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The translin–TRAX complex (C3PO) is a ribonuclease in tRNA processing

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Conserved translin–TRAX complexes, also known as C3POs, have been implicated in many biological processes, but how they function remains unclear. Recently, C3PO was shown to be an endoRNase that promotes RNA interference (RNAi) in animal cells. Here, we show that C3PO does not play a significant role in RNAi in the filamentous fungus *Neurospora crassa*. Instead, the *Neurospora* C3PO functions as an RNase that removes the 5′ pre-tRNA fragments after the processing of pre-tRNAs by RNase P. In addition, *translin* and *trax* mutants have elevated levels of tRNA and protein translation and are more resistant to a cell death–inducing agent. Finally, we show that C3PO is also involved in tRNA processing in mouse embryonic fibroblast cells. This study identifies the endogenous RNA substrates of C3PO and provides a potential explanation for its roles in apparently diverse biological processes.

Translin and its partner protein translin-associated protein X (TRAX) are highly conserved from fission yeast to human[1–3]. The two form a heteromeric complex and are proposed to be involved in many biological processes in different organisms, including normal cell growth, RNA metabolism, genome stability, neuronal development and spermatogenesis[4–8]. Although the translin and TRAX genes are not essential for cell survival, studies of *translin* (*tsn*) and *trax* (*trx*) mutants suggest that they are involved in cell proliferation in fission yeast, in motor response in *Drosophila melanogaster* and in spermatogenesis and behavior in mice[4,9–11]. The translin–TRAX complex is able to bind DNA and RNA[1,2,7,12], but how it acts to regulate diverse biological processes is unknown.

Recently, the translin–TRAX complex was biochemically purified as component 3 promoter of RISC (C3PO) from *Drosophila* and human cells[13,14]. C3PO is necessary for efficient RNAi mediated by small interfering RNAs (siRNAs)[13,14]. RNAi is a conserved eukaryotic gene-silencing mechanism mediated by small noncoding RNAs[15–18]. In RNAi pathways, siRNAs, which are generated by Dicer-mediated cleavage of double-stranded RNA (dsRNA), associate with Argonaute-family proteins and guide them to the RNA targets to regulate gene expression. Genetic depletion of C3PO impairs RNAi efficiency in both *Drosophila* and human cells. Notably, C3PO was demonstrated to be an RNA-specific endonuclease that can promote RNAi by removing the passenger strand of the siRNA duplex. Crystal structures of C3PO revealed that six translin and two TRAX subunits form an asymmetric octamer with the RNase catalytic residues located on the TRAX subunits[14,19]. However, it is unclear whether translin and TRAX also have a similar role in RNAi in other eukaryotic organisms. In addition, the functions of translin and TRAX in different biological processes suggest that the complex has other cellular functions, but the endogenous RNA substrates of the translin–TRAX complex as an endonuclease are not known.

The filamentous fungus *Neurospora crassa* is an important eukaryotic model system for RNAi studies[20,21]. We previously showed that QIP, an exonuclease, interacts with the Argonaute protein QDE-2 and removes the nicked passenger strand from the siRNA duplex[22]. Therefore, the role of C3PO in *Drosophila* and humans is similar to that of QIP in *Neurospora*. In addition to siRNAs, several other types of small RNAs (sRNAs) have been identified in *Neurospora*[23,24].

There is one translin (NCU06664, 239 amino acids) and one TRAX homolog (NCU06059, 349 amino acids) in the *Neurospora* genome, and they are highly conserved relative to their counterparts in *Drosophila* and humans. In this study, we set out to determine the function of C3PO in *Neurospora* by identifying its endogenous RNA substrates. We showed that *Neurospora* translin and TRAX do not have a significant role in RNAi. Instead, we discovered that lack of the *Neurospora* translin–TRAX complex (nC3PO) resulted in dramatic accumulation of pre-tRNA fragments in *Neurospora*. Genetic, molecular and biochemical experiments showed that the translin–TRAX complex acts as an RNase that removes 5′ pre-tRNA fragments after the processing of pre-tRNAs by RNase P. Moreover, *translin* and *trax* mutants have elevated tRNA levels, increased protein translation efficiency and increased resistance to a programmed cell death–inducing agent. This study reveals the endogenous substrates of the RNase activity of the translin–TRAX complex and provides a potential mechanism that explains its roles in many biological processes.

RESULTS

Neurospora translin and TRAX play no significant role in RNAi

To determine whether *Neurospora* translin and TRAX form a complex *in vivo*, a construct that expressed Myc-His–tagged TRAX (Myc-TRX) was transformed into a *trax*-knockout strain, and the Myc-TRX from this strain was purified by a nickel column, followed

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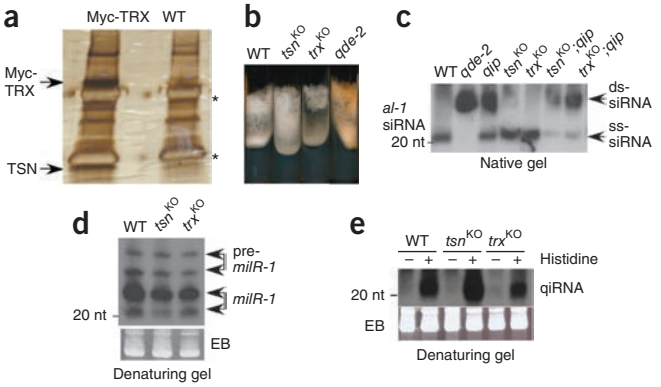

Figure 1 *Neurospora* translin and TRAX are not required for RNAi and siRNA passenger-strand removal. (**a**) Purification of the *Neurospora* translin–TRAX complex. SDS-PAGE gel shows the c-Myc immunoprecipitation products from the protein extracts of the wild-type and Myc-TRX strains. The two arrows indicate the bands identified by mass spectrometry as Myc-TRX and translin (TSN), respectively. The two asterisks indicate the IgG bands. (**b**) A photograph of *Neurospora* slants showing the gene silencing of *al-1* by the expression of the ds*al-1* construct in the indicated strains, which results in the orange-to-white color change of the cultures. Cultures were grown with QA (1×10^{-3} M). In the *qde-2* strain, the ds*al-1*-induced gene silencing was abolished. WT, wild-type. (**c**) Northern blot analyses of *al-1* siRNA of the indicated strains by a native gel. Cultures were grown with QA (1×10^{-3} M) and RNA samples from the indicated strains were used. The two arrows indicate the siRNA duplex (ds-siRNA) and single-stranded siRNA (ss-siRNA), respectively. (**d**,**e**) Northern blot analyses showing the expression profiles of (**d**) *milR-1* milRNAs and (**e**) qiRNA in the indicated strains. EB, ethidium bromide.

by immunoprecipitation using anti–c-Myc monoclonal antibody. The same purification procedure was performed using a wild-type strain that lacks the Myc-TRX. Two major specific protein bands (indicated by the arrows) were observed in the purification products of the Myc-TRX strain (**Fig. 1a**). Mass-spectrometry analysis identified the top band as Myc-TRX and the lower band as the *Neurospora* translin, indicating that, as for other eukaryotic organisms, *Neurospora* translin and TRAX form a complex.

