Tri-snRNP-associated proteins interact with subunits of the TRAMP and nuclear exosome complexes, linking RNA decay and pre-mRNA splicing

Anita Nag† and Joan A. Steitz*

Department of Molecular Biophysics and Biochemistry (MB&B); Howard Hughes Medical Institute (HHMI); Yale University School of Medicine; Boyer Center for Molecular Medicine; New Haven, CT USA

†Current address: Department of Biological Science; Florida State University; Tallahassee, FL USA

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Introduction

Nuclear machinery not only synthesizes and processes several different types of coding and non-coding RNA molecules, but also ensures production of functionally competent RNAs by selective degradation of aberrant species. More specifically, the nuclear RNA degradation apparatus is known to participate in the proper processing of snRNAs, snoRNAs,1 and rRNAs,2,3 as well as in the decay of cryptic transcripts.4 Moreover, it plays crucial roles in gene silencing5 and in the quality control of tRNAs6 and mRNAs.7-14

Two macromolecular complexes, the Trf4/Air2/Mtr4 Polyadenylation (TRAMP) complex and the nuclear exosome, contribute both to these processing events and to nuclear RNA surveillance.10,15-19 Many non-coding RNAs are generated by endonucleolytic cleavage, followed by 3'-polyadenylation and processing of the polyadenylated precursor.1 The polyadenylation (TRAMP) complex that marks RNAs for degradation or processing consists of a noncanonical poly(A) polymerase (Trf4/Trf5), a Zn knuckle-containing RNA binding protein (Air1/Air2), and an RNA helicase Mtr4.20-21 The nuclear exosome as responsible for degradation or processing contains a nuclear-specific subunit Rrp6 (PM/Scl-100 in humans) in addition to the nine-subunit exosome core.22-24 Mutations in yeast factors associated with RNA splicing (Prp2), with splicing and export (Sub2), with transcription (THO), and with polyadenylation (Rna14, Rna15) have revealed a critical role for the nuclear exosome-specific subunit Rrp6 in counteracting the generation of aberrant messages produced in these mutant cells.7,8,11,14

Communication between all these nuclear events—transcription, splicing, cleavage, polyadenylation, and nuclear RNA degradation—is pivotal to the formation of functional eukaryotic RNAs.25 The complex steps of splicing must be strictly orchestrated in order to avoid creating aberrant messages. Adding an extra layer of complexity is alternative splicing in higher eukaryotes, which can be important in disease (for review, see ref. 28). Although there are relatively few genes that undergo splicing in S. cerevisiae, a connection between the splicing and the nuclear...
decay machineries has been established. Bousquet-Antonelli et al. showed that levels of pre-mRNA remained low after disrupting splicing, either with a temperature-sensitive allele of Prp2 (a splicing factor required for the first transesterification step) or with a 3′-splice site mutation in the intron. However, inhibition of degradation by the nuclear exosome resulted in accumulation of both pre-mRNAs and spliced RNAs, suggesting a competition between splicing and degradation of pre-mRNAs.

In vertebrates, communication between splicing and the nuclear degradation machinery is likewise expected, but no such interactions have been reported. The TRAMP complex is conserved in T. brucei, D. melanogaster, S. cerevisiae and S. pombe, as is the exosome, including the nuclear-specific subunit Rrp6(PM/Scl-100). Yet, both the TRAMP complex and the exosome are poorly characterized in mammalian cells, and the current literature reports only a few investigations of the T4-mediated TRAMP complex and its connection with the Rrp6-mediated exosome complex. Two human proteins, T4-1 (pol) and T4-2 (papd5), share 37% identity and 56% similarity with the γT4-6 protein. Recently, Scheller et al. identified a role of papd5 in degradation of transcripts generated by RNA polymerase I. Finding a splicing factor associated with a putative nuclear RNA decay factor suggests potential regulation of RNA processing. Since we discovered the presence of a tri-snRNP protein Prp31 in the T4-mediated TRAMP complex, we sought to establish whether this interaction serves to recruit the nuclear decay machinery to the spliceosome. Therefore, we asked if other members of the nuclear RNA decay complex (Mtr4) or of the exosome machinery (PM/Scl-100) also associate with Prp31. Immunoprecipitation of Prp31 using an anti-Prp31 antibody (mouse polyclonal, Abnova) immunoprecipitated a small fraction of Prp31 from the nuclear extract and resulted in modest but clear coimmunoprecipitation of Mtr4 with RNase A treatment. In contrast, a rabbit polyclonal antibody raised against the C-terminus (aa484–497) of Prp31 failed to

