core RNAi machinery. Our biochemical studies indicate that the "slicer" mechanism plays a dominant role in *Drosophila* RISC assembly. C3PO, a Mg^{2+}-dependent endoribonuclease for which Trax is the catalytic subunit, promotes RISC activation by removing siRNA passenger strand cleavage products. The RNase activity of C3PO may be stimulated by Ago2-mediated nick in duplex siRNA and/or fraying of the ends of cleavage products. The exonuclease QIP may function in a similar manner in *Neurospora crassa* (*19*). This robust and progressive reconstitution system should greatly facilitate in-depth mechanistic studies of the assembly, function, and regulation of holo-RISC, the catalytic engine of RNAi.

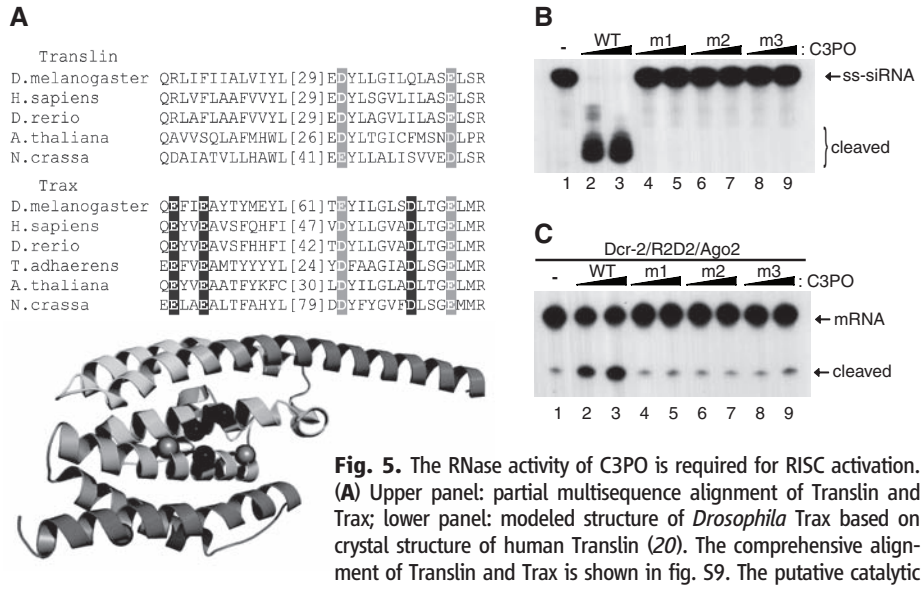

Fig. 5. The RNase activity of C3PO is required for RISC activation. (**A**) Upper panel: partial multisequence alignment of Translin and Trax; lower panel: modeled structure of *Drosophila* Trax based on crystal structure of human Translin (*20*). The comprehensive alignment of Translin and Trax is shown in fig. S9. The putative catalytic Asp and Glu residues are in black; two other highly conserved Glu residues are in gray. (**B**) RNase activity was compared between WT and each of three catalytic mutant C3PO complexes: m1 (Glu^{123} → Ala), m2 (Glu^{126} → Ala), and m3 (Asp^{204} → Ala). (**C**) RISC-enhancing activity was compared between WT and the three catalytic mutant C3PO complexes by assaying together with recombinant Dcr-2–R2D2 and Ago2.

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20. Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S10
References

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Effects of Antibiotics and a Proto-Oncogene Homolog on Destruction of Protein Translocator SecY

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Protein secretion occurs via translocation by the evolutionarily conserved Sec complex. LacZ hybrid proteins have long been used to study translocation in *Escherichia coli*. Some LacZ hybrids were thought to block secretion by physically jamming the Sec complex, leading to cell death. We found that jammed Sec complexes caused the degradation of essential translocator components by the protease FtsH. Increasing the amounts or the stability of the membrane protein YccA, a known inhibitor of FtsH, counteracted this destruction. Antibiotics that inhibit translation elongation also jammed the translocator and caused the degradation of translocator components, which may contribute to their effectiveness. Intriguingly, YccA is a functional homolog of the proto-oncogene product Bax Inhibitor-1, which may share a similar mechanism of action in regulating apoptosis upon prolonged secretion stress.

Protein translocation is a fundamental process that is essential for the delivery of most extracytoplasmic proteins to their final destination. This process is mediated by an evolutionarily conserved heterotrimeric membrane protein complex called the Sec61 complex (Sec61αβγ) in mammals and the Sec complex (SecY, -E, and -G) in prokaryotes (*1*). In *Escherichia coli*, two pathways target proteins to the Sec complex (*2*): the posttranslational Sec pathway, which targets most outer membrane (OM) and periplasmic proteins (*3*), and the cotranslational pathway, which is used primarily by inner membrane (IM) proteins, where the ribosome-nascent chain complex is targeted to the Sec complex by the signal recognition particle (SRP) (*4*). In both cases, proteins are initially directed to the SecY translocator via an amino-terminal signal sequence, which may or may not be cleaved upon translocation. The nature of this signal sequence determines which targeting pathway is used (*2*).

Genetic analysis of protein secretion is facilitated by *lacZ* (which specifies β-galactosidase) gene fusions (*5*, *6*). When the signal sequence of the OM protein LamB is fused to LacZ, the resulting hybrid protein is targeted for the posttranslational translocation pathway (*5*). Upon induction with maltose, large amounts of hybrid protein are made, and rapid folding of LacZ sequences in the cytoplasm causes a lethal jamming of the Sec complex (Fig. 1A), as evidenced by the accumulation in the cytoplasm of the precursor forms of wild-type secreted proteins (*7*). Under noninducing conditions (in the absence of maltose) in which low amounts of hybrid protein are made, lethal jamming does not occur. But because the hybrid protein is inefficiently secreted, some LacZ remains in the cytoplasm, where it is active, and strains carrying this fusion exhibit a Lac^+ phenotype. When the signal sequence of this hybrid is changed to increase its hydrophobicity, the resulting H*LamB-LacZ hybrid is directed instead to the alternative cotranslational SRP pathway (*4*). Because full-length LacZ is never exposed to the cytoplasm when secretion occurs cotranslationally, the H*LamB-LacZ hybrid is translocated efficiently to the periplasm, and jamming does not occur (Fig. 1B). In the oxidizing environment of the periplasm, LacZ misfolds. Thus, under noninducing conditions, strains carrying this fusion are Lac^-. Under inducing conditions, toxic hybrid protein aggregates accumulate in the periplasm so that even though no Sec complex jamming is observed, these strains are as maltose-sensitive as strains producing LamB-LacZ (*4*).

The Cpx two-component system regulates gene expression in response to misfolded proteins in the cell envelope. Dominant mutations, called *cpxA**, ac-

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Science

C3PO, an Endoribonuclease That Promotes RNAi by Facilitating RISC Activation

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RNA Wars

During RNA interference (RNAi), the Dicer endonuclease generates small interfering (si)RNAs that, with the help of the protein R2D2, are loaded into the siRNA-induced silencing complex (RISC). Using siRNAs as guides, RISC, and specifically its Argonaute subunit, targets complementary RNAs for destruction. In order to identify other components of the RISC complex, **Liu *et al.*** (p. 750) reconstituted the core RISC activity, using purified *Drosophila* Dicer, R2D2, and Ago-2. The protein C3PO (component 3 promoter of RISC), which consists of heterodimer of Translin and Translin-associated factor X (Trax), was found to enhance RISC activity in this system, and in vivo, with the Trax endonuclease activity activating RISC through the removal of siRNA passenger strand cleavage products.