To determine the role of translin and TRAX in dsRNA-triggered RNAi, a construct that can inducibly express dsRNA specific for the *albino-1* (*al-1*) gene in the presence of quinic acid (QA)[22,25] was introduced into wild-type, *translin*-knockout (*tsn*^{KO}) and *trax*-knockout (*trx*^{KO}) strains[26] at the *his-3* locus by homologous recombination. The resulting transformants were purified to obtain homokaryon strains. The silencing of *al-1* expression by RNAi blocks carotenoid biosynthesis, resulting in a color change from orange to white. Expression of the dsRNA specific for *al-1* resulted in a color change that was similar in the wild-type, *tsn*^{KO} and *trx*^{KO} strains (**Fig. 1b**), indicating that the nC3PO does not play a major role in dsRNA-induced RNAi in *Neurospora*.

Because of the role of C3PO in removing the siRNA passenger strand in animals, we compared the production and nature of *al-1* siRNA in the *tsn*^{KO} and *trx*^{KO} strains with those in wild-type and *qde-2* and *qip* mutants. As shown previously[22], almost all *al-1* siRNA produced in the wild-type strain was single-stranded, and only duplex siRNA, but no single-stranded siRNA, was present in the *qde-2* mutant (**Fig. 1c**). The vast majority of siRNA in the *qip* strain was in duplex form, confirming the role of QIP in siRNA passenger-strand removal. In contrast to the siRNA profile in the *qip* strain, all siRNA was single-stranded in both *tsn*^{KO} and *trx*^{KO} mutants. To examine the possibility that QIP and the nC3PO may play redundant roles in siRNA passenger-strand removal, we created *tsn*^{KO};*qip* and *trx*^{KO};*qip* double mutants and introduced the double-stranded *al-1* (ds*al-1*) construct into the strains by cotransformation. Although the levels of total *al-1* siRNA were lower in the transformants of the double mutants (likely because cotransformation triggered a partial silencing of the ds*al-1* construct)[27], the ratios of single-stranded siRNA to total siRNA were not statistically different between the *qip* single mutant and the *tsn*^{KO};*qip* and *trx*^{KO};*qip* double mutants (**Fig. 1c** and **Supplementary Fig. 1**). Although we cannot exclude the possibility that nC3PO plays a minor role in RNAi, these results indicate that translin and TRAX do not play a significant role in siRNA-mediated gene silencing in *Neurospora*.

We also examined the role of translin and TRAX in the production of *Neurospora* miRNA-like small RNAs (milRNAs)[24]. Although the maturation of *milR-1* milRNA, the most abundant milRNA in *Neurospora*, was completely blocked in the *qip* mutant[24], milRNA production was normal in the *tsn*^{KO} and *trx*^{KO} mutants (**Fig. 1d**). Similarly, the amounts of DNA damage–induced QDE-2–interacting small RNA (qiRNA)[23] in the *tsn*^{KO} and *trx*^{KO} mutants were comparable to those in the wild-type strain (**Fig. 1e**). These results indicate that translin and TRAX are not involved in the production of known types of *Neurospora* sRNAs.

Accumulation of sRNA species in the *tsn*^{KO} and *trx*^{KO} mutants

The lack of a significant role for translin and TRAX in RNAi and sRNA production in *Neurospora* and conservation of the translin–TRAX complex in eukaryotes suggest that translin and TRAX have other roles in *Neurospora*. To investigate this possibility, we compared the total sRNA profile of a wild-type *Neurospora* strain to those of the *tsn*^{KO} and *trx*^{KO} mutants in gels of the enriched sRNA samples stained with ethidium bromide. A ladder of ~18–50-nucleotide sRNA species accumulated to high levels in both the *tsn*^{KO} and *trx*^{KO} mutants but not in the wild-type and RNAi-mutant strains (*qde-2*, *dcl-1 dcl-2*, *qip* and *qde-1*) (**Fig. 2a**; data not shown). The most abundant species that accumulated in *tsn*^{KO} and *trx*^{KO} mutants were around 20 nucleotides (nt) in length. To examine whether the lack of the translin–TRAX complex in the *tsn*^{KO} and *trx*^{KO} mutants is responsible for the accumulation of these RNAs, constructs for expression of Myc-tagged wild-type translin (Myc-TSN) or Myc-TRX were transformed into the *tsn*^{KO} and *trx*^{KO} mutants, respectively. The expression of translin or TRAX rescued the phenotype in the *tsn*^{KO} and *trx*^{KO} mutants, indicated by the disappearance of the RNA ladder in these strains (**Fig. 2b**).

The accumulation of sRNA species and the proposed function of C3PO as an RNA-specific nuclease raise the possibility that these RNAs are the substrates of the translin–TRAX complex. To test this hypothesis, we created two Myc-TRX constructs in which RNase catalytic cysteine residues (Cys473 or Cys482)[13,14,19] were mutated to alanine (C473A or C482A) and introduced these constructs separately into the *trx*^{KO} mutant. Despite similar levels of expression of the mutant proteins and the wild-type protein, neither mutant construct rescued the phenotype of the *trx*^{KO} mutant (**Fig. 2c**,**d**), suggesting that the accumulated sRNA species in the *tsn*^{KO} and *trx*^{KO} mutants are the endogenous RNA substrates of the translin–TRAX complex in *Neurospora*.

Accumulation of pre-tRNA fragments in *tsn*^{KO} and *trx*^{KO} mutants

To identify the accumulated small RNA species in the *tsn*^{KO} and *trx*^{KO} mutants, we size-fractionated the sRNA samples of the wild-type and

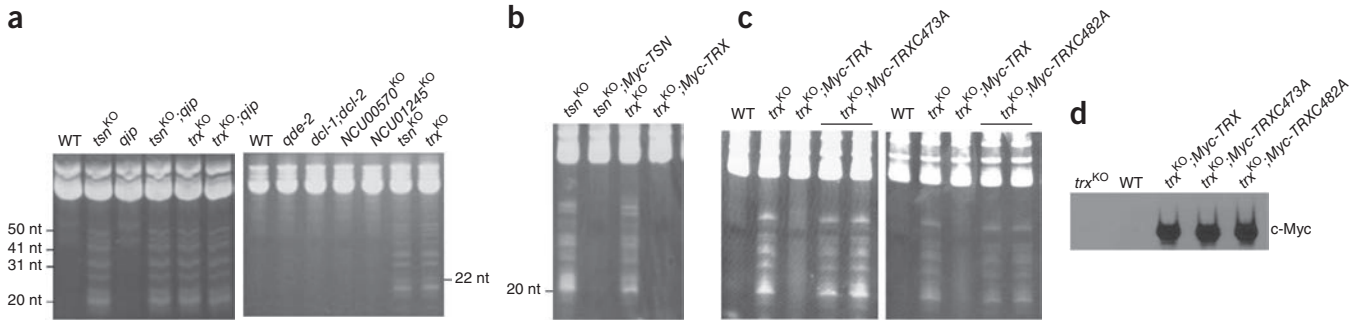

Figure 2 Accumulation of sRNA species in the *tsn*^{KO} and *trx*^{KO} strains is dependent on the catalytic activity of the translin–TRAX complex. (**a**–**c**) Ethidium bromide (EB)-stained denaturing PAGE gels showing the sRNA profiles in the indicated strains. Total RNA was used in **b** and **c**. *NCU00570*^{KO} and *NCU01245*^{KO} are knockout mutants for two genes with potential roles in RNA polymerase III–mediated transcription. (**d**) Western blot analysis with the anti–c-Myc antibody showing the expression of Myc-TRX in the indicated strains. The wild-type and the *trx*^{KO} strains were used as negative controls.

