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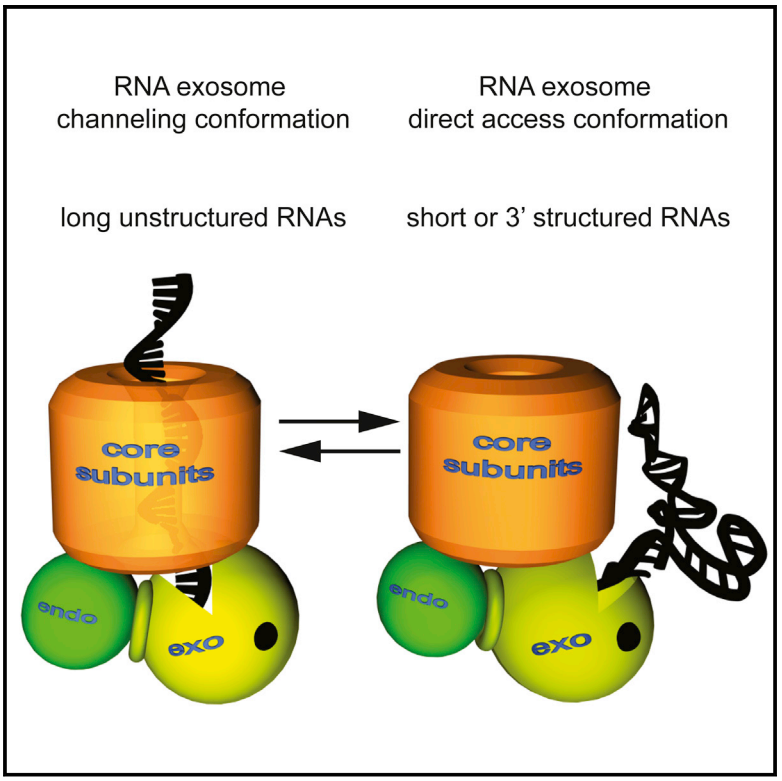
The RNA Exosome Channeling and Direct Access Conformations Have Distinct In Vivo Functions

Graphical Abstract



Highlights

- Functions of the channeling versus direct access RNA exosome conformations tested
- Specific substrates access RNA exosome directly, bypassing the central channel
- Direct access substrates include hypomodified tRNA and 5S rRNA

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In Brief

The RNA exosome adopts different conformations. In one conformation, RNA can pass through a central channel to access the 3′ exonuclease active site, whereas in the alternative conformation, RNA can access the active site directly. Han and van Hoof show that these routes are used by different RNA substrates.

Han & van Hoof, 2016, Cell Reports *16*, 3348–3358
September 20, 2016
http://dx.doi.org/10.1016/j.celrep.2016.08.059

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The RNA Exosome Channeling and Direct Access Conformations Have Distinct In Vivo Functions

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http://dx.doi.org/10.1016/j.celrep.2016.08.059

SUMMARY

The RNA exosome is a 3′–5′ ribonuclease complex that is composed of nine core subunits and an essential catalytic subunit, Rrp44. Two distinct conformations of Rrp44 were revealed in previous structural studies, suggesting that Rrp44 may change its conformation to exert its function. In the channeling conformation, ($Rrp44^{ch}$), RNA accesses the active site after traversing the central channel of the RNA exosome, whereas in the other conformation, ($Rrp44^{da}$), RNA gains direct access to the active site. Here, we show that the $Rrp44^{da}$ exosome is important for nuclear function of the RNA exosome. Defects caused by disrupting the direct access conformation are distinct from those caused by channel-occluding mutations, indicating specific functions for each conformation. Our genetic analyses provide in vivo evidence that the RNA exosome employs a direct-access route to recruit specific substrates, indicating that the RNA exosome uses alternative conformations to act on different RNA substrates.

INTRODUCTION

The RNA exosome is an essential 3′–5′ exoribonuclease complex with a wide variety of molecular functions (Chlebowski et al., 2013; Houseley and Tollervey, 2009; Januszyk and Lima, 2014; Makino et al., 2013b). In the nucleus, it processes 3′-ends of various RNA species and degrades aberrant RNAs (Porrua and Libri, 2013). In the cytoplasm, it is involved in regular mRNA turnover and mRNA surveillance (Klauer and van Hoof, 2012; Schaeffer et al., 2010). These molecular functions are best defined in yeast, but many are conserved in human cells (Staals and Pruijn, 2011). In human patients, different mutations that affect RNA exosome activity are associated with distinct human diseases: multiple myeloma (Weißbach et al., 2015), pontocerebellar hypoplasia (Boczonadi et al., 2014; Wan et al., 2012), trichohepatoenteric syndrome (Fabre et al., 2012; Hartley et al., 2010), and most recently, a distinct disorder that has not yet been named (Di Donato et al., 2016). A thorough investigation of the structure and function of the RNA exosome is essential to understand how the RNA exosome carries out all of these functions, and how RNA exosome defects cause these very diverse diseases.