Results

Mammalian homologs of TRAMP complex components T4-1 and Mtr4 associate with the tri-snRNP protein Prp31. To isolate factors associated with human T4-6 proteins, a FLAG-tagged T4-1 was expressed in HEK293 cells, followed by extract preparation and immunoprecipitation with anti-FLAG antibody in the presence of ribonuclease (RNase) A. Immunoprecipitated proteins were eluted usingFLAG peptide and were subjected to LC-MS/MS (Table S1). Surprisingly, in addition to many proteins that may be non-specific background (for example, Gem4 in Fig. 1A), we identified a tri-snRNP-specific splicing factor, Prp31, whose enrichment was verified by western blot analysis of the immunoprecipitate (IP) (Fig. 1A). Prp31 is part of the U4/U6 complex and plays an important role in the stability and integrity of the spliceosome. It is also linked to autosomal dominant retinitis pigmentosa, a genetic eye condition that progressively leads to incurable blindness.

Finding a splicing factor associated with a putative nuclear RNA decay factor suggests potential regulation of RNA processing. Since we discovered the presence of a tri-snRNP protein Prp31 in the T4-mediated TRAMP complex, we sought to establish whether this interaction serves to recruit the nuclear decay machinery to the spliceosome. Therefore, we asked if other members of the nuclear RNA decay complex (Mtr4) or of the exosome machinery (PM/Scl-100) also associate with Prp31. Immunoprecipitation of Prp31 using an anti-Prp31 antibody (rabbit polyclonal, Abnova) immunoprecipitated a small fraction of Prp31 from the nuclear extract and resulted in modest but clear coimmunoprecipitation of Mtr4 with RNase A treatment (Fig. 1B). In contrast, a rabbit polyclonal antibody raised against the C-terminus (aa484–497) of Prp31 failed to
crosslinking of cells to stabilize weak interactions. Immuno-
we expressed C-terminally FLAG-tagged Prp31 in HEK293 cells
the antibody heavy chain and is therefore difficult to visualize,
Since the major band of endogenous Prp31 runs very close to
interaction depends on the phosphorylation sites of Prp31.
Schneider et al. thoroughly characterized some of these phos-
phosphorylation sites on Prp31. Treatment of the extract with
alkaline phosphatase prior to immunoprecipitation with anti-
MR4 moderately reduced the intensity of the upper band (25% decrease) in the Prp31 blot (Fig. 1C, lane 8).

Prp31 interacts with both Mr4 and PM/Scl-100 and the
interaction depends on the phosphorylation sites of Prp31.
Since the major band of endogenous Prp31 runs very close to
the antibody heavy chain and is therefore difficult to visualize,
we expressed C-terminally FLAG-tagged Prp31 in HEK293 cells and performed coimmunoprecipitations after formaldehyde
Formaldehyde

\[
\begin{array}{ccccccccc}
5\% \text{ Input} & \text{FLAG-IP} & \text{Mtr4} & \text{Prp6} & \text{PM/Scl-100} & \text{PM/Scl-75} & \text{FLAG} \\
\hline
\text{Simple wash} & - & - & - & - & - & - \\
\text{METTL1-FLAG} & + & + & + & + & + & + \\
\text{PET107-FLAG} & + & + & + & + & + & + \\
\text{FLAG-IP} & + & + & + & + & + & + \\
\end{array}
\]

Figure 2. Prp31 forms a complex with both Mr4 and PM/Scl-100.
FLAG-tagged Prp31 was expressed in HEK293 cells and cell extracts were
prepared with or without prior formaldehyde crosslinking using RIPA
buffer followed by sonication. Anti-FLAG M2 conjugated agarose beads (10μl) were used for immunoprecipitation followed by western blot analysis for the tri-snRNP-associated protein Prp6 and the exosome-associated protein PM/Scl-100. PM/Scl-75 and Mr4, as indicated on the right. * represents an unknown non-specifically reacting protein. Both lanes 2 and 3 and lanes 6 and 7 were initially separated by one lane, which was removed for presentation.

