Visualization of distinct substrate-recruitment pathways in the yeast exosome by EM

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The eukaryotic exosome is a multisubunit complex typically composed of a catalytically inactive core and the Rrp44 protein, which contains 3′-to-5′ exo- and endo-RNase activities. RNA substrates have been shown to be recruited through the core to reach Rrp44’s exo-RNase (EXO) site. Using single-particle EM and biochemical analysis, we provide visual evidence that two distinct substrate-recruitment pathways exist. In the through-core route, channeling of the single-stranded substrates from the core to Rrp44 induces a characteristic conformational change in Rrp44. In the alternative direct-access route, this conformational change does not take place, and the RNA substrate is visualized to avoid the core and enter Rrp44’s EXO site directly. Our results provide mechanistic explanations for several RNA processing scenarios by the eukaryotic exosome and indicate substrate-specific modes of degradation by this complex.

The eukaryotic exosome is a multisubunit protein complex crucial for RNA maturation, surveillance and turnover1–7. The exosome core is composed of six RNase PH-like subunits (Rrp41, Rrp45, Rrp42, Rrp43, Mtr3 and Rrp46) and three capping subunits containing RNA-binding domains (Rrp4, Rrp40 and Csl4)8, in a similar architecture to that of the archaeal exosome9–11. However, the exonuclease activity of the core has been lost in yeast and humans8,12 but gains its RNase activity by binding a tenth subunit, Rrp44 (refs. 12,13).

Rrp44 contains multiple functional domains (Fig. 1a). Its PilT N-terminus (PIN) domain exhibits manganese-dependent endonuclease (ENDO) activity14–16. The C-terminal EXO region consists of tandem cold-shock domains (CSD1 and CSD2), a magnesium-dependent 3′-to-5′ EXO RNDB domain and an S1 domain. The overall architecture of Rrp44’s EXO region is similar to that of bacterial RNase II, but the recruitment of RNA to the two enzymes differs17–20. Also, whereas RNase II can process only single-stranded (ss) RNA, Rrp44 can unwind and degrade duplex RNA17, probably via elastic-based helicase-like activity21.

Rrp44 associates with the core complex by binding of the PIN domain to subunits Rrp41 and Rrp45 (refs. 18,22,23). In tune with a similar function of the archaeal exosome, RNA substrates with long 3′ ss overhangs are first channeled through the eukaryotic exosome core before being degraded by Rrp44 (refs. 22,24). RNA degradation and protection assays indicate that ~31–33 stretched nucleotides are required to reach Rrp44’s EXO site from the top of the exosome core’s RNA-binding subunits22. This through-core route is clearly demonstrated by the crystal structure of the yeast Rrp44–exosome (RE) in complex with an RNA substrate with a 5′ hairpin and long 3′ ss overhang18. However, degradation of hypomethylated yeast initiator methionine tRNA (tRNAiMet)6 and tRNAs with a double CCA motif at the 3′ end25 by Rrp44 implies the presence of an RNA-processing pathway that does not necessarily involve channeling through the core. Recent transcriptome data also suggest the presence of alternative routes bypassing the core for RNA substrates with shorter 3′ ss overhangs to be processed by the exosome26. The multiporous structure of the apo-RE complex provides the potential for RNA substrates to take multiple routes including the through-core and direct-access pathways to reach Rrp44’s EXO site22.

To further dissect the mechanism of RNA recruitment to the exosome, we performed biochemical and single-particle EM analysis on the Saccharomyces cerevisiae RE in concert with different RNA substrates. Our single-particle analysis revealed a substrate-induced conformational change of the complex upon RNA binding and substrate-specific alternative routes of RNA recruitment by the exosome complex.

RESULTS

RNase assays for both through-core and direct-access routes

The through-core route and direct-access route predict distinct outcomes in the processing of the 3′ ss overhang of a highly structured RNA substrate. Although the through-core route stalls when the long ss overhang is trimmed down to ~30 nt, the direct-access route bypasses this restriction and predicts a processed ss overhang of probably less than 10 nt. We used a set of molecular-ruler experiments to distinguish these two pathways, by fusing unstructured AU-rich sequences of various lengths at the 3′ end of the highly structured hepatitis delta virus (HDV) ribozyme (Supplementary Fig. 1a,b).

