The exosome-binding factors Rrp6 and Rrp47 form a composite surface for recruiting the Mtr4 helicase

Benjamin Schuch¹, Monika Feigenbutz², Debora L Makino¹, Sebastian Falk¹, Claire Basquin¹, Phil Mitchell²*, & Elena Conti¹**

Abstract

The exosome is a conserved multi-subunit ribonuclease complex that functions in 3’ end processing, turnover and surveillance of nuclear and cytoplasmic RNAs. In the yeast nucleus, the 10-subunit core complex of the exosome (Exo-10) physically and functionally interacts with the Rrp6 exoribonuclease and its associated cofactor Rrp47, the helicase Mtr4 and Mpp6. Here, we show that binding of Mtr4 to Exo-10 in vitro is dependent upon both Rrp6 and Rrp47, whereas Mpp6 binds directly and independently of other cofactors. Crystallographic analyses reveal that the N-terminal domains of Rrp6 and Rrp47 form a highly intertwined structural unit. Rrp6 and Rrp47 synergize to create a composite and conserved surface groove that binds the N-terminus of Mtr4. Mutation of conserved residues within Rrp6 and Mtr4 at the structural interface disrupts their interaction and inhibits growth of strains expressing a C-terminal GFP fusion of Mtr4. These studies provide detailed structural insight into the interaction between the Rrp6–Rrp47 complex and Mtr4, revealing an important link between Mtr4 and the core exosome.

Keywords nuclear exosome; RNA degradation; X-ray crystallography; yeast genetics

Subject Categories RNA Biology; Structural Biology

Introduction

Most cellular ribonucleic acids are transcribed in the nucleus as larger precursor molecules that are then processed to produce mature, functional RNAs. The maturation of ribosomal RNAs (rRNAs), small nuclear RNAs and nucleolar RNAs (snRNAs and snoRNAs) requires the trimming of the extended 3’ end of their nascent transcripts and the elimination of the excised RNA fragments (reviewed in Bernstein & Toth, 2012). These processes involve a complex of ribonucleases known as the RNA exosome (Allmang et al., 1999a). In addition to 3’ end processing, the exosome functions in RNA turnover and surveillance pathways (reviewed in Schmid & Jensen, 2008; Schaeffer et al., 2011; Chlebowski et al., 2013). In the nucleus, it mediates the turnover of precursor transfer RNAs (pre-tRNAs) and precursor messenger RNAs (pre-mRNAs) (Gudipati et al., 2012). The nuclear exosome also swiftly eliminates misprocessed iRNAs (Kadaba et al., 2004) and pre-mRNAs (Bousquet-Antonelli et al., 2000; Hilleren et al., 2001) as well as cryptic unstable transcripts (CUTs) generated by antisense and intergenic transcription (Wyers et al., 2005; Davis & Ares, 2006; Neil et al., 2009). In the cytoplasm, the exosome participates in the turnover of mature mRNAs (Anderson & Parker, 1998) and in quality-control pathways that eliminate defective mRNAs with premature stop codons (Mitchell & Tollervey, 2003) or without a stop codon (van Hoof et al., 2002).

The exosome core complex consists of ten subunits that are evolutionarily conserved and are essential in yeast (Allmang et al., 1999b). Nine of these subunits form a catalytically inactive barrel-like structure (Exo-9) (Liu et al., 2006; Dziembowski et al., 2007) that threads RNA substrates to the tenth subunit, Rrp44 (also known as Dis3) (Bonneau et al., 2009; Malet et al., 2010; Wasmuth & Lima, 2012; Makino et al., 2013a; Liu et al., 2014). Rrp44 is bound at the bottom of the Exo-9 barrel and contains a processive 3’–5’-exoribonuclease site and an endonuclease site (reviewed in Schneider & Tollervey, 2013; Makino et al., 2013b). While the Exo-10 core is found in both the nucleus and the cytoplasm, physical and genetic interactions have linked the exosome core to several cofactors that have specific subcellular localization (reviewed in Schneider & Tollervey, 2013). In the cytoplasm, the exosome functions together with the Ski complex, a multi-subunit assembly centered at the helicase Skl2 (Anderson & Parker, 1998; Araki et al., 2001; Halbach et al., 2013). In the yeast nucleus, the exosome is associated with a set of conserved proteins that include Rrp6 (known as PM/Scl-100 in humans), Rrp47 (also known as Lrp1 in yeast and as C1D in humans), Mpp6 and Mtr4 (also known as Doh1 in yeast) (reviewed in Butler & Mitchell, 2011).
Rrp6 contains a 3’–5’-exoribonuclease site. In contrast to Rrp44, the Rrp6 nuclelease functions in a distributive manner and stalls when encountering structured RNA sequences (Briggs et al., 1998; Burkard & Butler, 2000; Liu et al., 2006; Januszky et al., 2011). Rrp6 binds the exosome directly, near the top of the Exo-9 barrel (Cristodero et al., 2008; Makino et al., 2013a; Wasmuth et al., 2014). Although in vitro Rrp6 and Rrp44 can bind Exo-9 independently of each other, Exo-9 binding interconnects the enzymatic properties of the two ribonucleases (Liu et al., 2006; Wasmuth & Lima, 2012). The interplay between Rrp6 and Rrp44 also emerges from in vivo studies. During the maturation of 5.8S rRNA, Rrp44 degrades the 3’ end of the precursor to leave a processing intermediate that is then trimmed to the final product by Rrp6. This intermediate features a 3’ extension of 30 nucleotides (Briggs et al., 1998), a length that corresponds to the size of the internal channel of Exo-10 (Bonneau et al., 2009; Makino et al., 2013a). Rrp6 can carry out the last processing step even when separated from Exo-10 (Callahan & Butler, 2008).