The exo-9 core of the eukaryotic RNA exosome is composed of nine essential subunits and the overall architecture of this core is conserved among bacteria, archaea, and eukaryotes (Januszyk and Lima, 2010, 2014; Liu et al., 2006). The exo-9 core is formed by six RNase PH-like subunits that form a ring structure and are capped on one side by three RNA binding proteins. In the bacterial and archaeal enzymes, the catalytic active sites are located inside of the PH-ring and a single-stranded RNA is threaded into the central channel of this ring for degradation (Evguenieva-Hackenberg, 2010; Evguenieva-Hackenberg et al., 2014; Lin-Chao et al., 2007; Lorentzen et al., 2005). Unlike its bacterial and archaeal counter parts, the exo-9 core of the eukaryotic RNA exosome is catalytically inert. Instead, it interacts with two nucleases, Rrp44/Dis3 and Rrp6 (Bonneau et al., 2009; Butler and Mitchell, 2011; Dziembowski et al., 2007; Makino et al., 2013a; Malet et al., 2010; Wasmuth et al., 2014). Rrp44 is responsible for most of the 3′ to 5′ exoribonuclease activity of the RNA exosome (Dziembowski et al., 2007), while Rrp6 is restricted to the nucleus and its 3′ to 5′ exoribonuclease activity appears to be important for a subset of RNA exosome functions (Briggs et al., 1998; Butler and Mitchell, 2011). In addition to 3′–5′ exoribonuclease activity, Rrp44 has endoribonuclease activity that is mediated by a separate active site (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). While yeast has one RRP44/DIS3 gene, the human genome encodes three homologs Dis3, Dis3L1, and Dis3L2. Dis3 and Dis3L1 associate with the Exo-9 core in the nucleus and cytoplasm, respectively (Januszyk and Lima, 2014), and appear functionally very similar to the single yeast Rrp44 (Shiomi et al., 1998; Staals et al., 2010; Tomecki et al., 2010). In contrast, Dis3L2 is not known to be associated with the Exo-9 core, indicating that Rrp44 family members can have RNA exosome-independent functions (Chang et al., 2013; Lubas et al., 2013; Malecki et al., 2013).

The exo-9 core RNA exosome in association with Rrp44 has been studied by X-ray crystallography and electron microscopy (EM) (Bonneau et al., 2009; Liu et al., 2014, 2016; Makino et al., 2013a, 2015; Malet et al., 2010; Wang et al., 2007). These studies

revealed that the ability of RNA to bind inside the exo-9 ring is conserved between eukaryotic, bacterial, and archaeal enzymes. However, since there is no active site in the ring, an important difference is that in the eukaryotic enzyme the RNA substrate is thought to pass all the way through the exo-9 ring to access the Rrp44 active site. This channeling of RNA substrates in vitro requires a long (30 nt) unstructured 3′ end (Bonneau et al., 2009; Liu et al., 2014; Makino et al., 2013a; Malet et al., 2010). A different conformation of Rrp44 has also been described by both X-ray crystallography and EM (Bonneau et al., 2009; Liu et al., 2014, 2016; Makino et al., 2013a, 2015). This second conformation is seen in vitro in either the absence of RNA, or with very short (<12 nts) RNAs of unclear physiological relevance, and has been suggested to be important in vivo for acting on RNAs with highly structured 3′ ends (Liu et al., 2014). In this alternative conformation, RNA is thought to access the exonuclease active site of Rrp44 without going through the central channel. For convenience, we will refer to these two conformations as Rrp44^{ch} (channel) RNA exosome conformation and Rrp44^{da} (direct access) RNA exosome conformation, indicating the substrate recruitment site (Movie S1). The exo-9 core of the RNA exosome contains the same proteins for both conformations and undergoes only minor conformational change (Liu et al., 2014). Thus, the only significant difference between the da and ch conformations of the RNA exosome is a large rotation of the exonuclease domain of Rrp44, while the endonuclease domain and the exo-9 core are essentially identical in both conformations. The importance of the Rrp44^{ch} conformation is supported by the observation that mutations that sterically and electrostatically interfere with RNA channeling through exo-9 cause specific RNA processing and growth defects (Drazkowska et al., 2013; Malet et al., 2010; Wasmuth and Lima, 2012), but whether the Rrp44^{da}-exosome indeed allows direct access of substrates to the active site in vivo, whether the da conformation is only adopted when the RNA exosome is not RNA bound, or whether it is formed at all in vivo is not clear.