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With a C75U mutation introduced to prevent the HDV-ribozyme cleavage, 5'−end 32P-labeled substrates with a stable tertiary structure27−29 were properly refolded for processing assays (Supplementary Fig. 1c). We carried out RNA processing assays of these substrates by Rrp44 alone and RE complex under single-turnover conditions in low Mg2+ (100 μM), under which the RE was more active12. The low-Mg2+ condition, however, also resulted in the partial destabilization of the 5' portion of the HDV structure beyond the tightly folded pseudoknot (after nucleotide G40), thus increasing the length of the 3' ss overhang by 33 nt. All HDV RNAs were efficiently processed by both Rrp44 and the RE, to produce ~5-nt completely digested products as well as one or two predominant processing intermediates (Fig. 1b and Supplementary Fig. 1d). The EXO active site mutant (D551N, EX−) but not the ENDO active site mutant (D171N, EN−) (Supplementary Fig. 1e), results suggesting predominant exonuclease activity from Rrp44 and the RE complex in our assays. Mapping of the HDV ribozyme with RNase T1 and OH ladder (Supplementary Fig. 1a,f) indicated that the single processing intermediate in Rrp44-alone reactions represents a stalling event near residue G40 of HDV RNA, presumably because of the blockage of the direct-access pathway by the tightly folded 5' pseudoknot. Interestingly, the same processing intermediate was also present in RE reactions, thus suggesting that a similar direct-access route is present in the complex. Formation of the short processing intermediate in RE reactions, owing to excess free Rrp44, is unlikely because our purification procedure ensures a 1:1 molar ratio between Rrp44 and the exosome core (Supplementary Fig. 1g). The complex's integrity was further supported by EM (Supplementary Fig. 1h). In the RE reactions, an additional processing intermediate ~26 nt longer than the Rrp44-reaction intermediate was evident for all HDV RNAs, results consistent with a processing event by the through-core route (Fig. 1b and Supplementary Fig. 1d). The above results suggest that although the through-core route is predominant (~70%), a direct-access route is simultaneously present for the HDV RNA degradation.

Two distinct RE conformations with different RNA substrates

We further performed three-dimensional (3D) reconstruction of RE complexes incubated with ssRNA substrates of different length (Supplementary Table 1) under nondigesting conditions (with 5 mM EDTA present to sequester the catalytic metal ions), under which all RNA substrates were able to bind the RE without detectable degradation (Supplementary Fig. 2). Interestingly, the series of 3D reconstructions revealed two distinct conformations of the RE, depending on the ssRNA length (Fig. 1c,d). When incubated with RNA substrates shorter than 12 nt, the resulting EM reconstruction of the complexes revealed a conformation (RE-short) very similar to that of apo-RE (Fig. 1c). By contrast, when the RNA substrates were longer than 14 nt, the RE adopted a distinct conformation (RE-long) (Fig. 1d). The major difference occurred within the Rrp44 protein, which is more compact in the RE-long than in the RE-short conformation, but the structure of the core complex was almost unchanged between the two conformers (Table 1 and Supplementary Tables 2 and 3). This RNA-induced conformational change is not necessarily restricted to purely ssRNAs, because the same conformation of RE-long was also present in the RE−HDV40 complex (Fig. 1d).

We docked atomic models into the two conformations to rationalize the observed conformational changes. For the RE-short 3D reconstruction, the crystal structure of the human core exosome (PDB 2NN6) fits nicely in the map as a rigid body, whereas the crystal structures of Rrp44’s PIN domain and C-terminal EXO region

Table 1 Cross-correlation coefficients among different 3D models

|                | Apo-RE  | RNA08-RE | RNA10-RE | RNA12-RE | SA-RNA47-RE |
|----------------|---------|----------|----------|----------|-------------|
| RNA47−RE core | 0.9323  | 0.9265   | 0.9287   | 0.9135   | 0.8892*     |
| RNA47−RE Rrp44| 0.6822  | 0.6443   | 0.6573   | 0.6573   | 0.6849      |

The top row is the comparison among different models at the core; the bottom row is the comparison among different models at the Rrp44 portion.