Rrp47 and Rrp6 interact in vitro (Stead et al., 2007) and in vivo (Mitchell et al., 2003; Synovsky et al., 2009). Consistently, depletion of Rrp47 leads to defects in RNA processing and degradation that are similar to those observed in rpsA strains (Mitchell et al., 2003; Peng et al., 2003). The presence of Rrp6 protects Rrp47 from degradation in yeast and conversely Rrp47 stabilizes Rrp6 (Feigenbutz et al., 2013a,b; Stuparevic et al., 2013). Knock-out of either Rrp6 or Rrp47 is synthetically lethal with the absence of Mpp6, another factor that functions in the maturation of 5.8S rRNA, the degradation of CUTs and pre-mRNA surveillance (Milligan et al., 2008). The human orthologue of Mpp6 has been shown to interact with PM-Scl100–C1D (Rrp6–Rrp47) in co-immunoprecipitation experiments, and also with human Mtr4 (Schilders et al., 2007). Mtr4 is a Ski2-related RNA helicase (Jackson et al., 2010; Weir et al., 2010; Halbach et al., 2012) and is essential for viability in yeast (de la Cruz et al., 1998). Mtr4 is required for Rrp6-dependent and Rrp6-independent functions of the nuclear exosome (Jackson et al., 2010; Klauer & van Hoof, 2013) and is also part of the TRAMP complex (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). In human cells, Mtr4 has been shown to associate with either Mpp6 (Schilders et al., 2007) or Rrp6 (Lubas et al., 2011). In yeast, all genetic data suggest a close association between Mtr4 and the exosome, but no direct interaction has been reported thus far. In this work, we dissected the interaction network of the nuclear cofactors of the yeast exosome in vitro and identified the structural basis for how Rrp6 and Rrp47 assemble in a complex that directly recruits Mtr4.

**Results**

Saccharomyces cerevisiae Rrp6–Rrp47 recruits Mtr4 to the exosome

The domain organization of nuclear cofactors of the *S. cerevisiae* exosome is in several cases known from previous structural studies or can be extrapolated from sequence analysis (Fig 1A). The Rrp6 exoribonuclease is a modular protein of 733 residues. The Rrp6 N-terminal region (so-called PMC2NT) mediates the interaction with Rrp47 and is expected to be a folded domain (Stead et al., 2007). The central region encompasses the exoribonuclease (Exo domain), which includes the catalytic DEDD site and the regulatory HRDC domain (Midtgaard et al., 2006). The C-terminal region consists of mainly low-complexity sequences and contains both the Exo-9-binding segment and two nuclear localization signals (NLSs) (Callahan & Butler, 2008; Makino et al., 2013a). Rrp47 (184 residues) has an N-terminal domain that binds Rrp6 and a C-terminal low-complexity region rich in positively charged residues (Costello et al., 2011) (Fig 1A). The Mtr4 helicase (1073 residues) contains a low-complexity N-terminal region of 80 residues followed by a DEXH helicase core characterized by an insertion domain also known as the arch domain (Jackson et al., 2010; Weir et al., 2010) (Fig 1A). Mpp6 (186 residues) is a small basic protein without recognizable domains.

We recombinationally expressed and purified different versions of these proteins, incubated them in different combinations and analyzed the mixtures using size-exclusion chromatography to dissect their direct interactions (Fig 1B–G). In the case of Rrp6, we engineered a version of the protein that spans from the Rrp47-binding domain to the exosome-binding domain (Rrp6<sub>ANLS</sub>), as the inclusion of the very C-terminus resulted in an unstable sample that was quickly degraded. Rrp6<sub>ANLS</sub> co-eluted with full-length Rrp47 (Fig 1B, peak 1 in the size-exclusion chromatography profile on the left and lane 1 in the corresponding Coomassie gel on the right). Rrp6<sub>ANLS</sub>–Rrp47 did not interact with Mpp6 (Fig 1B, peaks and lanes 2 and 3). Instead, Rrp6<sub>ANLS</sub>–Rrp47 interacted with full-length Mtr4 (Fig 1B, peak and lane 2). Interestingly, Mtr4 required the Rrp6<sub>ANLS</sub>–Rrp47 complex to bind to Exo-9 (Fig 1C, compare peak and lane 1, with peaks and lanes 4 and 5). Rrp6 lacking the N-terminal Rrp47-binding domain (Rrp6<sub.AP</sub>) co-eluted with Exo-9 but lost most of the binding to Mtr4 (Fig 1C, peaks and lanes 5 and 6). The other nuclear exosome cofactor, Mpp6, interacted with Exo-9 both in the presence and in the absence of Rrp6<sub>ANLS</sub>–Rrp47 (Fig 1D, peak and lane 1, compare with peak and lane 2). Finally, Exo-9, Rrp6<sub>ANLS</sub>–Rrp47, Mtr4, Mpp6 and Rrp44 co-eluted in a single peak that

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**Figure 1. Direct interactions of the nuclear cofactors of the yeast exosome.**

A Schematic representation of the domain arrangement of the nuclear exosome cofactors from *Saccharomyces cerevisiae* used in this study. Gray-filled rectangles denote domains whose structures are known from previous studies: the central region of Rrp6 with the exonuclease (Exo) and HRDC domains (Midtgaard et al., 2006), the exosome-binding domain in the C-terminal region of Rrp6 (Makino et al., 2013a) and the entire helicase region of Mtr4 (Jackson et al., 2010; Weir et al., 2010). Colored rectangles highlight the N-terminal domains of Rrp6, Rrp47 and Mtr4 visualized in the structure of the ternary complex reported here. Truncation mutants engineered for the biochemical and structural analysis are indicated.

B–G Size-exclusion chromatography assay to assess formation of protein complexes. Purified samples (as indicated) were incubated and co-injected on an analytical size-exclusion column (Superdex 200 Increase 3.2/300, GE Healthcare, exclusion volume 0.8 ml). On the left are the overlays of the chromatograms (rel. AU and V<sub>r</sub>); the chromatograms show relative absorbance and retention volume of the proteins, respectively. On the right are the Coomassie-stained SDS–PAGE gels with samples from the corresponding peak fractions. The detailed analysis of the chromatography profiles is shown in Supplementary Table S1.
The N-terminal domains of Mtr4, Rrp6 and Rrp47 form a ternary complex in vitro

Consistent with previous studies (Stead et al., 2007; Costello et al., 2011; Dedic et al., 2014), we observed an interaction between the N-terminal regions of Rrp6 (Rrp6N, residues 1–111) and Rrp47 (Rrp47AC, residues 1–133) (Fig 1A). Rrp6N and Rrp47AC co-eluted with Mtr4 in size-exclusion assays (Fig 1E, peak and lane 1). Next, we assessed which part of Mtr4 is recognized by Rrp6–Rrp47. Rrp6N and Rrp47AC co-eluted with the N-terminal region of Mtr4 (Mtr4N) (Fig 1F, lane and peak 1), while no interaction was detected with the helicase domain (Mtr41–20) (Fig 1F, lane and peak 14). As a note, the cytoplasmic Ski2 helicase does not contain an analogous N-terminal sequence, and consistently, the cytoplasmic exosome complex does not contain Rrp6 and Rrp47. Finally, using limited proteolysis experiments, we could narrow down the Rrp6–binding domain of Rrp47 even further to residues 1–103 (Rrp47N) (Fig 1A and Supplementary Fig S1C).