In the absence of formaldehyde (Fig. 2, lane 8), indicating that the
ability of rabbit anti-Prp31 to coimmunoprecipitate Mr4 is
not simply due to a particularly weak interaction between Mr4 and Prp31. Moreover, FLAG-tagged Prp31 coimmunoprecipi-
tated the exosome subunits PM/Scl-100 (Rrp6), which is possibly
associated through Mr4, even in the absence of formaldehyde
crosslinking (Fig. 2, lanes 7 and 8). In contrast, Prp6, a U5-
associated protein that interacts directly with Prp31,37 was present
at much higher levels in the anti-FLAG IP after formaldehyde
treatment (compare lanes 7 and 4). Likewise, visualization of the
PM/Scl-75 component of the exosome in the FLAG-tagged
Prp31 IP was dependent on formaldehyde (lanes 7 and 8). Even
though the interactions of Prp6 and PM/Scl-75 seem weak, their
presence suggests involvement of mature and functionally relevant
complexes in this association. As a control, we used a FLAG-
tagged protein with known nuclear localization, METTL1, which was unable to pull down Mr4, Prp6, PM/Scl-100, or -75 (lane 6).

After confirming the interaction between Prp31 and Mr4, we wanted to establish whether Mr4 binding is specific for a
posttranslationally modified form of Prp31. Since Prp31 has
multiple potential phosphorylation sites, we introduced mutations
into each cluster of sites in two Prp31 variants deleted at the
C-terminus (aa483-1-438, lane 9). For details see the Table above.
Specifically, we first generated the truncated Prp31 (aa1-483) to ask whether
the antibody-binding surface of Prp31 located in the C-terminus
between aa484 and aa497 is required for the association
of Prp31 with Mr4. This would explain the lack of Mr4
coimmunoprecipitation with rabbit anti-Prp31 raised against this
epitope (Fig. 1). Second, we created a further deletion (leaving
aa1-438) of the C-terminus to remove several putative phospha-
rylation sites and also generated alanine mutations to disrupt
other sites: mutant 1 (mut1) carries alanine mutations at sites
205, 273, and 275, while mutant 2 (mut2) has additional
mutations to alanine in residues 439, 440, and 455. Mutants
1 and 2 were created in deletion mutant 1-483 (485mut1 and
483mut2), and mutant 1 was generated in the 1-438 (439mut1)
truncated Prp31 (Fig. 3A).

Figure 3A shows that both Prp31 deletion mutants (483 and
438aa long) bind Mtr4 comparably to the wild-type protein
(compare lanes 10 and 13 with lane 9), suggesting that neither
the antibody binding site (aa484-497) nor the C-terminal
phosphorylation sites (aa439-455) contribute to Mtr4 binding.
A similar profile was obtained for PM/Scl-100 binding (Fig. 3A).
However, mutations of the phosphorylation sites in the middle
of the 438aa truncated protein (Y205A, T273A, Y275A) resulted
in diminished binding of Mtr4 (lane 11), suggesting that
phosphorylation of these Prp31 residues strengthens the inter-
action with Mr4. As expected from lane 13, additional mutations
in the C-terminal phosphorylation sites (S439A, T440A, T455A)
did not hinder the binding capacity further (compare lanes 11 and
12). Similar to the mutations in the 438aa protein, muta-
tions of aa205, 273, and 275 in the 483aa Prp31 protein reduced
binding of Mtr4, but even more efficiently (lane 14). We there-
fore conclude that the phosphorylation sites in the middle of
the Prp31 molecule contribute significantly to its interaction with

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Figure 3. Interaction between Prp31 and Mtr4 is dependent on the phosphorylation sites of Prp31. (A, top) FLAG-tagged mutants of hPrp31, which include two different deletions at the C-terminus (aa1–483 and aa1–438), as well as the indicated amino acid substitutions at specific positions. (A, bottom) Binding of Prp31 to Mtr4 depends on putative phosphorylation sites in the middle of Prp31. Each Prp31 mutant was individually expressed in HEK293 cells followed by lystate preparation in RIPA buffer followed by sonication. FLAG-tagged Prp31 mutants and associated proteins were selected on anti-FLAG M2 agarose beads followed by western blotting to identify the interacting partners indicated on the right. A longer exposure did not reveal significant binding of Mtr4 and PM/Scl-100 in lane 14 (data not shown). (B) Relative binding of PM/Scl-100 and Mtr4 to each FLAG-tagged Prp31 protein is graphed. The western blot was analyzed using Image J; bound fractions of Prp31, PM/Scl-100, and Mtr4 to each FLAG-tagged Prp31 protein is graphed. The western blot was analyzed using Image J; bound fractions of Prp31, PM/Scl-100, and Mtr4 proteins were normalized to their respective inputs, and binding data were plotted for the normalized PM/Scl-100 and Mtr4 relative to the normalized FLAG proteins in the IP lanes. Results are presented as an average of two experiments except for the 438aa Prp31 determination, which was from one experiment.
Mtr4 and PM/Scl-100. Quantification of the western blot data are shown in Figure 3B.