tsn^{KO} strains, generated cDNA libraries of sRNAs (17–30 nt) and sequenced them using an Illumina/Solexa Genome Analyzer. We obtained 1,572,492 and 1,658,457 reads from the wild-type and *tsn*^{KO} libraries, respectively, that perfectly matched the *Neurospora* genome. After the sRNA sequences were mapped to the assembled *Neurospora* genome sequences, we compared the profiles of sRNA distributions between the wild-type strain and the *tsn*^{KO} mutant. Although the distribution of sRNAs appeared to be comparable in both strains for most of the *Neurospora* genome (**Supplementary Fig. 2**), there were significantly more sRNA reads in the *tsn*^{KO} mutants than in the wild type that were mapped to the regions immediately upstream of the predicted *Neurospora* tRNA genes (**Fig. 3**). There were more than twice as many sRNA reads within 100 nt upstream of the 5′ end of predicted tRNAs in the *tsn*^{KO} mutant than in the wild-type strain. It is important to note that although there is significantly more sRNA in the *tsn*^{KO} mutant than in the wild-type strain (as indicated by the ethidium bromide–stained gels), similar total sRNA reads were obtained from the two strains. Thus, the relative numbers of the sRNA reads from the *tsn*^{KO} mutant were underestimated, and therefore, the actual differences of the 5′ pre-tRNA sRNA reads between the *tsn*^{KO} mutant and the wild-type strains should be much greater. Although the 5′ ends of the wild-type sRNAs mapped to these regions showed no significant nucleotide preference, the sRNAs in the *tsn*^{KO} mutant exhibited a preference for 5′ adenosine (48%) and uridine (36%). Most of these sRNAs matched the sense strand of the predicted pre-tRNAs, but a significant fraction also matched the antisense strand. In the *tsn*^{KO} mutant, the antisense but not sense sRNAs had a strong preference for 5′ uridine (63%). The 5′-uridine preference of the antisense sRNAs is reminiscent of the *Neurospora* sRNAs produced by RNAi pathways. Detailed comparison of the sRNA distribution over the entire genome between the wild-type and *tsn*^{KO} strains revealed that there was a significant accumulation of sRNAs upstream of 56 predicted tRNA genes (**Supplementary Table 1**) in the *tsn*^{KO} strain, including those for 15 lysine tRNAs, 14 glutamic acid tRNAs and 11 aspartic acid tRNAs. In addition to the tRNA loci, there are 18 other loci with significant increases of sRNAs in the *tsn*^{KO} mutant (**Supplementary Table 2**). These sRNAs matched to predicted gene open-reading-frame regions, intergenic regions and repeat regions. For these sRNAs not originating from tRNA loci, no common functional and sequence motifs were observed.

tRNA genes are transcribed in eukaryotes by RNA polymerase III to produce tRNA precursors with 5′ and 3′ sequences that are removed to yield functional tRNAs[28–30]. The 5′ leader sequences of pre-tRNAs are cleaved by the endonuclease RNase P to generate mature 5′ ends of tRNAs, and the 3′ sequences are processed by tRNase Z and other enzymes. **Supplementary Figure 3a** shows the accumulation of sRNA in one representative tRNA locus (NCU1217, Lys tRNA). The secondary structure of the NCU12127 tRNA and the predicted pre-tRNA cleavage sites by RNase P and tRNase Z are shown in **Supplementary Figure 3b**. The accumulated RNAs mapped immediately upstream of the predicted mature Lys tRNA sequence. The exact match of the sRNA species to those of the 5′ leader sequences of the pre-tRNAs indicates that these RNAs are mostly pre-tRNA fragments that have been processed by RNase P.

Levels of *milR-1* milRNAs were comparable in both the wild-type and *tsn*^{KO} strains; however, the numbers of sRNA (sense and antisense) reads that matched to 5′ ends of pre-tRNAs for some tRNA genes were dramatically increased in the *tsn*^{KO} mutant (**Fig. 4a**,**b**). In addition, the levels of antisense sRNAs, although generally lower than those of the sense sRNAs, were similarly increased in the *tsn*^{KO} mutant. To confirm the deep-sequencing results, we performed northern blot analysis on a few randomly selected tRNAs, using probes that specifically detect the 5′ leader sequences of pre-tRNA transcripts (**Fig. 4c**,**d**). As expected, sRNAs containing the 5′ leader sequences accumulated to high levels in both *tsn*^{KO} and *trx*^{KO} mutants but were barely detectable in the wild-type strain. In addition, the expression of the Myc-TSN or Myc-TRX in the *tsn*^{KO} or *trx*^{KO} mutants, respectively, resulted in the decrease of pre-tRNA fragments to wild-type levels (**Fig. 4d**). The levels of pre-tRNA fragments in the *qip* mutants were comparable to levels in the wild-type

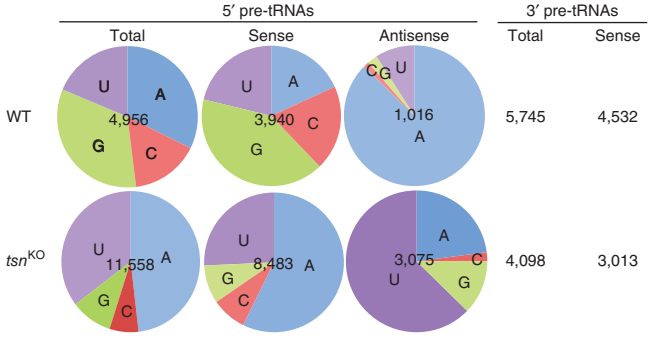

Figure 3 Deep sequencing of sRNAs reveals the accumulation of 5′ pre-tRNA fragments in the *tsn*^{KO} strain. Pie charts showing the relative numbers of sRNA reads and their 5′ nucleotide preferences from the 5′ and 3′ ends of the predicted *Neurospora* pre-tRNAs. Small RNAs matching within 100 nt upstream (5′) or downstream (3′) of predicted mature tRNA were included in the analyses.