*The comparison is calculated at the core region only, with SA density removed.
Figure 2 Comparison and analysis of the RE-short and RE-long 3D models. (a,b) Docking of crystal structures into the RE-short (a) and RE-long (b) 3D models. The front, back and bottom views of both docking models are shown. The color coding of the different subunits in the core complex follows the name list on the left in a. The cleft between CSD and RNB domains of Rrp44 that could recruit RNA substrate is marked with an arrow in a. (c,d) Front views of the RE-short (c) and RE-long (d) docking models, with CSD1 (green), CSD2 (purple), RNB (blue) and S1 (orange) domains labeled. The orange arrows in c show the rotation orientation for Rrp44’s C-terminal EXO region around the pivotal point (circled) from the RE-short to RE-long conformation. (e,f) Vertical section of the RE-short (e) and RE-long (f) docking models. The orange arrow in e indicates the CSD2 movement from the RE-short to RE-long state.

from the Rrp44–Rrp41–Rrp45 subcomplex structure (PDB 2WP8) fit well in the map as separate rigid bodies (Fig. 2a and Supplementary Video 1). In the docked model, the overall spatial relationship among Rrp44, Rrp41 and Rrp45 is very similar to that in the crystal structure of the subcomplex except that the PIN domain moves ~10 Å away from Rrp44’s EXO region. For the RE-long 3D reconstruction, we took the recently published crystal structure of the yeast RE in complex with a partial hairpin RNA substrate (PDB 4IFD) and docked it in the map as a rigid body. The crystal structure fit with good agreement into the 3D envelope of the RE-long structure, except that the PIN domain of Rrp44 needed to be shifted by about 10 Å closer to the EXO region (Fig. 2b and Supplementary Video 2). The high level of agreement between the two independently determined structures indicates the strong biological relevance of these two conformations. Compared to the crystal structure, the EM reconstructions all had a substantially weaker density for Csl4 on top of the core, in agreement with previous observations that Csl4 is prone to dissociation from the complex during purification.

Close examination of the two models revealed that the major structural change occurring in Rrp44 concerns a rigid-body rotation (~120 degrees) of its EXO region from the RE-short to RE-long state (r.m.s. deviation of 4.3 Å between the two atomic models, as determined with ProFit (http://www.bioinf.org.uk/software/profit/); Fig. 2c). In the RE-short conformation, there are two solvent-exposed openings in Rrp44: the opening between the PIN and the CSD domains and a major cleft between the CSD1 and the RNB domain that makes the EXO site exposed to solvent (Fig. 2a). This cleft has been shown to bind ssRNA substrates that enter the EXO site for degradation. The two openings are separated by the CSD domains in the center, with the CSD2 domain blocking the way from the core channel’s bottom exit to Rrp44’s EXO site (Fig. 2c,e). In contrast, in the RE-long conformation, the Rrp44’s region undergoes a dramatic rotation as it rolls against the bottom of the core and pivots around the CSD1 domain, so that the CSD2 domain moves away from the core channel while the RNB domain moves closer to the core channel (Fig. 2d,e and Supplementary Video 3). As a result, the cleft between the CSD1 and the RNB domain moves just to the core channel’s bottom exit, thus forming a continuous route for ssRNA substrates to thread from the core to Rrp44’s EXO site. In agreement with this notion, the RNA molecule from the crystal structure fits nicely within the central channel (Fig. 2f).

Our series of 3D reconstructions revealed that only ssRNAs longer than 14 nt induce the dramatic conformational change in Rrp44 within the RE, whereas ssRNAs shorter than 12 nt maintain the same apo-RE conformation. Such a dramatic structural transition in response to the ssRNAs’ length agrees with the crystal structure of the RNA–RE complex, in which interpretable electron density for the RNA substrate’s extreme 3’ end extends from the bottom exit of the core to Rrp44’s EXO site. In the crystal structure, RNA nucleotides −13 to −15 interact with the Rrp45 and Rrp41 subunits, whereas nucleotides −1 to −12 interact entirely with Rrp44. This indicates that ssRNA substrates with enough length to connect the core to Rrp44 are ready to induce Rrp44’s dramatic rearrangement.