Mtr4 associates with other components of the tri-snRNP independent of Prp31. Since Prp31 is part of the U4/U6.U5 tri-snRNP, we asked whether Mtr4 interaction extends to other components of the tri-snRNP complex. We used available antibodies to probe the interactions of endogenous (rather than tagged) tri-snRNP proteins (Prp4 and Prp6) with Prp3, Prp31, Mtr4, PM/Scl-100, and PM/Scl-75 in nuclear extract treated with RNaseA (Fig. 4A). The anti-Prp3 IP contained significant amounts of Mtr4 (Fig. 4A, lane 4). Similarly, anti-Mtr4 appears to coimmunoprecipitate Prp4 (Fig. 4B, lane 4), a conclusion that is confirmed below (see Fig. 5). In contrast, none of the proteins detectably coimmunoprecipitated Prp6 (Fig. 4A, lane 6). 35 S-labeled Prp31 did not bind to the mixture of GST-Mtr4 products (lanes 1 and 2), which is compatible with the results of the depletion experiment shown in Figure 6. However, since the comparable experiment with Prp3 did not reveal binding to GST-Mtr4 above the background level (data not shown), we cannot rule out additional direct interactions with other di-snRNP proteins.

To determine whether Mtr4’s interaction with Prp3 and Prp4 proteins of the U4/U6 di-snRNP complex depends on Prp31, we performed immunoprecipitation of Prp3 from Prp31-depleted nuclear extract. Prp3 was chosen since it showed more efficient coimmunoprecipitation of Mtr4 than the other di-snRNP proteins (Fig. 4A, lane 6). 35 S-labeled Prp31 did not bind to the mixture of GST-Mtr4 products (Fig. 5, lane 3), which is compatible with the results of the depletion experiment shown in Figure 6. However, since the comparable experiment with Prp3 did not reveal binding to GST-Mtr4 above the background level (data not shown), we cannot rule out additional direct interactions with other di-snRNP proteins.

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150 mM NaCl, a condition in which Prp4 and Prp3 maintain their interaction. The resulting Prp31-depleted supernatant, as well as a mock-depleted supernatant, were subjected to immunoprecipitation by anti-Prp3 antibody. In both cases, Mtr4 appeared well as a mock-depleted supernatant, were subjected to immunoprecipitation by anti-Prp3 antibody. In both cases, Mtr4 appeared consistently present when high salt conditions were used during the IP.

**Discussion**

We have demonstrated that components of the nuclear decay machinery, Mtr4 and PM/Scl-100, interact with several U4/U6-associated proteins (Prp31, Prp3 and Prp4) of the tri-snRNP complex within the spliceosome. These findings suggest a possible mechanism for recruitment of the decay machinery to the pre-mRNA processing apparatus to ensure generation of properly spliced RNA and decay of aberrant transcripts.

Communication between the splicing and decay machineries was anticipated from genetic interactions observed between the splicing factor Prp2 and subunits of the exosome in *S. cerevisiae.* Specifically, whereas a temperature-sensitive allele of Prp2 showed decreased levels of spliced RNA without an increase in the unspliced precursor at the non-permissive temperature, when combined with a deletion of Rpt6, unspliced precursor levels increased. This result suggested that unspliced precursors generated because of a defect in splicing were being degraded by the nuclear exosome complex. Likewise, defects in the THO/Sub2 complex, which is involved in mRNP biogenesis and export, have been reported to result in degradation of a specific pool of mRNAs by a pathway that includes both the TRAMP and exosome complexes. Finally, defects in mRNA cleavage and polyadenylation likewise result in decay of long-read-through transcripts by an Rpt6-dependent mechanism.