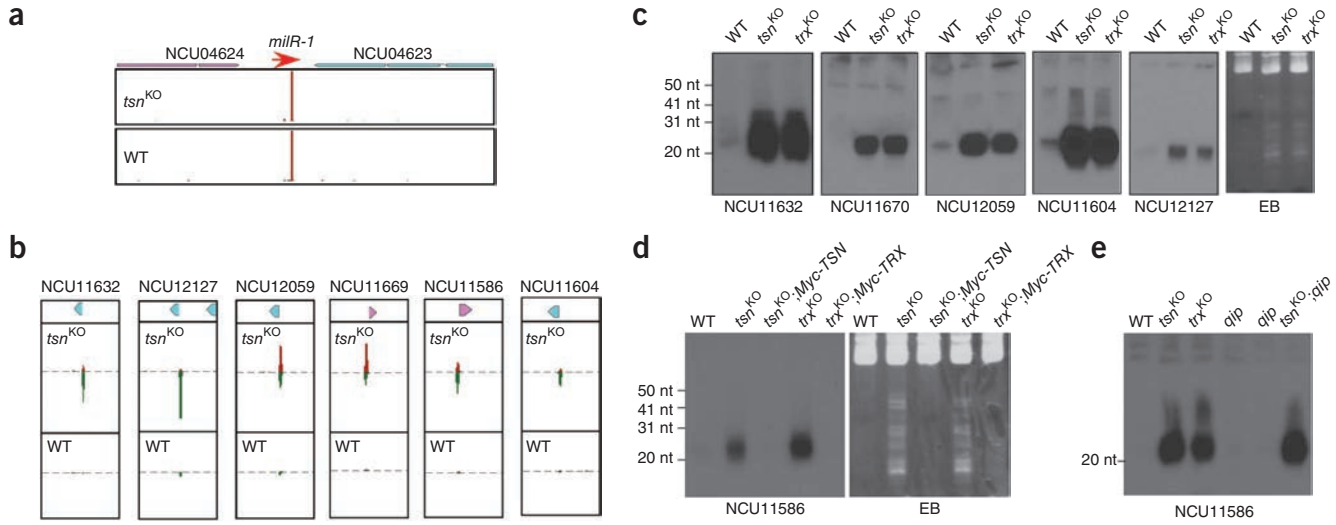

Figure 4 High levels of 5′ pre-tRNA fragments in the *tsn*^{KO} and *trx*^{KO} strains. (**a**,**b**) Comparison of sRNA distributions at the (**a**) *milR-1* and (**b**) representative tRNA loci between the wild-type and *tsn*^{KO} strains. Numbers of sRNAs (logarithmic scale) in 10-nt sliding windows are shown. Small RNAs matched to either Watson (red) or Crick (green) strand of DNA are shown. (**c**–**e**) Northern blot analyses of 5′ pre-tRNA fragments of selected tRNAs in the indicated strains. EB, ethidium bromide.

strain and were not further increased in the *tsn;qip* double mutant (**Fig. 4e**), further indicating that QIP and the nC3PO have distinct roles in different cellular processes. Although most of the pre-tRNA signals were seen around 20–22 nt, higher-molecular-weight signals were observed for some probes, suggesting that pre-tRNA fragments are the major sRNA species seen on the ethidium bromide–stained gels in the *tsn*^{KO} and *trx*^{KO} strains.

The antisense-specific sRNAs are products of RNAi

The antisense sRNAs complementary to some of the pre-tRNA fragments were also dramatically increased in the *tsn*^{KO} and *trx*^{KO} mutants (**Fig. 5a**). To examine whether the antisense sRNAs are the products of the RNAi pathway, we generated *dcl-2*^{RIP}*;tsn*^{KO} double mutants (RIP denotes repeat-induced point mutation). DCL-2 is the major Dicer protein in *Neurospora*[31]. The antisense-specific sRNA was completely abolished in the *dcl-2*^{RIP}*;tsn*^{KO} mutant (**Fig. 5b**). In addition, a high-molecular-weight RNA smear accumulated in the *dcl-2*^{RIP}*;tsn*^{KO} mutant (**Supplementary Fig. 4**), suggesting that it is dsRNA. Furthermore, the sense-specific sRNA was also modestly reduced in the *dcl-2;tsn*^{KO} mutant (**Fig. 5c**), indicating that the antisense sRNAs are produced by Dicer. To examine whether QDE-1, an RdRP (RNA-dependent RNA polymerase) that is essential for transgene-induced gene silencing in *Neurospora* (quelling)[32,33], is required for the production of antisense sRNAs, a *qde-1;tsn*^{KO} double mutant was generated. The levels of the antisense-specific sRNA were comparable in both the wild-type and *qde-1;tsn*^{KO} strains (**Fig. 5d**), indicating that QDE-1 is not required for the production of antisense-specific RNA. It is likely either that SAD-1 and RRP-3 (refs. 25,34), the two other RdRPs in *Neurospora*, are involved in converting the pre-tRNA fragments into dsRNAs or that these RdRPs may play redundant roles. Furthermore, immunoprecipitation of Myc-QDE-2 from a *trx*^{KO};Myc-QDE-2 strain showed that the NCU11586-specific sRNA is associated with Myc-QDE-2, indicating that at least some of the sRNAs are incorporated into the Argonaute protein (**Fig. 5e**). Together, these results demonstrate that the antisense sRNAs are produced by RNAi using the sense sRNA as templates. It is important to note that the small ladders shown by ethidium bromide–stained gel (**Fig. 5b**, bottom) in the *tsn*^{KO} and *dcl-2;tsn*^{KO} double mutants were similar, indicating that the majority of sRNAs that accumulated in the *tsn*^{KO} mutant are cleavage products of pre-tRNAs by RNase and are not Dicer products.

5′ pre-tRNA fragments are substrates of nC3PO

Both the *Drosophila* and human translin–TRAX complexes act as single-stranded RNA endonucleases *in vitro*[13,14], suggesting that the

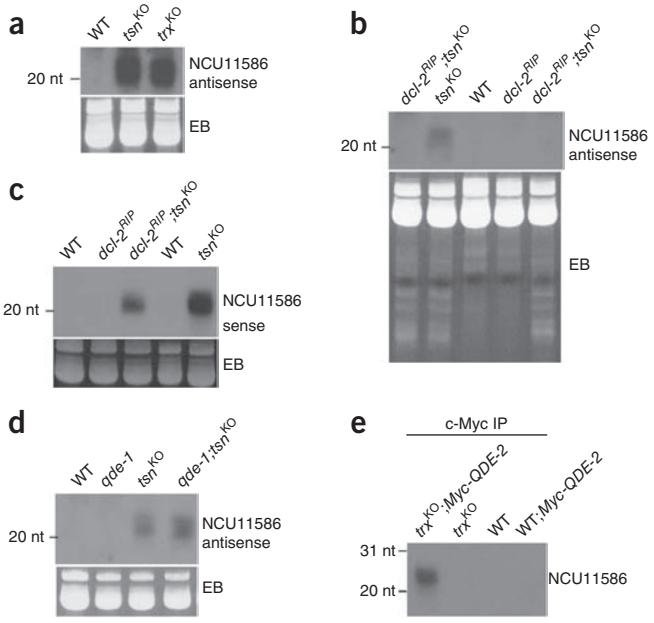

Figure 5 Dicer is required for the production of the antisense-specific sRNAs. (**a**–**d**) Northern analyses showing the levels of the antisense and sense sRNAs that match to the 5′ pre-tRNA fragment of NCU11586 in the indicated strains. Ethidium bromide (EB)-stained gels of the sRNAs are shown below. (**e**) Immunoprecipitation (IP) by the anti–c-Myc antibody followed by northern blot analysis showing that Myc-QDE-2 associated with the 5′ pre-tRNA fragments.