EM of RE in complex with RNAs with long 3’ overhangs

In order to visualize the RNA-recruitment pathways to the exosome, we preformed complexes between streptavidin (SA) and 5’-biotinylated ssRNA oligonucleotides of various lengths (SA–RNAs; Supplementary Table 1 and Supplementary Fig. 2b), incubated the SA–RNAs with RE complexes under nondigesting conditions and sought to locate the SA density in the resulting negative-stain EM reconstructions as an indicator of the ssRNA substrates’ 5’ ends. Single-particle two-dimensional (2D) classification of such samples allowed us to detect additional globular densities attached to the RE (Fig. 3a), the sizes of which agree well with a SA tetramer. There were substantially more class averages showing the additional SA densities for the ssRNA substrates longer than 36 nt but very few with SA densities for the ssRNA substrates shorter than 24 nt (Fig. 3b). This result was in agreement with pulldown assays using RE complexes as bait to detect horseradish peroxidase (HRP)-conjugated SA–RNA binding (Supplementary Fig. 3a). The observation reflects that SA prevents RNAs with short 3’ ss overhangs (<24 nt) from forming stable complexes with the RE but does not affect those with long enough 3’ ss overhangs (>36 nt) to thread through the core’s entry site to Rrp44’s EXO site to form a stable complex. We performed 2D classification and 3D reconstruction of the SA–RNA47–RE and SA–RNA50–RE and revealed a clear shape of the RE with a globular density attached to the entry side of the core complex in proximity to the Rrp4 protein (Fig. 3c).
The dimension of the globular density agrees very well with the size of a SA tetramer, so that we can unambiguously dock a SA atomic model within it (Fig. 3d,e). Interestingly, the 3D model shows the same conformational state of Rrp44 seen in the RE-long conformation (Fig. 3g,h), as verified by comparison of the 2D class averages with reprojections from the 3D models of RE-short and RE-long conformations (Supplementary Fig. 3b,c). This further proves that RNAs’ passage through the core channel and reaching Rrp44 are responsible for the RE’s conformational change. That RNAs with very different sequences, such as the 50-mer and 47-mer, behave similarly in their interactions with the RE underscores the importance of the single-stranded nature of RNA substrates over their specific sequences.

**EM of RE in complex with RNAs with short 3′ ss overhangs**

As we have revealed, in contrast to the longer RNA substrates, SA-labeled RNA substrates shorter than 24 nt do not seem to form a very stable complex with the RE in the nondigestive condition in the presence of 5 mM EDTA (Supplementary Fig. 3a), because SA prevents the relatively short ss overhang from reaching Rrp44’s EXO site through the core. However, in the presence of 2 mM MgCl₂, under which the RE is active for RNA processing, we found that SA–RNA substrates with a 3′ ss overhang shorter than 24 nt can also form reasonably stable complexes with the RE that can be characterized by EM (Fig. 4a). We were able to detect additional densities corresponding to the dimension of a SA in a portion of class averages from the SA–RNA24–RE sample, albeit fewer than in the SA–RNA47–RE. The RE portion of 2D class averages of SA–RNA24–RE compared reasonably well with reprojections of the 3D model of apo-RE, thus allowing us to assign each class average to a specific Euler angle in 3D space. The comparison between the 2D class averages and corresponding reprojections showed almost no SA additional density appearing at the top of the core complex.
in contrast to that for the SA–RNA47–RE sample, but it did occasionally show additional SA densities attached to other parts of the RE. Unfortunately, our efforts to obtain a 3D reconstruction from these 2D class averages were not successful, presumably because: (i) The 2D class averages with clear SA densities were not enough to cover the full set of views necessary for 3D reconstruction; and (ii) There may exist more than one SA-labeling site on the RE. We back-projected the 2D class averages into 3D space, with their SA densities as rods, on the basis of the Euler angle assigned to each 2D class average (Supplementary Fig. 4). This allowed us to capture two major cross-points of SA back-projection rods around the RE 3D model, thus allowing us to locate the most-probable SA locations attached to the RE in 3D space (Fig. 4b).

In the SA–RNA24–RE sample compared with the SA–RNA47–RE sample, the SA density appeared mostly close to Rrp44. The locations identified are around the RNB domain, facing its front and back sides. Both locations are close to the cleft between Rrp44’s CSD and RNB domains, which has been shown by crystallography to be able to recruit short ssRNAs17,18,22 (Fig. 4c). Therefore, the SA densities directly attached to these two sites provide strong evidence of RNA substrates’ direct access to Rrp44 by bypassing the core. In correlation with the RNase assays of the HDV-ribozyme substrates by Rrp44 and the RE, this observation suggests that Rrp44 maintains the capability to directly recruit RNAs when in complex with the core. We also detected a minor population of 2D class averages with the SA density near Rrp44’s PIN domain, results implying that the PIN domain’s endonuclease site may also recruit RNA substrates directly.