Our results offer a possible scenario for recruiting the exosome to the mammalian spliceosome through the U4/U6.U5 tri-snRNP complex, adding to our understanding of the mammalian decay process. Prp31 exhibited an RNaseA insensitive association with Mtr4 (Figs. 1–3). This interaction does not seem to be direct and is not itself principally responsible for the association between the tri-snRNP and TRAMP complexes since anti-Prp3 antibodies can immunoprecipitate Mtr4 from a Prp31-depleted extract (Fig. 6). Moreover, baculovirally expressed GST-Mtr4 binds in vitro translated Prp4 (Fig. 5), suggesting that this is one of the principal interactions. Interestingly, mutations in three potential phosphorylation sites (Y205, Y273, and Y275) of Prp31 were able to disrupt association of Prp31 with Mtr4 significantly. The X-ray crystal structure of Prp31 shows that residue Y205 does not interact with the 15.5k protein of the U4 snRNP but is exposed and could bind some other component. Residues Y273 and Y275 are located in a loop within a cleft that is in close proximity to the U4 snRNA binding site; however, the side chains point away from the U4 RNA. Although the mechanism by which phosphorylation enhances Prp31 interaction with Mtr4 is not clear, it is possible that as 205, 273 and 275 contribute to stability of the U4/U6 complex and that other proteins of this complex bridge the interaction of Prp31 with Mtr4. Recent work from the Lührmann laboratory suggests that phosphorylation of Prp31 by the Prp4 kinase induces assembly and stabilization of the spliceosomal B complex. Our work supports the idea that even though Mtr4 directly binds to the tri-snRNP through Prp4, phosphorylated Prp31 enhances the stability of the interaction between the spliceosome and the exosome. Prp31 is evolutionarily conserved and the human protein shares 25% identity and 60% similarity with its homolog in budding yeast. Interestingly, the Prp31 homolog in *S. cerevisiae* is conserved in some residues including Y273 but lacks many other tyrosine, threonine, and serine residues present in hPrp31. Budding yeast also lacks the Prp4 kinase that is known to be responsible for phosphorylating both Prp31 and Prp6 in humans. Thus, even though our Y205A, Y273A and Y275A triple mutant of human Prp31 is significantly deficient in binding Mtr4, budding yeast must exist an alternative way of recruiting the exosome complex. Perhaps with the lower complexity of splicing in yeast, fewer contacts are needed between the decay and the splicing machineries.
Mass spectrometric analysis of spliceosomal proteins at various stages has demonstrated recruitment of Mtr4 to the B complex and its continuous association with the C complex (supplementary data, ref.43). The presence of exosome subunits in purified spliceosomes has also been reported (supplementary data, ref.48). Since activation of the spliceosomal B complex results in the dissociation of many U4/U6-associated proteins, including Prp4, Prp3, and Prp31, the presence of Mtr4 in the C complex suggests that its association with the active spliceosome is achieved through some other constituent(s), possibly hnRNP.49 Whereas the tri-snRNP may act as the platform for initial recruitment of the nuclear exosome-mediated decay complex to spliceosomes, other proteins such as transcription factors can cotranscriptionally recruit the nuclear exosome to a pre-mRNA. For example, Andrusi et al. demonstrated interactions between the nuclear exosome subunits dRrp6 and dSbp6, elongation factor dSpt5, and RNA polymerase II in Drosophila.50 Using Chrominomus and Drosophila, Helsel et al. showed that the core exosome subunit Rrp4 is associated with polytene chromosomes and that this association is dependent on hnRNP M.51

Observations that the exosome can interact with multiple machineries suggest that production of a fully functional mRNA may be ensured through several different checkpoints. The exosome makes several contacts with the transcription and RNA processing machineries, but whether these interactions are mediated by the TRAMP complex is unknown. Our results show that both the TRAMP component Mtr4 and the exosome subunit PM/Scl-100 (and -75) exhibit similar binding interactions with the U4/U6 complex. In addition, we cannot rule out the possibility that multiple members of the TRAMP complex make separate contacts with one or more tri-snRNP components. Our mass spectrometric analysis was unable to detect any Mtr4 in an anti-Trf4-1 IP. The absence of Mtr4 may be due to technical reasons, as evidenced by the inconsistent presence of exosome components in the spliceosome analyzed by mass spectrometry.44 Our understanding is limited by the absence of a fully-characterized human TRAMP complex. Further work will be necessary to understand the contribution of different Trf4 homologs and their isoforms to functional TRAMP complexes, and to the stability and integrity of these complexes in the absence of an RNA substrate. It will be interesting to learn how and when the decay proteins dissociate after completion of a splicing event. Their association with the splicing machinery may simply reflect a coupled process whereby newly excised introns are rapidly degraded. Communi-
cation between cytoplasmic nonsense-mediated decay and alterna-
tive splicing is one avenue for achieving proper gene expression.26