Structure determination of the Rrp6N–Rrp47N–Mtr4N complex

Alone, Rrp47AC eluted as an apparent oligomer in size-exclusion chromatography (Fig 1G, peak 4), consistent with previous reports of its oligomeric nature when in isolation (Feigenbutz et al., 2013b). Upon co-expression, however, the Rrp6N–Rrp47AC complex eluted with a predominant peak corresponding to the expected molecular weight of a 1:1 complex (Fig 1G, peak 5). Rrp6N–Rrp47AC crystallized in a tetragonal space group with three independent binary complexes in the asymmetric unit (Supplementary Fig S2A). The structure was determined by combining phases from single-wavelength anomalous dispersion (SAD) experiments using crystals containing tantalum bromide and crystals of selenomethionine-substituted protein. The model has been refined to 2.35 Å resolution with an Rfree of 22.4%, Rfactor of 18.4% and good stereochemistry (Table 1).

Table 1. Crystallographic statistics.

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<td>0.043 (0.254)</td>
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Values in parentheses correspond to the highest resolution shell.
for crystallization and mediated lattice contacts. The model has been refined to 2.4 Å resolution with an $R_{\text{free}}$ of 24.3%, $R_{\text{factor}}$ of 20.1% and good geometry (Table 1). The atomic models of Rrp6 and Rrp47 are very similar in the three ternary complexes in the asymmetric unit and are also very similar when compared to the structure of the binary complex (root mean square deviation (rmsd) of 1.35 Å over 184 α-carbon atoms). The main difference is that in one of the three copies of Rrp6N–Rrp47D, twenty more residues of the C-terminal helix of Rrp47 are well ordered as a result of lattice contacts with a symmetry-related molecule (compare Fig 2A and B, left panel). The atomic model of Mtr4 shows well-defined electron density from residue 4 up to residue 17 (Fig 2B and Supplementary Fig S2C).

The Rrp6–Rrp47 interaction: an intertwined structure

The Rrp6N–Rrp47N complex has a compact α-helical fold (Fig 2B). At the secondary structure level, Rrp6N and Rrp47N are remarkably similar. They both consist of three long α-helices ($α_1, α_2$ and $α_3$)
with a short α-helix (αshort) between α1 and α2. The stretch of Rrp6N encompassing the first helix-turn-helix (α1–αshort–α2) can be superposed to the equivalent stretch of Rrp47N (rmsd of 1.99 Å over 39 αC atoms) (Fig 2C). The main topological difference between the two proteins is that helix α3 is oriented antiparallel to α2 in the case of Rrp47N, while it points in the opposite direction in the case of Rrp6N (Fig 2C and topology in D).

The secondary structure elements of Rrp6N and Rrp47N are highly intertwined (Fig 2B and D). The first helix-turn-helix of Rrp6N interdigitates with the first helix-turn-helix of Rrp47N, forming a heterodimeric 4-helix bundle (Figs 2D and 3A). The α3 helices of Rrp6N and Rrp47N pack against the side of the bundle that is lined by the α2 helices (Figs 2D and 3B). Evolutionarily conserved residues of the α1 and α2 helices form an extensive hydrophobic core in the center of the bundle (Figs 3A, 4A and B). The interactions between the α2 and α3 helices are also extensive, apolar and conserved (Figs 3B, 4A and B). The interaction of Rrp6N with Rrp47N buries 5,560 Å² (i.e., more than 33%) of the surface area of the two proteins. The complex appears to be further stabilized by inter-molecular salt bridges present on the outer surface of the heterodimer. Finally, the structure of the binary complex shows that the C-terminal helix of Rrp47 protrudes out of the globular core of Rrp6N–Rrp47AC, extending about 30 Å into the solvent (Fig 2A). The C-terminal region of Rrp47 that has been reported to interact with proteins involved in snoRNP assembly (Costello et al., 2011) would likely extend even further.

**Mtr4 binds Rrp6–Rrp47 via evolutionarily conserved interactions**

The overall structure of Rrp6N–Rrp47N resembles a turn of a superhelix with a conserved concave surface that is formed by the α1 helix of Rrp6 and the α2 and α3 helices of Rrp47 and that provides the binding site for Mtr4N (Figs 2B and 5A). Mtr4N binds as a short α-helix (residues 6–11), with extended segments at both ends. Apolar residues of Mtr4 (Leu6, Phe7, Val9, Phe10, Val15 and Leu17) contact hydrophobic residues of Rrp6 (Leu10, Ile14 and Val17) and Rrp47 (Tyr10, Tyr55, Phe62, Leu77, Leu80 and Met87) (Fig 5B). In addition, Mtr4 Glu12 forms a salt bridge with Rrp6 Arg18, while Mtr4 Asp5 interacts electrostatically with Lys84 of Rrp47. We tested the effect of mutating a set of the conserved interacting residues in vitro binding assays. Isothermal titration calorimetry (ITC) experiments showed that Rrp6N–Rrp47AC bound to the Mtr4N peptide with a $K_d$ of 1.3μM, but no binding to an Mtr4N F7A, F10A mutant was detected (Fig 5C, left and central panel). Conversely, an Rrp6N I14E, R18E–Rrp47AC mutant showed no binding to wild-type Mtr4N by ITC (Fig 5C, right panel). We note that there are also additional conserved residues, including Asp23 and Phe30 in the Rrp6 $\alpha_{short}$ helix and Rrp6 Glu90 and Asp97 on the convex surface of the superhelix (Fig 5A). These residues do not contact Mtr4N in the structure, and consistently, their mutation did not affect binding to Mtr4N in ITC experiments (Supplementary Fig S3A).