**Time-resolved EM of RNA degradation by the RE**

To directly monitor the exosome’s RNA-processing reaction, we mixed RE with SA-labeled RNA47 under the processing condition (2 mM Mg2+ in buffer) and took samples at different reaction time points for single-particle EM analysis as done above. This revealed 10–30% of 2D class averages with clear additional densities corresponding to RE-bound SAs. We grouped the actively processing SA–RNA–RE complex into three major families on the basis of the locations of SA in the 2D class averages (Fig. 5): (i) particles with a single SA density at the top of the RE, corresponding to the complex recruiting RNA substrates via the through-core pathway (Fig. 5a); (ii) particles with one single SA density around the bottom part of the RE, corresponding to the complex recruiting RNA substrates by the direct-access pathway (Fig. 5b); and (iii) particles with two SA densities attached to the RE at the top and bottom, corresponding to the complex recruiting RNA substrates via both pathways simultaneously (Fig. 5c).

We examined the distribution of particles within each family during the reaction (Fig. 5d). Because RNA47 has a long ss overhang, it is expected to be recruited to the EXO site of Rrp44 primarily via the through-core route. Indeed, in all the reaction time points, family 1 was the most populated of the three families. In agreement with processive degradation, the population of family 1 particles decreased as the reaction time went on, results indicative of the release of shortened SA–RNA substrates from the core channel. In contrast, family 2, albeit less populated than family 1, remained at roughly the same percentage at all reaction time points. Together with a similar assay of SA–RNA24 substrates (Supplementary Fig. 5 and Supplementary Note), this suggests that the direct-access route of RNA-substrate recruitment is less competitive than is the through-core route on RNAs with long 3′ ss overhangs, but it works as a distinct route toward deeper processing for RNA substrates with short 3′ ss overhangs. The very minor population of family 3 particles at the beginning of the reaction may reflect competition between the through-core and direct-access routes for RNA recruitment for some RE complexes.

The results above suggest that the RE may adopt a mixture of states during the processing of RNA substrates. In agreement with this, 3D reconstruction from a full set of particle images taken of RE complexes in the presence of both RNA and Mg2+ showed a much weaker density of the Rrp44 region (Supplementary Fig. 6), results indicative of a highly mobile nature of Rrp44 during RNA processing. Supervised classification of the above data set revealed the coexistence of roughly 30% RE-short and 70% of RE-long conformers (Supplementary Fig. 6), thus verifying the coexistence of, and possible dynamic transition between, the two recruitment pathways.

**Cryo-EM reconstruction of RE in complex with tRNA substrates**

tRNAs are major substrates under the quality control of the exosome and related complexes such as TRAMP, which adds short poly(A) tails (6–15 nt) to the 3′ ends of RNAs before processing30–32. To examine the RE’s transactions with tRNA substrates, we produced various tRNAs with different 3′-end poly(A) tails from 0 nt to 20 nt by using *in vitro* transcription (lacking post-transcriptional modifications) and performed degradation assays with Rrp44 and RE complexes. tRNAs with tails of at least 10 nt were efficiently processed by Rrp44, whereas...
the tail-less tRNA produced only faint degradation products (Fig. 6a). These results differ slightly from those in another published report, and this is probably because our assays used a four-fold-higher concentration of Mg$^{2+}$ to prevent unfolding of the tRNA. The removal of the 3′ ss overhang from the tRNA by the RE occurs only when the overhang is 20 nt or longer (Fig. 6a). Because an intermediate overhang length of 20 nt is too short for channeling through the core but is longer than is required for degradation by Rrp44 alone, this suggests that direct-access products can be generated by the RE and that the core slightly modifies the pathway of the RNA to Rrp44.

In order to directly visualize tRNAs’ interaction with the RE, we performed cryo-EM 3D analysis of a frozen-hydrated specimen of the RE in complex with unmodified yeast tRNA$^{Met}$. Using the single-particle 3D maximum-likelihood classification method, we classified the data set and reconstructed two major conformers, one as a model of apo-RE and the other as a model of the RE with clear additional density attached to the pocket formed by Rrp44, Rrp45 and Rrp43 at the predicted direct-access route (Fig. 6b). The additional density of the latter model can be fitted with the atomic model of a tRNA (PDB 4TNA) with its 3’ overhang inserted into the RE complex 26 and 27. The physiological role of the through-core route for RNA degradation has been firmly established from both in vitro and in vivo assays.
The core complex 26. The same work also revealed substantial sub-

Further cleaved by the direct-access pathway after retracting from

May first be processed via the through-core pathway and then be

Processing, which removes the 3′-end overhangs and is responsible for the processing of different regions of the same

Figure 7 A hypothetical model for the degradation routes of RNA

Substrates with 3′-ss overhangs have been released from the central channel due to

The tension built in the complex (4). It may be further processed via the

Direct-access route leads to either further cleavage of the 3′-end tail and

Release of the processed RNA or full degradation of the RNA substrate (6).