The large rotation of the exoribonuclease domain results in a different side of Rrp44 facing exo-9 in the two conformations. In this study, we show that the amino acid residues at the interface between Rrp44^{da} and exo-9 are important for association between Rrp44 and exo-9, and thus that the da conformation is relevant in vivo. Furthermore, we show that specific RNA processing and degradation effects result from disrupting the rrp44^{da}-exosome and thus provide evidence that this conformation is used for specific RNA exosome functions. Shortly after the RNA exosome was discovered, two models for its overall function were proposed (Mitchell and Tollervey, 2000; van Hoof and Parker, 1999). The proteasome-like model proposed that to be degraded, a substrate had to access a central channel of an overall ring-shape structure, and many aspects of this model have since proven correct (Makino et al., 2013b). The now disfavored allosteric activation model was suggested as an alternative, and one important aspect of it was that the RNA exosome adopts different conformations for different functions. Here, we provide evidence for the importance of alternative conformations. Our results unite key tenets of both models, with a ch conformation that closely fits the proteasome-like model and an alternative conformation that is required for specific functions.

RESULTS

Identification of Residues in the Exonuclease Domain of Rrp44 that Contribute to Interaction with the RNA Exosome Core

Comparison of multiple X-ray crystallography and EM studies of the RNA exosome suggests that the endonuclease domain does not undergo major conformational changes between the da conformation and the ch conformation of the RNA exosome (Figures 1A–1C; Movie S1; Bonneau et al., 2009; Liu et al., 2014, 2016; Makino et al., 2013a, 2015). In contrast, the exonuclease domain forms two distinct Rrp44-exosome interfaces with the core subunits Rrp41 and Rrp45 (Figures 1B and 1C; Movie S1). Initial experiments suggested that the exonuclease domain has noncatalytic functions, possibly including contributing to exo-9 interaction (Figure S1; Supplemental Information). Structural studies indicate that such exonuclease domain interaction with exo-9 would be specific to one or the other conformation. Five conserved residues of Rrp44 (R439, R440, H466, L500, and D602) appear to be important for exonuclease domain interaction with exo-9 in the da conformation (Figures 1B and S2A; highlighted in green in Movie S1). Mutation of these residues would specifically disrupt the interaction of Rrp44^{da} with the RNA exosome core because they do not seem to participate in the Rrp44^{ch}-exosome core interaction and are largely solvent exposed in Rrp44^{ch} (Figure 1C, red spheres; Movie S1). Therefore, we constructed a mutant allele of *RRP44*, *rrp44-da*, in which these five residues are changed to alanine and used co-immunoprecipitation to test for effects on exo-9 interaction.

Rrp44-TAP variants were immunoprecipitated from a yeast strain, which expresses Myc-tagged Rrp43 (one of the exo-9 subunits), and western blot was performed to detect co-precipitation of Rrp43-Myc (Figure 1D). Furthermore, we performed these experiments under low, medium, and high stringency conditions (no NaCl, 50 mM NaCl, and 1 M NaCl). Under the high stringency condition, similar to what we used previously (Schaeffer et al., 2012), the amount of Rrp43-Myc that co-purified with Rrp44-da-TAP or the previously analyzed Rrp44-CR3-TAP was reproducibly reduced compared to wild-type Rrp44-TAP, suggesting that residues in both the endonuclease and exonuclease domains are important for the interaction of Rrp44 with the RNA exosome core (Figure 1D). The CR3 motif within the endonuclease domain forms a zinc coordination site that is important for the proper positioning of the YRD motif that directly interacts with the exo-9 core (Makino et al., 2013a; Schaeffer et al., 2012). Rrp44-yrd-TAP, in which the YRD motif is changed to alanines, showed no detectable co-purified Rrp43-Myc at high stringency, consistent with the idea that the YRD motif directly interacts, while the CR3 motif has a less important role by positioning the YRD residues. Under medium stringency conditions, wild-type Rrp44, Rrp44-CR3, and Rrp44-da reproducibly co-immunoprecipitated approximately equal amounts of Rrp43-myc, while Rrp44-yrd co-purified strongly reduced amounts. Finally, under low stringency conditions, all three mutant forms of Rrp44-TAP co-purified Rrp43-myc. These data indicate that residues in both the endonuclease and exonuclease domains contribute to interaction with the core RNA exosome, although the contribution of the R439, R440,