**Impact of Rrp6N–Rrp47AC on RNA binding and degradation**

The concave surface of Rrp6N–Rrp47N that binds Mtr4N is highly positively charged (Fig 6A). Given these electrostatic properties and previous reports that Rrp47 binds RNA (Stead et al., 2007), we tested whether the interaction between Rrp6 and Rrp47 might also serve as an RNA-binding site (at least in the absence of Mtr4). Since the structure of the N-terminal region of Rrp6 is expected to be unfolded in the absence of Rrp47, we compared Rrp6N–Rrp47AC, extending about 30 Å into the solvent (Fig 2A). The C-terminal region of Rrp47 that has been reported to interact with proteins involved in snoRNP assembly (Costello et al., 2011) would likely extend even further.

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Structure of the Rrp6-Rrp47-Mtr4 interaction

A

B

C

Figure 4. Structure-based sequence alignments of Rrp6α, Rrp47α, and Mtr4α.

A-C The alignments of Rrp6 (A), Rrp47 (B) and Mtr4 (C) include orthologs from the representative species Saccharomyces cerevisiae (S.c.), Neurospora crassa (N.c.), Homo sapiens (H.s.), Drosophila melanogaster (D.m.) and Danio rerio (D.r.), based on a comprehensive alignment. The secondary structure elements are shown above the sequences. Conserved residues are highlighted in color. Colored circles above the sequences identify residues involved in the interaction with Rrp6 (red circles), with Rrp47 (orange circles) and with Mtr4 (blue circles). Circles of two colors indicate residues involved in interactions with two partners in the ternary complex. Residues targeted for mutagenesis are highlighted with a black square.

protein–protein interaction site for Mtr4α rather than an RNA-binding site. Rrp61α, Rrp62, D296N and Rrp61α, D296N-Rrp47AC bound RNA with a similar affinity in the low micromolar range (Fig 6B). In RNA degradation assays, the Rrp61α and Rrp61α, D296N-Rrp47AC complex showed some-what lower activity as compared to that of Rrp61α, D296N (Fig 6C), indicating that the Rrp61α-Rrp47AC module subtly downregulates the enzymatic properties of the Rrp6 ribonuclease. Although the rationale for this effect is currently unclear, similar observations have been recently reported (Barbosa et al., 2014; Dedic et al., 2014).

We carried out a set of degradation assays of Rrp61α, D296N-Rrp47AC in the presence of Mtr4, with and without the other subunits of the nuclear exosome complex (Supplementary Fig S3B). We first tested a double-stranded substrate with a short 3' overhang (10 nucleotides) that from previous work is known to be inaccessible to the Rrp44 exoribonuclease when in the context of Exo-9 (Bonneau et al., 2009) (Supplementary Fig S3B, upper panel). We also tested a double-stranded substrate with a long 3' overhang (35 nucleotides) that is accessible to the processive exoribonuclease activity of Rrp44 (Supplementary Fig S3, lower panel). We found that the Rrp6-Rrp47 degradation properties on these substrates were not affected by the presence of Mtr4 (Supplementary Fig S3B). Although we saw no significant effect of Mtr4 on the degradation of these substrates.
by either Rrp6 or Rrp44, we caution that it is possible that the helicase domain of Mtr4 might operate in the context of more complex RNA structures.

Structure-based mutations in the Rrp6 N-terminal domain result in 5.8S rRNA processing defects in vivo

Loss-of-function rrp6 and rrp47 mutants show strong defects in the 3’ processing of 5.8S rRNA and box C/D snoRNAs (Briggs et al., 1998; Allmang et al., 1999a; Mitchell et al., 2003). These mutants accumulate the 5’ external transcribed spacer (5’ ETS) fragment that is released during early processing of the pre-rRNA transcript, as well as truncated fragments of U3, 5S and snR13 as a result of impaired RNA surveillance processes (Briggs et al., 1998; Allmang et al., 1999a; Mitchell et al., 2003). To address the importance of the interaction between Mtr4 and the Rrp6–Rrp47 heterodimer in vivo, we designed specific rrp6 and rrp47 mutants based on the structure of the Rrp6N–Rrp47N–Mtr4N complex and analyzed the levels and...
Figure 6.
Figure 6. Impact of Rrp6Δ–Rrp47Δ on ribonuclease activity.

A Surface representation of Rrp6Δ–Rrp47Δ colored according to electrostatic potential (blue for electropositive and red for electronegative). Mtr4Δ is shown in gray, with negatively charged residues in a stick representation. The molecule is viewed in the same orientation as in Fig 5A.

B Quantitative measurements of RNA-binding affinities in solution by fluorescence anisotropy using fluorescein-labeled homopolymeric substrates. The data were fitted to a binding equation describing a single-site binding model to obtain the dissociation constants (Kd). The best fit was plotted as a solid line. The Kd and their corresponding errors are the mean and standard deviation of a minimum of 3 independent experiments and are compiled in the table (top panel).

C Nuclease activity of the indicated Rrp6 and Rrp6–Rrp47 constructs toward single-stranded RNAs and duplex RNAs with 3′ overhangs. Substrates were designed to have a 17-base pair GC-rich duplex (ds17), corresponding to the 3′ end of tRNA 30 (Vincent & Deutscher, 2006, Lorentzen et al, 2008), and a 3′ overhang of 10 adenine nucleotides (A10). The RNAs were 5′-end-labeled with [γ-32P]ATP, and the reaction products were resolved on 20% denaturing PAGE and visualized with a phosphorimager. A Coomassie-stained gel with the wild-type and nuclease-deficient mutant proteins used in the RNase activity and RNA affinity assays (B) is shown on the right.

Structure of the Rrp6–Rrp47–Mtr4 Interaction

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Mtr4 and Rrp6 mutants show synergistic effects in vivo

C-terminally tagged Mtr4 fusion proteins can support cell growth (Ghaemmaghami et al, 2003; Huh et al, 2003). Indeed, we observed no clear growth defect for an mtr4-gfp strain when compared to an isogenic wild-type strain. Northern analyses of RNA isolated from the mtr4-gfp strain during growth in either minimal or rich medium did not show significant accumulation of the 5.8S + 30 fragment or the 3′ extended forms of snoRNAs that are characteristic of Rrp6Δ mutants (Briggs et al, 1998; Allmang et al, 1999a). However, the Northern analyses showed defects in the degradation of other structured RNAs, with a clear accumulation of the 5′ ETS fragment that has been previously shown to accumulate upon Mtr4/Dob1 depletion (de la Cruz et al, 1998) (see Fig 7A, lanes 7 and 8). These results indicated that the presence of the C-terminal GFP protein partially compromises the function of Mtr4, without causing a general effect on Rrp6-dependent processing or degradation pathways. We therefore generated an rrp6Δ allele in the haploid mtr4-gfp strain and tested for genetic interactions between the mtr4-gfp allele and the rrp6 mutants.