The most recent yeast genetics analysis revealed that the central channel

Of the exosome core is essential for the function and viability of

Budding yeast.35,36 But this does not preclude the presence of an

Alternative RNA-recruitment route, which could be very important

In processing a subset of RNA substrates. The existence of a possible
direct-access pathway for RNA recruitment and processing by the RE

Was first hypothesized on the basis of our EM reconstruction of the

RE complex23 and was also implied from genetic and biochemical work from others,6,25 but no direct observation of such a route was available. Our data here provide the first direct visual evidence, to our

Knowledge, of such an RNA-recruitment pathway that bypasses the core channel. Compared to the through-core route, the direct-access route has a weaker affinity for RNA yet contributes substantially to

RNA processing even at the beginning of a reaction. The direct-

Access route is likely to have a more dominant role in the processing

Or degradation of RNA substrates with 3′-ss overhangs shorter than

30 nt, for both single-stranded and structured RNAs. This agrees with the

Transcriptome analysis that reported that structured RNAs with

Short 3′-ss overhangs may be released from the central channel due to

The tension built in the complex (4). It may be further processed via the

Direct-access route leads to either further cleavage of the 3′-end tail and

Release of the processed RNA or full degradation of the RNA substrate (6).

Methods

Methods and any associated references are available in the online

Version of the paper.

Accession codes. The 3D reconstruction electron density maps obtained by negative-stain EM have been deposited into the Electron Microscopy Data Bank under accession codes EMD-2491 (RE–RNA08), EMD-2492 (RE–RNA10), EMD-2493 (RE–RNA12),

EMD-2494 (RE–RNA14), EMD-2495 (RE–RNA16), EMD-2496 (RE–RNA24), EMD-2497 (RE–RNA36), EMD-2498 (RE–RNA47),

EMD-2499 (RE–SA–RNA47), EMD-2500 (RE–SA–RNA50) and

EMD-2522 (RE–HDV40). The 3D reconstruction electron density

Maps obtained by cryo-EM have been deposited into the Electron Microscopy Data Bank under accession codes EMD-2501 (RE–tRNA) and EMD-2502 (apo-RE), respectively.

Note: Any Supplementary Information and Source Data files are available in the online

Version of the paper.

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Author contributions

J.-J.L., X.L., C.-Y.N. and H.-W.W. performed EM and single-particle analysis. J.-J.L., M.A.B., X.L. and C.-Y.N. purified the exosome complex. M.A.B. and A.K. performed RNA degradation assays of wild-type and mutant complexes. J.-J.L., M.A.B., X.L., A.K. and H.-W.W. planned the experiments. J.-J.L., M.A.B., A.K. and H.-W.W. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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ONLINE METHODS

Tandem affinity purification (TAP) and reconstitution of S. cerevisiae exosomes. Purification of the core and Rrp4–exosome was done as described with minor modification. Briefly, ~500 g of TAP–Rrp46 ΔRrp6 yeast cells were lysed with bead-beaters, and after ultracentrifugation the supernatant was loaded onto 3 ml of Rabbit IgG resin (Sigma–Aldrich). The resin was extensively washed with high-salt buffer (500 mM NaCl), and the Rrp44–exosome was eluted by column TEV cleavage to remove the protein A tag. For purifying the core exosome, Rrp44 was removed from the complex by washing with 40 ml of 1.2 M MgCl₂. The mutant Rrp44–exosome complexes were formed by mixing of a 50% amount of purified recombinant mutant Rrp44 (EX-, D551N; EN-, D117N; or DM, D551N D117N) with the core exosome at 4 °C overnight. All Rrp44–exosome complexes used in the biochemical assays, wild type or mutant, were further purified on an ion-exchange Mono Q 5/50 GL column (GE Healthcare), on which any excess Rrp44 and endogenous nucleic acids were well separated from the Rrp44–exosome complexes (Supplementary Fig. 1g). Samples were flash frozen in liquid nitrogen and stored at −80 °C until later use.

RNA degradation assays. HDV and tRNA substrates were produced by RNA polymerase T7–based in vitro transcription. RNA was separated on a 10% polyacrylamide/8 M urea gel and RNA visualized by UV shadowing. The RNA band was excised from the gel, crushed, and eluted in nuclease-deionized water at 4 °C overnight.