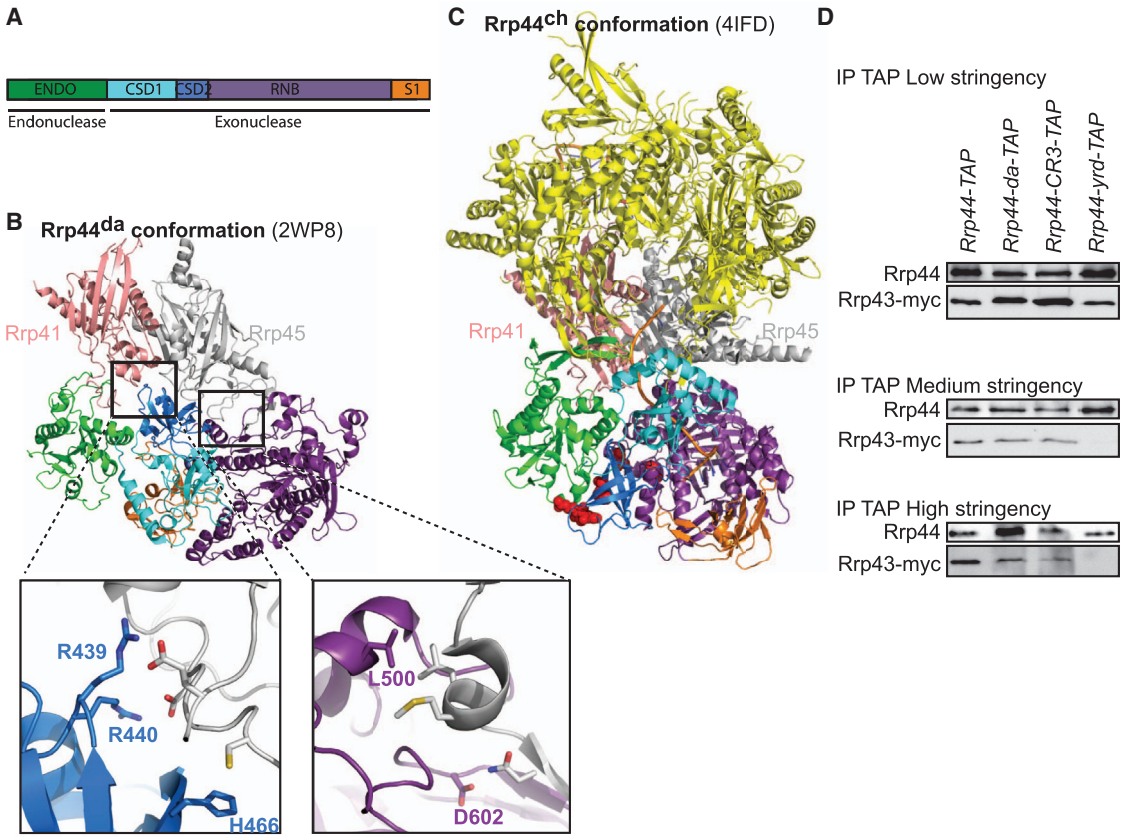

Figure 1. Identification of Residues Important for the da Conformation of the RNA Exosome

(A) Domain organization of Rrp44. endonuclease domain: ENDO; cold shock domain 1/2: CSD1/2; RNase II family catalytic domain: RNB; S1 RNA binding domain: S1. The domains are color coded as in (B) and (C).

(B) Five conserved residues (R439, R440, H466, L500, and D602) that are important for the formation of the Rrp44^{da}-conformation.

(C) The five residues (shown as red spheres) are located on the bottom and exposed to solvent in the Rrp44^{ch}-conformation. The cartoon versions of the X-ray crystal structures were generated by MacPyMol (Schrödinger).

(D) Mutations in the five residues of (B) disrupt the Rrp44-exosome interaction. The TAP-tagged variants of Rrp44 were immunoprecipitated at different wash conditions, and western blot was conducted by using α-Protein A and α-Myc antibodies.