Attempts to delete the RRP6 gene in the mtr4-gfp strain directly were unsuccessful, but correct integrants were isolated when the mtr4-gfp strain had been transformed with aURA3 plasmid encoding a functional Rrp6 fusion protein (Allmang et al, 1999b). Notably, deletion of the RRP6 gene was also achieved in mtr4-gfp strains expressing rrp6Δ27R,F30R and rrp6ΔE90R,D97R variants of the Rrp6 fusion protein but not the rrp6ΔI4E,R18E mutant. To determine whether the mtr4-gfp rrp6ΔI4E,R18E double mutant is synthetic lethal, wild-type and rrp6ΔI4E,R18E mutant alleles were subcloned into the LEU2 plasmid pRS415 and transformed into the mtr4-gfp rrp6Δ plasmid shuffle strain and the resulting transformants were tested for growth on medium containing 5-fluoroorotic acid (5 FOA). While transformation with the wild-type RRP6 genes gave rise to viable colonies on 5 FOA medium, no growth was observed for the mtr4-gfp rrp6Δ strain after transformation with a plasmid encoding the rrp6ΔI4E,R18E mutant or the cloning vector (Fig 8A). We concluded that the mtr4-gfp strain is dependent upon Rrp6 for cell growth.

To determine whether the Rrp6/Mtr4 interaction in yeast is blocked by the rrp6ΔI4E,R18E mutation, pull-downs were performed on lysates from mtr4-gfp strains expressing plasmid-encoded, zz epitope-tagged wild-type or mutant Rrp6 fusion proteins (in addition to the endogenously encoded Rrp6). Mtr4-gfp was bound to the wild-type zz-Rrp6 protein, but not the I4E, R18E mutant (Fig 8B, left panel). We then addressed whether mutation of the N-terminal region of Mtr4 also causes a block in the Rrp6/Mtr4 interaction in vivo. Immobilized zz-Rrp6 protein retained the wild-type Mtr4-gfp protein, whereas binding of the mtr4-gfp F7A,F10A mutant was only slightly above background levels (Fig 8B, right panel). These data support the conclusion that the structurally defined Rrp6Δ–Rrp47Δ–Mtr4Δ complex forms the principal interaction between Rrp6 and Mtr4 in yeast.

If the synthetic lethal phenotype observed for the mtr4-gfp rrp6ΔI4E,R18E mutant (Fig 8A) is due to loss of interaction between Mtr4 and Rrp6, a strong synergistic effect would be predicted upon introduction of the F7A, F10A mutation in the mtr4-gfp mutant. Indeed, both the mtr4-gfp and the mtr4F7A,F10A mutants grew comparably to the wild-type strain, whereas the strain expressing an Mtr4-gfp fusion protein bearing the F7A, F10A mutation was nonviable (Fig 8C). We concluded that the strong synergistic
Figure 7. Structure-based mutations in the Rrp6 N-terminal domain result in 5.8S rRNA processing defects in vivo.

A Northern blot analyses of RNA from rrp6, mtr4-gfp and mtr4-gfp rrp6 double mutants. Strains were grown in selective minimal medium, unless indicated otherwise. Consecutive hybridizations of a single blot are shown; panels shown for lanes 1–6 and 7–11 are juxtaposed from a single image. The major RNAs detected by each probe are indicated on the right. Asterisks indicate truncated RNA fragments of U3, snR13 and 5S. The amount of ‘5.8S+30’ RNA, the 5’ ETS fragment and the major U3 and 5S degradation fragments in each mutant (indicated beneath the appropriate panel) is expressed relative to the rrp47-zz strain in lanes 1–6 and to the mtr4-gfp strain during growth in YPD in lanes 7–11.

B Spot growth assays of rrp6 mutants. Serial dilutions of pre-cultures were spotted onto selective minimal medium plates and incubated at 30 or 37°C. The plates were photographed after 3 days.

C Western analyses of rrp6 mutants. (Upper panels) Expression levels of Rrp6 and the Rrp47-zz fusion protein in the rrp6 mutants. (Lower panels) Mtr4-gfp fusion protein expression levels in the mtr4-gfp rrp6 double mutants. Pgk1 levels were analyzed as a loading control in each case.
effects observed in strains expressing a C-terminal Mtr4-gfp fusion protein in combination with either the Rrp6 I14E, R18E mutation (that impairs the interaction with the N-terminus of Mtr4) or the Mtr4 F7A, F10A mutation (that impairs the interaction with the N-terminal domains of Rrp6–Rrp47) are due to loss of binding. The very C-terminus of Mtr4 is embedded within the base of the DExH core of the helicase, where the 3’ end of an RNA substrate is expected to emerge after unwinding (Jackson et al., 2010; Weir et al., 2010).

Collectively, these data suggest that the base of Mtr4 is also engaged in interactions within the nuclear exosome complex and that linking the C-terminus of Mtr4 to a GFP protein weakens this interaction.

Northern analyses of the viable mtr4-gfp rrp6D27R,F30R and mtr4-gfp rrp6E90R,D97R double mutants revealed a strong synergistic block in the degradation of some RNAs, including the 5’ ETS fragment and...
Here, we show that the N-terminal domains of Rrp6 and Rrp47 are required for these effects, however, has remained unclear. The exosome-mediated RNA processing and turnover pathways. The truncated fragments of U3, snR13 and 5S rRNA (Fig 7A, lanes 9 and 10). In contrast, the defect in 5.8S rRNA maturation seen in the mtr4-gfp rrp6 mutants may be defective in RNA surveillance mechanisms, rather than 5.8S rRNA or snoRNA processing.

**Discussion**

The interaction of Rrp6 and Rrp47 is known to stabilize the individual proteins in vivo and to influence their function in exosome-mediated RNA processing and turnover pathways. The molecular basis for these effects, however, has remained unclear. Here, we show that the N-terminal domains of Rrp6 and Rrp47 assemble into a globular heterodimer with an elaborate architecture formed by intertwined pairs of α-helices. The interlocked Rrp6N–Rrp47N structure explains why heterodimer formation leads to stabilization and to functional interdependence. In isolation, the individual proteins are expected to be partially unfolded and aggregated. As a 1:1 complex, they form a composite molecular surface for the direct recruitment of the N-terminal region of Mtr4, which binds via a set of evolutionarily conserved interactions. Together with the observation that Rrp6 and Rrp47 assemble only after they are independently imported into the nucleus (Feigenbutz et al., 2013b), these results rationalize how the cell might avoid the untimely recruitment of Mtr4 to the exosome in the cytoplasm.