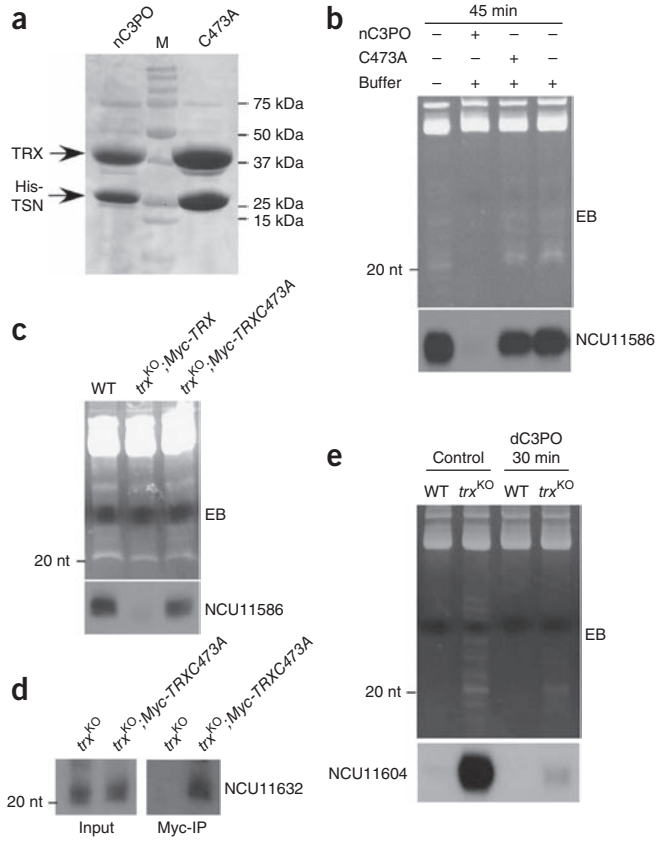

Figure 6 The pre-tRNA fragments are substrates of the *Neurospora* translin–TRAX complex. (**a**) A Coomassie Blue–stained gel showing the purified recombinant wild-type *Neurospora* translin–TRAX complex (nC3PO) and the nC3PO with the TRAX catalytic point mutation (C473A). TRX, TRAX; TSN, translin; M, marker. (**b**) The recombinant nC3PO can digest the 5′ pre-tRNA fragments *in vitro*. EB-stained gel of the sRNAs (top) and northern blot analysis (bottom) of RNA treated *in vitro* with nC3PO and with nC3PO C473A are shown. The sRNA preparation from the *trx*^{KO} strain was used as the RNA substrate. EB, ethidium bromide. (**c**) The nC3PO partially purified from *Neurospora* can digest 5′ pre-tRNA fragments *in vitro*. EB-stained gel of the sRNAs and northern blot analysis of *trx*^{KO} sRNA samples treated with the c-Myc immunoprecipitates from the indicated strains are shown. (**d**) Immunoprecipitation of Myc-TRX (C473A) from the *trx*^{KO};Myc-TRXC473A strain showing that the catalytically dead nC3PO associated with 5′ pre-tRNA. The *trx*^{KO} strain expressing no Myc-tagged protein was used as a negative control. (**e**) The recombinant *Drosophila* C3PO can digest 5′ pre-tRNA fragments *in vitro*. EB-stained gel of the sRNAs (top) and northern blot analyses (bottom) of sRNA treated *in vitro* with *Drosophila* C3PO (dC3PO) for 30 min are shown. The sRNA preparation from the *trx*^{KO} strain was used as the RNA substrate.

pre-tRNA fragments that accumulated in the *Neurospora tsn*^{KO} and *trx*^{KO} mutants are the *in vivo* substrates of the translin–TRAX complex. To test this hypothesis, we coexpressed the *Neurospora* TRAX and the histidine-tagged translin in *Escherichia coli* and purified the recombinant *Neurospora* nC3PO by a TALON metal-affinity column and a Superose 6 gel-filtration column. In addition, we also expressed and purified the mutant complex that contains the C473A TRAX mutation. As expected, the recombinant translin and TRAX form a complex in *E. coli* (**Fig. 6a**). We then examined the ability of recombinant nC3PO to cleave the *Neurospora* pre-tRNA fragments *in vitro*. The addition of the wild-type nC3PO to the RNA sample of the *tsn*^{KO} mutant resulted in the disappearance of the sRNAs on the ethidium bromide–stained gel without significant effects on the high-molecular-weight RNA bands (**Fig. 6b**). In addition, northern blot analysis showed that the level of the 5′ tRNA fragment of NCU11586 was dramatically decreased by the treatment. In contrast, the C473A mutant nC3PO had no effects on the sRNA ladder seen on the ethidium bromide–stained gel and on the 5′ pre-tRNA fragment level. This result demonstrates that the nC3PO is indeed an RNase that can degrade the pre-tRNA fragments.

To show that the endogenous nC3PO from *Neurospora* has a similar activity, we immunoprecipitated Myc-TRX from the *trx*^{KO};Myc-TRX strain and Myc-TRXC473A from the *trx*^{KO};Myc-TRXC473A strain. A wild-type strain that does not express Myc-tagged protein was used as a negative control for the immunoprecipitation. The immunoprecipitates from the three strains were separately incubated with the RNA samples of the *tsn*^{KO} mutant. The immunoprecipitates from the *trx*^{KO};Myc-TRX strain, but not from the wild-type and *trx*^{KO};Myc-TRXC473A strains, showed a decrease of the sRNA ladder on the ethidium bromide–stained gel and the level of the 5′ pre-tRNA fragment (**Fig. 6c**).

We then examined whether the nC3PO is associated with 5′ pre-tRNA fragments *in vivo*. Because the C473A mutation of TRAX is catalytically inactive, we reasoned that the nC3PO-RNA association can be stabilized in the *trx*^{KO};Myc-TRXC473A mutant. After c-Myc immunoprecipitation, the Myc-TRXC473A–associated sRNA was examined by northern blot analysis. The pre-tRNA fragments were specifically pulled down by the immunoprecipitation in the *trx*^{KO};Myc-TRXC473A strain, indicating that nC3PO associates with pre-tRNA fragments *in vivo* (**Fig. 6d**). Taken together, our results demonstrate that the 5′ pre-tRNA fragments are the *in-vivo* substrates of nC3PO.