The purified RNAs were dephosphorylated with FastAP alkaline phosphatase (Fermentas), phenol extracted, ethanol precipitated, and dissolved in nuclease-free water. RNA was 5’-end labeled with polynucleotide kinase (New England BioLabs) and 2 μl of 3,000 Ci/mmol [γ-32P]ATP (Perkin Elmer), purified on 10% polyacrylamide/8 M gels, eluted and ethanol precipitated. RNA pellets were washed with 80% ethanol, air dried, dissolved in a final volume of 30 μl nuclease-free water, and stored at −20 °C until use.

Immediately before use, RNA substrates were refolded in 2× reaction buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, and twice the concentration of MgCl₂ used in the assay) by heating to 80 °C for 5 min and subsequent snap cooling at 4 °C. All reactions were performed in 1× reaction buffer, with a molar ratio of protein to RNA of 2:1, and incubated at 30 °C for indicated time points. The reactions were terminated by addition of an equal volume of formamide plus 10 mM EDTA and heating of the solution at 65 °C for 10 min. The reaction products were analyzed on 16% acrylamide/8M Urea sequencing gels in 0.5× TBE and quantified by a Typhoon phosphorimager (GE Healthcare).

Preparation of exosome-coupled IgG resin beads. An amount of 100 ml supernatant of TAP–Rrp46 ΔRrp6 yeast lysate was incubated with rabbit IgG affinity resin beads (Sigma–Aldrich) at 4 °C for 2 h with constant stirring. The resin was then centrifuged at 500 r.p.m. for 2 min and washed three times each with 10 ml washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% glycerol with 2 mM DTT, 2 mM EDTA and 1 tablet of complete EDTA-free protease inhibitor mixture from Roche Applied Science). The resin was then washed three times each with 10 ml of high-salt buffer (washing buffer plus 250 mM NaCl), and the beads were re-equilibrated three times with 5 ml washing buffer.

Gel-shift and pulldown assays. Streptavidin (SA) was purchased from Sigma, and HRP-coupled streptavidin (HRP-SA) was from Abicon. For gel-shift assays, proteins and RNAs were mixed together in a 4:1 molar ratio in nuclease-free water and incubated on ice for 5 min, then run on a 0.5% acrylamide native gel in 0.5x TBE buffer. The gels were subsequently stained with ethidium bromide and imaged in a regular UV-illuminated gel scanner.

For pulldown assays, HRP-SA and RNAs were mixed together in a 4:1 molar ratio and incubated on ice for 5 min. 1 μl of HRP-SA–RNA complex (2.5 μM) was incubated with 30 μl of exosome-coupled IgG resin in 500 μl of reaction buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM DTT) at room temperature (~22 °C) for 15 min with occasional stirring. The mixture was centrifuged at 500 r.p.m. for 2 min, and the resin was washed three times, each with 1.5 ml reaction buffer. 2 μl supernatant or beads were loaded on nitrocellulose film. The film was subsequently incubated in ECA agent for HRP signal development as in normal western blotting.

Electron microscopy sample preparation. The RNA–RE complex for EM analysis was prepared by mixing of RNA and RE in a 1:2.1 molar ratio in a nondigestive buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 1 mM DTT at room temperature for 15 min. For preparation of SA–RNA–RE sample in nondigestive condition, SA and biotinylated RNAs were first mixed together in nuclease-free water and incubated on ice for 15 min in a 2:1 molar ratio and diluted to a final concentration of 6 μM of RNA. Subsequently, the SA–RNA mixture was incubated with RE complexes as described above in a molar ratio of 1:2:1. All samples were diluted at a final concentration of ~80 nM of the exosome in the nondigestive buffer and negatively stained in 2% (v/w) uranyl acetate solution after the standard deep-stain procedure on holey carbon–coated EM copper grids covered with a thin layer of continuous carbon. For SA–RNA–RE samples in magnesium condition, everything followed the same procedure except that the nondigestive reaction buffer was replaced by a magnesium buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2 mM MgCl₂) and the incubation time of SA–RNA and RE was 5 min.