The Rrp6N–Rrp47N heterodimer does not undergo significant conformational changes upon Mtr4N binding and thus appears to function as a rather rigid platform. The structural analysis and in vivo data suggest that this platform also contains docking sites for other interaction partners whose identities remain to be explored. It is also likely that Rrp6, Rrp47 and Mtr4 are engaged in additional, albeit weaker, contacts in the context of the nuclear exosome complex. Although we did not observe direct interactions between Rrp6–Rrp47 and Mpp6 and between Mtr4 and Mpp6, as has been reported for the human orthologues (Schilders et al., 2007), it is possible that the yeast proteins might engage in analogous contacts but without the high affinity required for detection in the in vitro reconstitution assays we used. Indeed, synthetic lethal mutations in *S. cerevisiae* support the presence of redundant interactions among the yeast cofactors of the nuclear exosome (Milligan et al., 2008; Garland et al., 2013 and Fig 8A). The presence of additional weak interactions would also rationalize why Mtr4 would be able to carry out at least part of its functions in vivo in the absence of Rrp6 and Rrp47, as can be inferred from the severity of the corresponding knockout studies (Briggs et al., 1998; de la Cruz et al., 1998; Mitchell et al., 2003). The emerging picture is that Rrp6, Rrp47, Mpp6 and Mtr4 assemble together with Exo-10 with a combination of high-affinity interactions and additional intermolecular contacts to form a functional nuclear complex (Fig 9). Understanding how the nuclear exosome complex is structured and how it coordinates the multiple catalytic activities of core components and cofactors awaits future studies.

**Materials and Methods**

**Protein purification**

*Saccharomyces cerevisiae* Mtr4 full-length, Mtr4ΔN5 and the Neurospora crassa Mtr4 orthologue FRH were expressed and purified according to the protocol in Weir et al. (2010). Exo-9 and Rrp44 were expressed and purified as in Makino et al., 2013a. All other *S. cerevisiae* proteins purified in this study were expressed recombinantly using *E. coli* BL21-Gold (DE3) pLysS cells (Stratagene) grown in TB medium and induced overnight at 18°C. Rrp6N, Rrp6ΔN and Mtr4ΔN were expressed as His-tagged proteins. Rrp6N–Rrp47N, Rrp6N–Rrp47AC and Rrp6ANL5–Rrp47 complexes were co-expressed such that the Rrp6 constructs bear a N-terminal His tag while the Rrp47 constructs are untagged. The proteins were purified using cobalt-based (or nickel for Mtr480) affinity chromatography, followed by cleavage of the His tag with the appropriate protease (human rhinovirus 3C protease for Rrp6N, Rrp6N–Rrp47N, Rrp6N–Rrp47AC, tobacco etch virus (TEV) protease for Mtr4ΔN, Rrp6ΔNLS–Rrp47 and small ubiquitin-like modifier (SUMO) protease for Rrp6ΔN5). Protease-treated Rrp6ΔN5 was loaded on the affinity column a second time to remove uncleaved species. After affinity purification, all samples (with the exception of Mtr4ΔN) were subjected to
anion exchange chromatography (HiTrap Q HP, GE Healthcare). Rrp6\textsubscript{N} and Rrp6\textsubscript{NLS}-Rrp47 were further purified over a HiTrap Heparin Sepharose HP column (GE Healthcare).

Size-exclusion chromatography (SEC) on a Superdex 200 or Superdex 75 column (GE Healthcare) was performed as a final step of purification for all proteins. Rrp6\textsubscript{N} and complexes of this construct were finally purified in buffer A (20 mM Tris pH 7.5, 100 mM NaCl) supplemented with reducing agents. An additional 10% glycerol was added to the size-exclusion buffer when purifying Rrp6\textsubscript{N} and Rrp6\textsubscript{NLS}-Rrp47. Mtr4\textsubscript{N} was purified in buffer A supplemented with an additional 50 mM NaCl and 2 mM DTT. Size exclusion for Rrp6\textsubscript{N} was performed in buffer B (20 mM MES pH 6.0, 250 mM NaCl, 10% glycerol, 2 mM DTT). Rrp6 mutants were verified by DNA sequencing and purified using the protocol for the wild-type protein. Yeast Rrp47\textsubscript{AC} was purified over a HiTrap Heparin Sepharose HP column, and processed using XDS (Kabsch, 2010) and Aimless (Evans et al., 2004) using the anomalous Ta signal. Identifiable \(\alpha\)-helices were manually placed with Coot (Emsley et al., 2010) and used as a starting model for SAD-MR by exploiting the anomalous Se signal using the program PHENIX AutoSol (Terwilliger et al., 2009). After manual chain tracing, the model was completed with Coot and refined against the native data using phenix.refine (Afonine et al., 2012). The Rrp6\textsubscript{N}-Rrp47\textsubscript{N}-Mtr4\textsubscript{N} structure was solved by SAD-MR using Phaser (McCoy et al., 2007) with parts of the Rrp6\textsubscript{N}-Rrp47\textsubscript{AC} model as a search model and AutoSol using the anomalous signal from yttrium. Merohedral twinning generated by a twofold axis perpendicular to a crystallographic threefold axis of the trigonal space group became apparent by the poor quality of the electron density for one of the three copies of the complex in the asymmetric unit. The twin law (\(h\),\(-l\),\(k\)) and twinning fraction (0.5, perfect twin) was determined using phenix.xtriage (Adams et al., 2010). After manual tracing of the Mtr4\textsubscript{N} sequence, the model was completed using Coot and refined against twinned data using phenix.refine. Several Yttrium ions form clusters surrounded by electron density that likely corresponds to a negatively charged loop of Rrp6 (residues 63–73). In the Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} structure, the same loop has only partial electron density and was not modeled. Although it is possible that in two copies of the binary complex, this loop is engaged with another molecule in the asymmetric unit, this is a crystal lattice artifact as it occurs neither in the third copy nor in the ternary complex.