H466, L500, and D602 residues in the exonuclease domain is not as important as the contribution of the YRD motif in the endonuclease domain. In addition, the contribution of the R439, R440, H466, L500, and D602 residues for exo-9 interaction suggests the presence of the da conformation in vivo.

The RNA Exosome da Conformation Is Required for Its Normal Function

To determine whether the Rrp44^{da} conformation is required for the function of the RNA exosome in vivo, we tested the growth of the *rrp44-da* mutant using a plasmid shuffle assay. Briefly, an *RRP44* deletion strain that carries a wild-type *RRP44* allele on a plasmid with a *URA3* marker was transformed with a second plasmid carrying a wild-type *RRP44* or *rrp44* mutant alleles and a *LEU2* marker. Resulting transformants were plated on 5FOA containing media that selects for cells that have lost the *RRP44*, *URA3* plasmid as well as on control media. The strain transformed with *rrp44-CR3* or *rrp44-yrd* grew slowly after losing the wild-type *RRP44* gene, which is consistent with previous studies (Figure 2; Schaeffer et al., 2012; Schaeffer and van Hoof, 2011). Importantly, *rrp44-da* also caused a growth defect compared to wild-type, although this growth defect was less severe than that of *rrp44-CR3* or *rrp44-yrd*. The slow growth of the *rrp44-da* strain is not due to reduced expression of the mutant allele, because western blot analysis shows that the mutant and wild-type allele expressed from a plasmid are expressed at similar levels to each other and to the endogenous Rrp44 (Figure 2). The slow growth of the *rrp44-da* strain suggests that the da conformation is required for RNA exosome function. Interestingly, combining the *rrp44-da* mutation with either *rrp44-CR3* or *rrp44-yrd* resulted in lethality (Figure 2), suggesting that the two contact sites with exo-9 are partially redundant (see Discussion).

Because the *rrp44-CR3-da* and *rrp44-yrd-da* alleles were lethal, we could not assess whether these proteins are expressed at the normal level. We thus repeated the analysis with TAP-tagged plasmids. The plasmid shuffle assay with *RRP44-TAP* variants confirmed that *rrp44-CR3-da* and *rrp44-yrd-da* alleles were lethal. Importantly, when introduced into a wild-type strain (that contains the endogenous *RRP44* gene), the Rrp44-CR3-da-TAP and Rrp44-yrd-da-TAP proteins were detected by

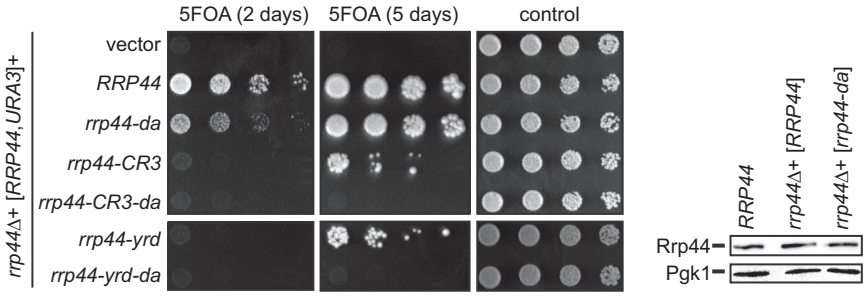

Figure 2. The Rrp44^{da}-Exosome Interface and the CR3/YRD RNA Exosome Interface Are Partially Redundant

rrp44-da causes a slow growth phenotype and is synthetic lethal with *rrp44-CR3* and *rrp44-YRD*. An *rrp44*Δ strain carrying a wild-type *RRP44* allele in a *URA3* plasmid was transformed with *LEU2* plasmids carrying *RRP44* variants. The transformants were serially diluted and spotted on 5FOA and SC-LEU-URA (control) media. The western blot indicates that the plasmid-encoded *rrp44-da* allele is expressed at the same level as endogenous Rrp44 (first lane) or plasmid-encoded wild-type Rrp44 (second lane).

western blot, ruling out the possibility that the lethal phenotype is due to the lack of expression (Figure S3). Taken together, these data suggest that both the five residues mutated in *rrp44-da* and the CR3/YRD motif are important for the function of the RNA exosome and that they are partially redundant. In addition, the importance of the five residues indicates that the Rrp44^{da}-exosome contributes to the essential function of the RNA exosome.