Time-resolved electron microscopy sample preparation. To monitor the structure of SA–RNA being processed by the RE in a degradation reaction, we first mixed 4 μl of SA–RNAs produced as described above with 2 μl of 3× reaction buffer (150 mM Tris–HCl, pH 8.0, 450 mM NaCl, 6 mM MgCl₂) at 30 °C. Subsequently, we added 4 μl of RE complexes (2.5 μM dissolved in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2 mM MgCl₂) to the reaction system to initiate the reaction. At given reaction time points, the reaction was terminated with the addition of 10 μl termination buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA) and immediately cooled on ice. 4 μl of the final solution was then negatively stained.

Electron microscopy and image processing. We examined all the negative-stained specimens on an FEI Tecnai–F20 electron microscope operated at 200 kV acceleration voltage at 50,000 nominal magnification with a range of defocus from 0.8 to 1.2 μm. The electron micrographs were taken on a Gatan Ultrascan 4000 4k × 4k CCD camera.

The digital micrographs taken from the CCD camera were then processed with the EMAN2 package to semiautomatically pick the individual particle images of the complexes and with the IMAGIC-4D package to determine and correct the contrast transfer functions by the phase-flip method. For each sample, 20,000–50,000 negatively stained particle images were analyzed two-dimensionally with IMAGIC-4D. Briefly, we performed iterative multivariate statistical analysis (MSA) and multireference alignment (MRA) cycles on the particle stacks with the IMAGIC-4D package to obtain 2D class averages as described previously. We performed 3D reconstruction of all the samples with projection-matching refinement of particle stacks against the same initial model of apo-RE (EMD-1439, ref. 23) low-pass filtered to 70 Å in SPIDER as described previously. The resolution of final reconstruction was estimated with the Fourier shell correlation (FSC) algorithm at criteria of 0.5. The number of particles contributing to and the resolution of each 3D reconstruction (measured by FSC 0.5 criteria) are summarized in Supplementary Table 4. The distinct differences among the final reconstructions indicated that our results are free of model bias. This was also verified by a totally different initial model of an elongated bar converging to the same structures of the RE sample after about 30 rounds of projection-matching refining iterations. The model bias–free nature of the RE sample is very likely to be due to its strong intrinsic structural features in the high-contrast negative-stained images.

The docking of atomic models was performed by UCSF Chimera with fit-model-in-map functions. All the 3D models in the figures are rendered in UCSF Chimera.

Cryo-electron microscopy of RE in complex with tRNA. We incubated 250 nM RE with 1 μM unmodified yeast tRNA in reaction buffer (50 mM Tris–HCl, 150 mM NaCl and 2 mM MgCl₂) on ice for 10 min. 4 μl of the reaction solution was then applied to glow-discharged C-flat grids (1.2/1.3) covered with a layer of continuous carbon with a thickness of ~4 nm. The grids were then blotted and plunged into liquid ethane in a FEI Vitrobot Mark IV. The frozen-hydrated grids were subsequently examined under a FEI Titan Krios electron microscope.
operated at 120-kV acceleration voltage. Micrographs of RE complexes were collected with AutoEMation43 installed on the microscope at low-dose condition with a dose of ~20 electrons/Å² and a defocus value ranging from −1.2 to −4 µm. The micrographs were collected on a FEI Eagle CCD camera with a pixel size of 1.5 Å. We used the EMAN2 package to perform raw micrograph screening, CTF determination, and semi-automatic particle picking39 and obtained a data set of about 61,000 particle images. We then performed 3D refinement and classification of the particle images with the Relion package44. After the first 25 iterations of classification, we identified two major classes of particles (accounting for more than 56,000 images) with good quality of reconstruction out of six classes. We further performed 3D refinement of the two classes and got a final reconstruction of the RE–tRNA complex from about 28,800 particles and of the apo-RE complex from about 27,200 particles, respectively. The numbers of particles contributing to and the resolution of each 3D reconstruction (measured by FSC 0.5 criteria) are summarized in Supplementary Table 4.

Back-projection analysis of SA–RNA24–RE complex. For back-projection analysis of the SA–RNA24–RE complex, we calculated the Euler angle of the 2D class averages by first masking off the SA densities in the 2D class averages and then matching them to re-projections of the 3D model of apo-RE. The corresponding view of the 3D model of apo-RE matching to a 2D class average was then displayed in UCSF Chimera. A 3D model of a rod perpendicular to the view was added in UCSF Chimera to represent the back-projection of the corresponding SA density in the 2D class average. By applying this procedure to all the views of the 3D model matching to the 2D class averages with SA density, we finally visualized the cross-point of all the rods, assuming that the most crossed points have a higher probability to represent the location of SA in the 3D model.

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