**Crystallization and structure determination**

Crystals of yeast Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} were grown at 20°C by sitting-drop vapor diffusion from drops formed by equal volumes of protein (at 18 mg/ml in size-exclusion buffer comprising 20 mM Tris, 100 mM NaCl and 1 mM DTT) and of crystallization solution (1.8 M (NH\(_4\))\(_2\)SO\(_4\), 125 mM NaCl and 100 mM Na-cacodylate pH 5.8). For heavy-atom derivatization, native Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} crystals (grown in 2.05 M (NH\(_4\))\(_2\)SO\(_4\), 125 mM NaCl and 100 mM Na-cacodylate pH 6.4) were soaked for 15 min in crystallization solution supplemented with 1 mM Ta\(_{5}\)Br\(_{14}\) prior to cryoprotection. Optimized Se-Met-derivatized crystals were obtained in 2.0 M (NH\(_4\))\(_2\)SO\(_4\), 200 mM NaCl and 100 mM Na-cacodylate pH 6.2. Crystals were cryoprotected with crystallization solution supplemented with 24% glycerol for the native crystals and 17.5% glycerol for the Ta soaked and Se-Met-derivatized crystals prior to cryo-cooling and data collection.

Yeast Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} complex was mixed with a 1.5-fold molar excess of Mtr4\textsubscript{N} (synthesized peptide (H)-MDSTDLDVFEEFTPVELPTK-(NH\(_2\)); D20K substitution for synthesis strategy and solubility reasons) and 5 mM YCl\(_3\) and incubated at room temperature for 10 min. Crystals of the Rrp6\textsubscript{N}–Rrp47\textsubscript{N}–Mtr4\textsubscript{N} complex were grown at 20°C by sitting-drop vapor diffusion from drops formed by equal volumes of complex (at 27 mg/ml in size-exclusion buffer comprising 20 mM Tris, 100 mM NaCl and 0.5 mM TCEP) and crystallization solution (12% PEG 1000, 0.1 M imidazole pH 7.5 and 0.125 M calcium acetate). Crystals were cryoprotected in 19% PEG 1000, 0.1 M imidazole pH 7.5, 0.125 M calcium acetate, 5 mM YCl\(_3\) and 12% glycerol and supplemented with 0.5 mM Mtr4\textsubscript{N} peptide. Data were collected at the ID32-3 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and at the PXII and PXIII beamlines of the Swiss Light Source (SLS) (Villigen, Switzerland) and processed using XDS (Kabsch, 2010) and Aimless (Evans & Murshudov, 2013).

The Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} structure was solved at low resolution (5.2 Å) by SAD with SHELX (Sheldrick, 2008) and HKL2MAP (Pape & Schneider, 2004) using the anomalous Ta signal. The resolution of the electron density for one of the three copies of the complex in the asymmetric unit. The twin law (\(h\),\(-l\),\(k\)) and twinning fraction (0.5, perfect twin) was determined using phenix.xtriage (Adams et al., 2010). After manual tracing of the Mtr4\textsubscript{N} sequence, the model was completed using Coot and refined against twinned data using phenix.refine. Several Yttrium ions form clusters surrounded by electron density that likely corresponds to a negatively charged loop of Rrp6 (residues 63–73). In the Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} structure, the same loop has only partial electron density and was not modeled. Although it is possible that in two copies of the binary complex, this loop is engaged with another molecule in the asymmetric unit, this is a crystal lattice artifact as it occurs neither in the third copy nor in the ternary complex.

**Size-exclusion chromatography assay**

Equimolar amounts of purified proteins as indicated (500 pmol for Fig 1C and D, 700 pmol otherwise) were diluted in a total injection volume of 25 µl in SEC buffer A supplemented with 2 mM DTT. Samples were incubated for 1 h on ice to allow complex formation. Increase in particle size upon complex formation was assayed by comparing the retention volumes in SEC on a Superdex 200 Increase 3.2/300 (GE Healthcare). Composition of the SEC peak fractions were analyzed by SDS-PAGE and visualized by Coomassie staining (percentage of the SDS–PAGE depended on protein sample size).

**Fluorescence anisotropy**

Fluorescence anisotropy measurements were performed with a 5′-6-carboxy-fluorescein (6-FAM)-labeled poly(A)\(_{35}\) or poly(U)\(_{30}\) RNA at 20°C in 50 µl reactions on a Genios Pro (Tecan). The RNA was dissolved to a concentration of 10 nM (1 nM for Rrp6\textsubscript{N}, D296N–Rrp47) and incubated with Rrp6 or the Rrp6–Rrp47 complexes at different concentrations in a buffer containing 20 mM Tris pH 7.5, 100 mM NaCl and 1 mM DTT. The excitation and emission wavelengths were 485 nm and 535 nm, respectively. Each titration point was measured three times using ten reads with an integration time of 40 µs. The data were analyzed by nonlinear regression fitting using the BIOEQS software (Royer, 1993).

**Isothermal titration calorimetry (ITC)**

Rrp6\textsubscript{N}–Rrp47\textsubscript{AC} wild-type and mutant proteins were dialyzed overnight in the same buffer that was used to dissolve the lyophilized
Mtr4N peptide (20 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM TCEP). ITC experiments were carried out at 20°C with a iTC-200 MicroCal calorimeter (GE healthcare). The MicroCal cell was filled with Rrp6N-Rrp47NC at 50 µM concentration and stirred at 800 rpm. For each titration, Mtr4N was injected into the cell 20 times in 2 µl volumes per injection at the same intervals of time (4 min). The concentration of Mtr4N in the syringe (500 µM) was 10 times the concentration of the protein sample in the cell. The released heat was obtained by integrating the calorimetric output curves and was corrected for the effect of dilution by subtraction of the value of the last injection as background. As control for all ITC measurements, the injectant was titrated into buffer. The $K_d$ values and binding ratios were calculated with the Origin (V7) software supplied with the calorimeter. We used the same protocol to measure the $K_d$ of the Rrp6 and the Mtr4 mutants.