Materials and Methods

**Plasmids and cloning.** Primers used to generate cDNAs for Trf4-1, Mtr4, and Prp31 are listed in Table S2 along with the primers that were used for introducing mutations in Prp31. hTrf4-1 cDNA was generated by primers AN101 and AN102 and was introduced into the HindIII site of pcDNA3, carrying a FLAG tag upstream. METTL1-FLAG is described in Alexandrov et al.45 Primers AN189 and AN190 were then used to generate Prp31-FLAG cDNA with a C-terminal FLAG sequence, which was inserted in the BamH1-Xhol site. AN202, AN203, AN204, and AN205 were used to introduce the Y205A, Y273A, and Y275A mutations (mut1), whereas AN206, AN207, AN210, and AN211 were used to generate additional S456A mutations (mut2) into the C-terminally FLAG-tagged Prp31 by site-directed mutagenesis. Primers AN189/AN225 and AN189/ AN226 were used to generate truncated Prp31 proteins, aa1–483 and aa1–438. Mtr4 cDNA was generated using AN110 and AN232 primers and was inserted into the Xhol site of the pGEX-6P-3 construct (GE Life Healthcare Sciences). Plasmids used for in vitro transcription/translation of Prp proteins were obtained from Open Biosystems. Extract preparation and immunoprecipitation. FLAG-tagged proteins were expressed in HEK293 cells by transfecting appropriate plasmids. 48 h later, cells were harvested and washed with ice-cold PBS. Cells were then incubated in 1 packed cell volume (PCV) of buffer A (10 mM Hepes, 1.5 mM MgCl2, 100 mM KCl, 0.1 mM DTT), followed by passage through a 25 G/0.8 needle attached to a 1 ml syringe 8 times. The cell lysate was centrifuged at 12000 g. The supernatant was discarded and the pellet that contains nuclei was collected. The pellet was resuspended with 3/5 PCV of buffer C (20 mM Hepes, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.1 mM DTT, and 1% protease inhibitor cocktail from Calbio-
chem) for 15 min on ice. Finally, the nuclear fraction was centrifu-
gated at 12000 g for 5 min and the supernatant collected for immunoprecipitation. Extract from four 10 cm plates (approxima-
tely 20 × 106 cells) was mixed with 40 µl FLAG-M2 beads (Sigma) for immunoprecipitation for 2–4 h at 4°C. Beads were washed with 20 volumes of wash buffer (20 mM Tris pH7.5, 0.5 mM MgCl2, 0.5 M NaCl and 0.1% NP-40) 5 times. Proteins were eluted using 60 µg FLAG peptide. LC-MS/MS was analyzed by the protein core facility at Columbia University Medical Center. For formaldehyde cross-linking experiments, approximately 5 × 106 cells (one 90% confluent 10 cm plate) were incubated with 0.1% formaldehyde for 10 min at 37°C. Cells were washed with ice-cold PBS to eliminate extra formaldehyde and the cross-linking reaction was quenched with 0.5 µl 0.25 M glycine for 5 min. The cells were washed twice with ice-cold PBS and resuspended in RIPA buffer containing protease inhibitor (Calbiochem) and 1 mM DTT. After incubation on ice for 10 min, the cell suspension was sonicated 3 times for 10 sec at 30% efficiency. The lysate was centrifuged at 10000 rpm for 10 min and the supernatant was used for immunoprecipitation. Proteins were precipitated overnight at 4°C using 20 µl FLAG-M2 beads in the presence of 50 µg BSA and 10 µg RNaseA. The IP was washed with 1 ml RIPA buffer 8 times and proteins were eluted with SDS buffer. Experiments without crosslinking were done exactly the same way but without formaldehyde and glycine. We were able to obtain significant binding between
FLAG-tagged Prp31 and Mrt4 reproducibly only when cell lysates were prepared in RIPA buffer followed by sonication. An extract prepared by the milder Dignam protocol failed to show interaction.

Prp31 immunodepletion. An amount of 40 μl Dignam nuclear extract was adjusted to 700 mM NaCl, followed by immunodepletion using 6 mg protein A beads bound to anti-Prp31 antibody for 2 h. Supernatant from the depletion was incubated with protein A beads without antibody for 30 min. A mock depletion was performed in parallel using rabbit preimmune serum. Depleted extract was then divided into two sets for anti-Prp31 and rabbit preimmune serum.

Beads were washed with 500 μl NET-2 buffer. Proteins were eluted with SDS sample buffer and were run on an 8% gel followed by probing with anti-Prp31 antibody.

GST-Mr4 expression and immunoprecipitation. The GST-Mr4 expression plasmid (see Plasmids and cloning) was transformed into Rosetta cells and protein was expressed at 37°C for 3 h following IPTG treatment. Proteins were isolated following translation System, Promega)35 S-Met radiolabeled protein from

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