Because of the conservation of translin–TRAX complexes in eukaryotes, we also examined whether recombinant *Drosophila* C3PO (dC3PO) has a similar activity toward the 5′ pre-tRNA fragments. The addition of recombinant dC3PO to RNA samples of the *tsn*^{KO} or *trx*^{KO} mutants also resulted in a rapid disappearance of the sRNA ladder and the 5′ pre-tRNA fragments (**Fig. 6e**), suggesting that both the *Drosophila* and *Neurospora* translin–TRAX complexes have similar activities toward 5′ pre-tRNA fragments.

tsn^{KO} and *trx*^{KO} mutant phenotypes

The developmental phenotypes of the *tsn*^{KO} and *trx*^{KO} strains were similar to that of the wild-type strain under normal culture conditions. Because the nC3PO regulates the processing of tRNAs in *Neurospora*, we examined the tRNA expression levels of genes that are regulated by the complex. The tRNA levels of six selected genes were all significantly elevated in both the *tsn*^{KO} and *trx*^{KO} mutants (**Fig. 7a**), suggesting that the nC3PO suppresses tRNA production in *Neurospora*. It is possible that, in addition to 5′-end pre-tRNA fragments, tRNAs and pre-tRNAs are also substrates for the translin–TRAX complex.

Because of the essential role of tRNAs in general protein translation, we then compared the amounts of protein synthesized during a 30-min pulse of [^{35}S]methionine in the wild-type strains and the mutants. We observed modestly but significantly (~30%) greater ^{35}S incorporation in both the *tsn*^{KO} and *trx*^{KO} mutant strains as compared to the wild-type strain (**Fig. 7b**). Consistent with the elevated protein translation efficiency, both mutants also displayed modestly higher cell growth compared to the wild-type strain (**Fig. 7c**). Similarly, cell proliferation rates were previously found to be elevated in *tsn*^{KO} and *trx*^{KO} mutants of fission yeast[4]. We also examined the sensitivities of the *tsn*^{KO} and *trx*^{KO} mutant strains to a series of stress-inducing agents and found that both mutants showed increased resistance to the programmed cell death–inducing agent phytosphingosine[35] (**Fig. 7d**).

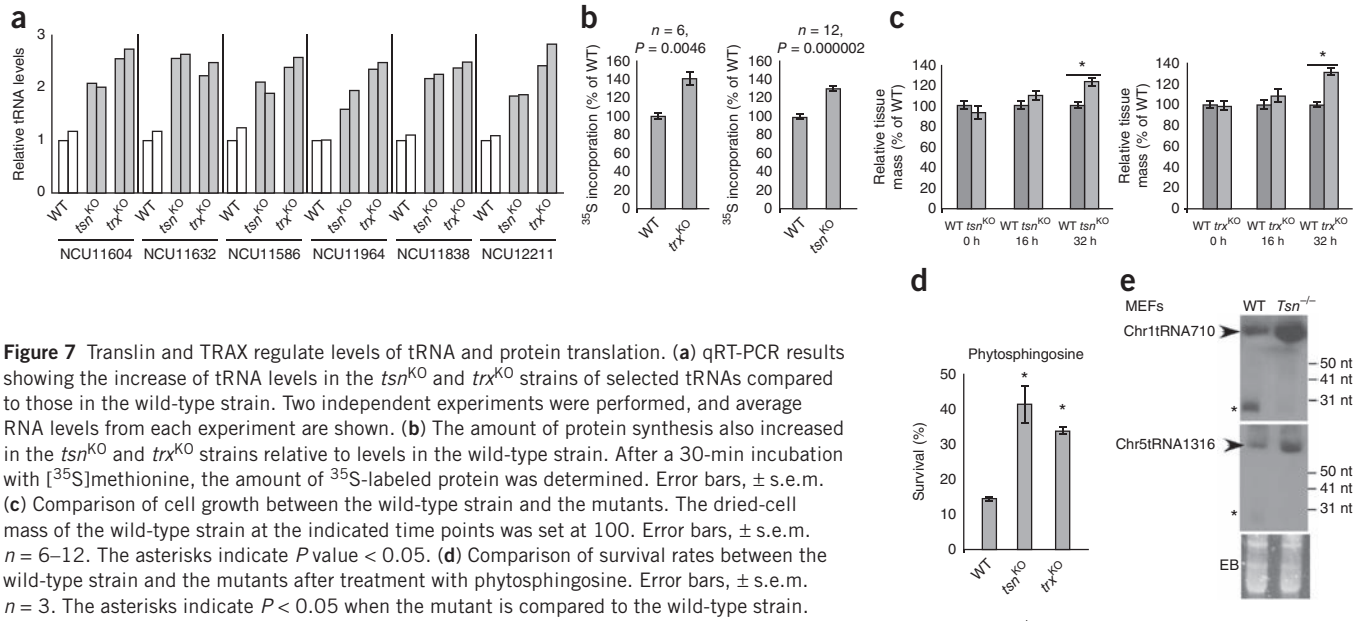

Figure 7 Translin and TRAX regulate levels of tRNA and protein translation. (**a**) qRT-PCR results showing the increase of tRNA levels in the *tsn*^{KO} and *trx*^{KO} strains of selected tRNAs compared to those in the wild-type strain. Two independent experiments were performed, and average RNA levels from each experiment are shown. (**b**) The amount of protein synthesis also increased in the *tsn*^{KO} and *trx*^{KO} strains relative to levels in the wild-type strain. After a 30-min incubation with [^{35}S]methionine, the amount of ^{35}S-labeled protein was determined. Error bars, ± s.e.m. (**c**) Comparison of cell growth between the wild-type strain and the mutants. The dried-cell mass of the wild-type strain at the indicated time points was set at 100. Error bars, ± s.e.m. n = 6–12. The asterisks indicate P value < 0.05. (**d**) Comparison of survival rates between the wild-type strain and the mutants after treatment with phytosphingosine. Error bars, ± s.e.m. n = 3. The asterisks indicate P < 0.05 when the mutant is compared to the wild-type strain. (**e**) Northern blot analysis showing the levels of the mouse tRNAs (indicated by the arrows) in the wild-type and *Tsn*$^{-/-}$ MEF cells. The asterisks indicate a sRNA species in the wild-type but not in the *Tsn*$^{-/-}$ MEF cells. Chr1tRNA710 and Chr5tRNA1316, *Mus musculus* tRNAs.

Together, these results suggest that the nC3PO regulates general protein translation efficiency and cell proliferation through its role in tRNA processing.