**Nuclease assay**

The exonuclease activity assay in Fig 6C was carried out at 30°C in a buffer containing 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM magnesium diacetate, 10% (v/v) glycerol, 0.1% (v/v) NP-40 and 1 mM DTT. The reactions contained protein at a final concentration of 2 nM, while the concentration of RNA substrates was 200 nM. Substrates were verified by native gel electrophoresis. Two-microliter aliquots from a 10 µl total reaction volume were taken at indicated time points and quenched by addition of 14 µl loading dye consisting of 10 mM EDTA, 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanol FF in formamide. The ‘0’ time point was taken before adding the protein. Reaction products were boiled for 5 min immediately before being resolved on a 20% acrylamide gel containing 50% urea. After transfer to Hybond N+ labeled oligonucleotide probes complementary to the ITS2 region of the pre-rRNA transcript (tgagaaggaaatgacctgct), the 5′ ETS region of the pre-rRNA (ctgcgttctctgtgagc), snR38 (agagaggttacctattattacccattcagacagggataactg), U3 (tctgcttcacctgctgttct), snR13 (ccacgcttcagacagggataactg), Mtr4::GFP, and a PCR amplicon (aaacgaccagaacaacctcttgg). Hybridized blots were placed under phosphor storage screens, and the data were captured using a Personal Molecular Imager (Bio-Rad). Nonsaturated images were adjusted for signal level and window using ImageJ (NIH, USA). RNA hybridization signals were quantified using ImageJ and normalized to the expression level of 5S rRNA.

**Plasmids and yeast strains**

Yeast expression plasmids either encoding an N-terminal zz fusion of Rrp6 under the control of the RRP4 promoter (Allmang et al., 1999b), or containing a genomic clone of the RRP6 gene and lacking the CEN6 element from the vector backbone (Feigenbutz et al., 2013a), have been reported previously. Expression of the N-terminal zz-Rrp6 fusion protein from the RRP4 promoter is comparable to the endogenous expression level of the C-terminal TAP-tagged protein (Stead et al., 2007). The RRP47 genomic clone used in this study is described in Costello et al. (Costello et al., 2011). The MTR4 construct pAv675 (Jackson et al., 2010) was kindly provided by Ambro van Hoof (University of Texas Health Science Center, Houston). The construct encoding mtr4-gfp was cloned in yeast by homologous recombination, using HindIII linearized pAv675 (after deletion of the HindIII polylinker site) and a PCR amplicon encompassing the mtr4-gfp::HIS3 allele. The isolated plasmid was confirmed by sequencing the mtr4/gfp junction along the complete length of the PCR product. Point mutations were introduced into the RRP6, RRP47 and MTR4 ORFs in these constructs by site-directed mutagenesis using the Quikchange kit (Agilent Technologies) and validated by sequence analysis. Plasmid inserts encoding the N-terminal epitope-tagged wild-type RRP6 and rrp614E,rrp618E mutants were subcloned into pRS415 (Stratagene) for the plasmid shuffle assay. The rrp6::KANMX4 allele was amplified by PCR and integrated into the mtr4-gfp strain by homologous recombination after initial transformation with plasmids encoding zz-Rrp6 fusion proteins. Correct integrants were identified by PCR amplification of genomic DNA.

The mtr4-gfp strain (Huh et al., 2003) was obtained from Invitrogen. The mtr4 plasmid shuffle strain yAv1151 (Jackson et al., 2010) was kindly provided by Ambro van Hoof. Isogenic wild-type and rrp6Δ strains were obtained from Euroscarf (University of Frankfurt, Germany). The rrp47-zz and rrp47-zz rrp6Δ strains have been previously reported (Mitchell et al., 2003). Yeast strains were routinely cultured at 30°C in SD selective minimal medium (2% glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base) supplemented with appropriate amino acids and bases or in YPD medium (2% glucose, 2% peptone, 1% yeast extract). Spot growth assays were performed on selective solid minimal medium or medium containing 5-fluoroorotic acid (5 FOA) using tenfold serial dilutions of freshly saturated pre-cultures. Plates were photographed after incubation for 3 days.

**RNA analyses**

Total cellular RNA was isolated from strains harvested during early log growth and resolved by electrophoresis through 8% polyacrylamide gels containing 50% urea. After transfer to Hybond N+ membranes (GE Healthcare), the RNA was hybridized with 5′-[32P]-labeled oligonucleotide probes complementary to the ITS2 region of the pre-rRNA transcript (tgagaaggaaatgacctgct), the 5′ ETS region of the pre-rRNA (ctgcgttctctgtgagc), snR38 (agagaggttacctattattacccattcagacagggataactg), U3 (tctgcttcacctgctgttct), snR13 (ccacgcttcagacagggataactg), Mtr4::GFP, and a PCR amplicon (aaacgaccagaacaacctcttgg). Hybridized blots were placed under phosphor storage screens, and the data were captured using a Personal Molecular Imager (Bio-Rad). Nonsaturated images were adjusted for signal level and window using ImageJ (NIH, USA). RNA hybridization signals were quantified using ImageJ and normalized to the expression level of 5S rRNA.

**Protein analyses**

For protein expression analyses, total cellular protein was prepared by alkaline/SDS lysis followed by TCA precipitation. Extracts were resolved by SDS-PAGE, transferred to Hybond C extra membranes (GE Healthcare) and incubated with a rabbit anti-Rrp6 antisera (Mitchell et al., 2003) or mouse anti-Pgk1 (clone 22C5D8, Life Technologies) primary antibody, followed by either goat anti-rabbit (A4914, Sigma) or goat anti-mouse (1706516, Bio-Rad) HRP-conjugated secondary antibodies. The Mtr4-gfp fusion protein was detected using the anti-gfp antibody. The Rrp47-zz fusion protein was detected directly using the PAP antibody conjugate (P1291, Sigma). ECL images were captured using a G:Box iChemi XL system (Syngene) and adjusted for the signal level and window using ImageJ.

For the pull-down experiments, yeast were lysed in 50 mM HEPES pH 7.4, 50 mM KCl, 5 mM MgCl2, 10% glycerol and 1 mM PMSF. Lysates were clarified by centrifugation at 13,000 g for 30 min, normalized for A230 units and passed through -200 µl IgG sepharose fast flow beads (GE Healthcare). The beads were washed...
5 times with 1 ml wash buffer (50 mM HEPES pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM DTT), and the retained material was eluted with 0.5 M acetic acid. Eluates were resolved through 10% SDS–PAGE gels and analyzed by Western blotting, as described above.

Data deposition

The coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 4WFH for the Rrp6N–Rrp47ΔC structure and 4WFD for the Rrp6N–Rrp47ΔN–Mtr4ΔN structure.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

BS carried out the structural analyses; BS, DM and SF, the in vitro biochemical analyses; CB, the biophysical analyses; and MF and PM, the in vivo experiments. BS, EC and PM wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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