C3PO is involved in tRNA processing in mice

The 5′ pre-tRNA fragments in mammalian tRNAs are generally much smaller than those in *Neurospora*[36,37], and they could not be detected by conventional analyses (data not shown). To examine whether the role of C3PO in tRNA processing is conserved in mammals, we performed northern blot analyses and qRT-PCR analyses to compare the levels of some mouse tRNAs between wild-type and *translin*-knockout (*Tsn*$^{-/-}$)mouse embryonic fiboblast (MEF) cells[14] (**Fig. 7e** and **Supplementary Fig. 5**). We found that the levels of six mouse tRNAs were significantly elevated in the *Tsn*$^{-/-}$ cells. In addition, northern blot analysis showed that an ~25 nt, tRNA-derived sRNA species in the wild-type cells was abolished in the *Tsn*$^{-/-}$ cells, suggesting that it was a C3PO cleavage product from the corresponding tRNA. These results suggest that, as in *Neurospora*, C3PO is also involved in tRNA processing in mammals.

DISCUSSION

tRNAs play essential roles in protein synthesis. The maturation of tRNAs requires accurate processing of pre-tRNAs at both 5′ and 3′ ends. RNase P has long been known to be responsible for the processing of pre-tRNAs at the 5′end[28–30], but how the cleaved 5′ RNA fragments are further processed was not known. In this study, we identified the highly conserved translin–TRAX complex as a major RNase that digests the 5′ pre-tRNA fragments in *Neurospora*. This conclusion is supported by genetic and biochemical evidence. First, the RNase P–cleaved 5′ pre-tRNA fragments accumulated to very high levels in both *tsn*^{KO} and *trx*^{KO} mutants. Second, the catalytic residues of the translin–TRAX complex were essential for processing of pre-tRNA fragments. Third, the recombinant nC3PO degraded the pre-tRNA fragments *in vitro* and associated with pre-tRNA fragments in cells. Because of the conservation of translin–TRAX complexes and tRNA processing pathways in eukaryotes, our results suggest that translin–TRAX complexes may have a similar function in other organisms. Supporting this notion, we showed that C3PO is also involved in tRNA processing in MEFs.

Although we observed the accumulation of 5′ pre-tRNA fragments in the *tsn*^{KO} and *trx*^{KO} mutants for 56 predicted *Neurospora* tRNAs (out of 413)[38], this is likely an underestimation of tRNAs regulated by translin–TRAX, because the coverage of our sRNA deep sequencing was limited to RNAs of 17–30 nt. Thus, 5′ pre-tRNAs that were larger or smaller were not included in our analyses. Nonetheless, it is likely that other RNases are also involved in degradation of 5′ pre-tRNA fragments. It is also worth noting that some tRNAs can be transcribed without 5′ leader sequences and do not require RNase P for maturation[39]. In addition to the pre-tRNA fragments, sRNA accumulation was found for 18 other non-tRNA loci (ORFs and intergenic and repeat regions), suggesting that nC3PO has additional non-tRNA substrates.

tRNA-derived RNA fragments or sRNAs are a major class of sRNA species in sRNA deep sequencing studies in organisms from fungi to animals[24,40,41]. Although most of these sRNAs are generated by RNAi-independent processing events, some had been demonstrated to be the products of Dicer processing[42]. Interestingly, one of the human tRNA-derived RNA fragments was found to be highly expressed in cancer cell lines, and its downregulation impaired cell proliferation[40]. On the other hand, angiogenin-induced tRNA fragments were shown to inhibit protein translation[43]. These results indicate that tRNA-derived sRNAs are not random degradation products and can have important biological functions.

Translin and TRAX are highly conserved in eukaryotic organisms from fungi to humans. In addition to the role of the translin–TRAX complex in human and *Drosophila* dsRNA-triggered RNAi pathways[13,14,19], both proteins have been implicated in many biological processes, including normal cell growth, genome stability, neuronal development and spermatogenesis, in different organisms[4–8,44]. These functions of the translin–TRAX complex suggest that it has functions other than dsRNA-triggered RNAi and has endogenous RNA targets. Our study identified 5′ pre-tRNA fragments as the major substrates for the translin–TRAX complex in *Neurospora*. The lack of a signfi-cant RNAi function in *Neurospora* for translin and TRAX suggests

that their role in pre-tRNA processing is more ancient than their role in RNAi. In addition, we found that in the tsn^{KO} and trx^{KO} mutants, tRNA levels, protein translation efficiency and cell growth were elevated. The increase in levels of protein translation we observed in the tsn^{KO} and trx^{KO} mutants is consistent with the increase of cell proliferation rates of tsn^{KO} and trx^{KO} mutant cells observed in fission yeast[4]. In addition, both translin and TRAX are known to be required for normal cell proliferation of mouse embryonic stem cells[5]. Because the changes in tRNA levels can differentially affect expression of different proteins, the roles of translin and TRAX in tRNA processing and other RNA processing may provide a potential explanation for its many biological roles in different organisms.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.L. designed, conducted and interpreted experiments. W.G. performed sRNA sequencing and analyses. C.L. prepared RNA from MEFs. C.C.M. and Q.L. interpreted experimental results. Y.L. designed and interpreted experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Strains and growth conditions. The wild-type strain used in this study was FGSC 4200(a). Mutant strains, including *qde-1*, *qde-2*, *qde-2* (D664A), *qde-3*, *qip*, *dcl-2* and a *dcl-1;dcl-2* double mutant (dcl^{DKO}), were generated in previous studies[22,25]. tsn^{KO} (FGSC12632, *A*; FGSC 12633, *a*) and trx^{KO} (NCU06059, FGSC13557, *a*) were obtained from the Fungal Genetic Stock Center. Myc-tagged protein–expressing strains, including Myc-His-translin, Myc-His-TRAX and Myc-His-TRAX (C473A, C482A) strains, were created in this study. The protein-expression constructs were transformed into the indicated *Neurospora* strains by homologous recombination at the *his-3* locus, and homokaryon strains were obtained by microconidial isolation[45]. The expression of transgenes at the *his-3* locus is under the control of the *qa-2* inducible promoter[46]. The tsn^{KO} and trx^{KO} *his-3* recipient strains were created by crossing a tsn^{KO} or a trx^{KO} strain to a *his-3* wild-type strain (FGSC6103). The double and triple mutants were obtained by crossing the corresponding single or double mutants. The d*sal-1* construct was introduced into the tsn^{KO}*;qip* and trx^{KO}*;qip* double-mutant strains by cotransformation with pBT6 plasmid (bearing the benomyl-resistant β-tubulin gene). Liquid cultures were grown in minimal medium (1× Vogel's, 2% glucose) at 30 °C overnight and then at room temperature with shaking at 130 r.p.m. for 24 h[47]. For liquid cultures containing QA, 0.01 M QA, pH 5.8, was added to the liquid culture medium containing 1× Vogel's, 0.1% glucose and 0.17% arginine.

Purification and cloning of sRNAs. sRNAs (~17–30 nt) were purified on 16% PAGE/7 M urea gels from wild-type and tsn^{KO} total RNA samples[22]. RNAs from regions of interest were eluted from the gels in elution buffer containing 10 mM Tris-Cl, pH 7.5, 1 mM EDTA and 0.3 M NaCl. RNAs were precipitated with 20 μg glycogen and 1 volume of isopropanol. RNA was incubated for 1 h at 37 °C with 1 U/μl Calf Intestinal Phosphatase (NEB) and 1 U/μl SUPERase in Buffer 3 (NEB), extracted twice with phenol and precipitated with ethanol. RNA and two 18-nt and 26-nt synthetic RNA markers were incubated with 10 μM of preadenylated DNA oligo (5′-AppCTGTAGGCACCATCAAT/ddC-3′), 1 unit/μl of SUPERase•In, 10% DMSO and 2 unit/μl T4 RNA ligase (Takara Bio Inc., 2050A) in 10 μl ligation buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.06 μg/μl BSA, 10 mM DTT). The 3′-ligated RNAs were phosphorylated with 1 unit/μl polynucleotide kinase in buffer 3 (NEB) containing 1 unit/μl SUPERase•In and 2 mM ATP at 37 °C for 1 h, extracted with phenol, precipitated with ethanol and 5′-ligated with 2 unit/μl T4 RNA ligase and 30 μM 5′ linker 5′-GTTCTACAGTCCGACGATC-3′ in 10 μl 1X buffer containing 1 unit/μl SUPERase•In, 0.1 μg/μl BSA and 10% DMSO. cDNA was synthesized using SuperScript III with RT oligo 5′-ATTGATGGTGCCTAC AG-3′. The cDNA was PCR amplified with oligonucleotides containing Illumina/Solexa linkers. Deep sequencing was performed on HiScanSQ (Illumina).

All sRNA analyses were performed with custom Perl (5.8.6) scripts on a chromosome genome assembly based on *N. crassa* assembly 7 (ref. 48; available upon request from M. Freitag, Oregon State University, Corvallis, Oregon, USA)[24], rDNA sequence[23] and Version 3 gene predictions for the *N. crassa* assembly 7 release including mitochondria sequence (Broad Institute). Gene annotations, structural RNAs and repeats in version 3 gene predictions were mapped and converted to sequence coordinates in the chromosome genome assembly. Small RNA reads with perfect matches were mapped to the genome, mitochondrial DNA sequence and rDNA sequence. A single match returned a unique genomic locus, which was defined by a chromosome number, the start and the end of the matched sequence on the chromosome, and its orientation. To calculate sRNA reads derived from a single genomic locus, the sRNA reads were normalized to the total number of matched genomic loci (repeat-normalized reads). The density of sRNA is presented using non-overlapping 10-nt sliding windows along the Watson or Crick strands of each chromosome (**Fig. 4a**,**b**). Immunopurification of the Myc-TRX, Myc-TRX(C473A) and Myc-QDE-2 ribonucleoprotein complexes was performed as previously described[22].

Northern blot analyses and quantitative RT-PCR assay. Equal amounts of enriched *Neurospora* sRNA[22] (12.5–25 μg) or total RNA of MEFs[14] (40 μg) from different RNA samples were separated on 16% denaturing polyacrylamide/7 M urea gels and transferred onto a Hybond-NX membrane (GE Healthcare). An RNA ladder (Ambion) or a mixture of primers of different sizes were used as size markers. Cross-linking of RNA to Hybond-NX was performed using a carbodiimide-mediated cross-linking method at 60 °C for 2 h as described[49], followed by baking at 80 °C for 2 h. For native gel blots, UV cross-linking was used. Hybridization was performed according to the manufacturer's instructions in ULTRAhyb oligo hybridization buffer (Ambion) for StareFire (IDT) labeled DNA probes or in ULTRAhyb hybridization buffer (Ambion) for T7-transcribed riboprobes. qRT-PCR were performed as previously described[25]. The Starfire probe sequences and all primer sequences are listed in **Supplementary Table 3**.

Preparation of recombinant *Neurospora* C3PO. The cDNAs of translin, TRAX and TRAX (C473A) were cloned in pETDuet-1 (Duet expression vector, Novagen) with modifications. The expression constructs were transformed into *E. coli* BL21 (DE3), which were grown in LB medium containing 100 μg/ml ampicillin to OD_{600} of 0.6 at 37 °C and then induced with 0.2–0.4 mM IPTG. The cells were resuspended in Buffer A (10 mM HEPES, pH 7.4, 10 mM KOAc, 2 mM $Mg(OAc)_2$, 5 mM β-mercaptoethanol) with freshly added protease inhibitors and 5 mM imidazole, and sonicated on ice. The supernatant was then run through TALON metal-affinity resin (Clontech) at 4 °C, sequentially washed with Buffer B (Buffer A with 1 M NaCl) with 5 mM imidazole, Buffer A with 5 mM imidazole, and Buffer A with 20 mM imidazole, and then eluted with Buffer A containing 150 mM imidazole. The nC3PO fraction was further purified by a Superose 6 gel filtration column.

Nuclease cleavage assay. The RNA cleavage assay was carried out as previously described[13]. Reactions containing recombinant *Drosophila* C3PO (0.4–1.8 μg) and the enriched *Neurospora* sRNA (25 μg) were incubated at 30 °C for the indicated time.

Measurement of protein translation. Conidia from 5- to 7-day-old cultures were isolated and inoculated into 150-mm Petri dishes containing 1× Vogel's minimum medium with 2% glucose and were incubated at room temperature for 2 d under constant light. Mycelial discs of 11–13 mm were cut from the mycelial mats, cultured in 1× Vogel's minimum medium with 2% glucose for 16 h at room temperature and then metabolically labeled with 1 μCi/ml EXPRE35S35S protein labeling mix (PerkinElmer) for 30 min. The protein extracts were then prepared as previously described[50,51], and 50 μg of total protein were precipitated with 10% TCA on filter paper 413 (VWR) for 30 min. The filter papers were then washed with 10% TCA twice for 5–15 min each and then with 1:1 ethanol/diethyl ether followed by drying and a second wash with diethyl ether or 100% ethanol and drying. The dried filter papers were immersed in 5 ml scintillation fluid and ^{35}S signals were counted. For control cultures, the protein synthesis inhibitor cycloheximide (10 μg/ml) was added before the labeling.

Measurement of cell growth. Conidia from 5- to 7-day-old *Neurospora* slants were inoculated into 150-mm petri dishes containing 1× Vogel's minimum medium with 2% glucose and were incubated at room temperature for 2 d under constant light. Mycelial discs of 7 mm were cut from the mycelial mats and cultured in 1× Vogel's minimum medium with 2% glucose at room temperature. Cultures were collected at the indicated time points (**Fig. 7c**) and dried in an oven at 80 °C for 1 d before the weight of the dried cell mass was determined.

Measurement of sensitivity to phytosphingosine. Measurement of phytosphingosine resistance was performed as previously described with modifications[35]. Conidia at 1×10^6 conidia/ml were germinated at room temperature shaker for about 5 h and then treated with phytosphingosine (Santa Cruz Biotechnology; 10 μg/ml in ethanol) for 2 h, with ethanol treatment as the control. Aliquots of the cultures were then diluted and spread onto fresh sorbose-containing plates and incubated at 30 °C until colonies appeared, and the colonies were counted and compared with the control to determine the survival rates.

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