The RNA Exosome da Conformation Utilizes Both the Exo- and Endoribonuclease Activities

Given that the RNA exosome possesses both exo- and endonuclease activities, we tested what activities require the Rrp44^{da}-exosome conformation. For this experiment, we used well-characterized mutations that generate an RNA exosome with only endonuclease activity (*rrp44-exo*$^{-}$) or an RNA exosome with only exonuclease activity (*rrp44-endo*$^{-}$) (Dziembowski et al., 2007; Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Introduction of the *rrp44-da* mutation into the RNA exosome with only endonuclease activity resulted in lethality (Figure 3A). This suggests that the Rrp44^{da} exo-9 interface is required for endonuclease activity. Similarly, introducing the *rrp44-da* mutation into the RNA exosome with only exonuclease activity resulted in severe growth defect (Figure 3A). Therefore, the Rrp44^{da} conformation is also required for exonuclease activity. A similar experiment using TAP-tagged variants showed the same lethal phenotype, and the expression level of the TAP-tagged proteins was comparable to TAP-tagged wild-type Rrp44, suggesting that the lethality is not due to failure to express the variant (Figure S3). This indicates that disrupting the da RNA exosome conformation affects both the exo- and endonuclease activities of the RNA exosome.

The RNA Exosome da Conformation Is Important for Nuclear Functions but May Be Dispensable in the Cytoplasm

The RNA exosome is present in both the nucleus and cytoplasm (Januszyk and Lima, 2014). The nuclear form of the RNA exosome is essential, while the cytoplasmic form is not (Anderson and Parker, 1998; Mitchell et al., 1997). Thus, the analysis of growth and viability described above assesses the essential nuclear function of the RNA exosome and suggests that the Rrp44^{da} conformation is important for nuclear function of the RNA exosome. This predicts that the *rrp44-da* mutation may show genetic interactions with mutations of nuclear RNA exosome co-factors, such as Rrp6. Rrp6 is an additional exonuclease that associates with the RNA exosome in the nucleus, but also has noncatalytic roles including mediating interactions with additional co-factors such as Rrp47 and Mtr4 (Butler and Mitchell, 2011; Feigenbutz et al., 2013; Schuch et al., 2014). As expected, the *rrp44-da* mutation shows a synthetic growth defect with *rrp6Δ* (Figure 3B), confirming that the Rrp44^{da} conformation is important for the nuclear functions of the RNA exosome.

To investigate whether the Rrp44^{da} conformation is also essential for the function of the cytoplasmic RNA exosome, we carried out two experiments. First, we tested for genetic interactions with the *dcp1-2* mutation. The cytoplasmic RNA exosome functions in one of two general mRNA decay pathways, and the cytoplasmic RNA exosome is not essential because of redundancy between these pathways (Anderson and Parker, 1998). Therefore, the cytoplasmic RNA exosome becomes essential if the alternative pathway is inactivated. *dcp1-2* is a temperature sensitive mutation in the alternative pathway, such that the cytoplasmic RNA exosome is essential in a *dcp1-2* strain incubated at the restrictive temperature (Schaeffer and van Hoof, 2011). *rrp44-da* did not show a significant growth defect when combined with *dcp1-2* (Figure 3C). This is in contrast with *rrp44-CR3*, which is synthetic lethal with *dcp1-2* at the restrictive temperature as previously shown (Schaeffer et al., 2012). The *rrp44-yrd* mutation is also synthetic lethal with *dcp1-2* as expected. This suggests that the Rrp44^{da} conformation is not essential for mRNA degradation by the cytoplasmic RNA exosome.

In addition to its function in general mRNA decay, the cytoplasmic RNA exosome is required for the rapid degradation of specific aberrant mRNAs (Frischmeyer et al., 2002; Klauer and van Hoof, 2012; Meaux and Van Hoof, 2006; van Hoof et al., 2002). Thus, in the second experiment, we tested the effect of the *rrp44-da* mutation on this mRNA quality control function. The *his3-nonstop* reporter mRNA lacks a stop codon and therefore is rapidly degraded by the cytoplasmic RNA exosome (van Hoof et al., 2002). Mutations that inactivate the cytoplasmic RNA exosome stabilize the *his3-nonstop* mRNA, which allows the cell to synthesize sufficient histidine to grow in the absence of added histidine. As previously reported (Schaeffer et al., 2012; Schaeffer and van Hoof, 2011), the *rrp44-CR3* mutation allows a *his3-nonstop* strain to grow in the absence of added histidine, indicating a defect in cytoplasmic RNA exosome function (Figure 3D, left two images). As expected, the *rrp44-yrd* mutation

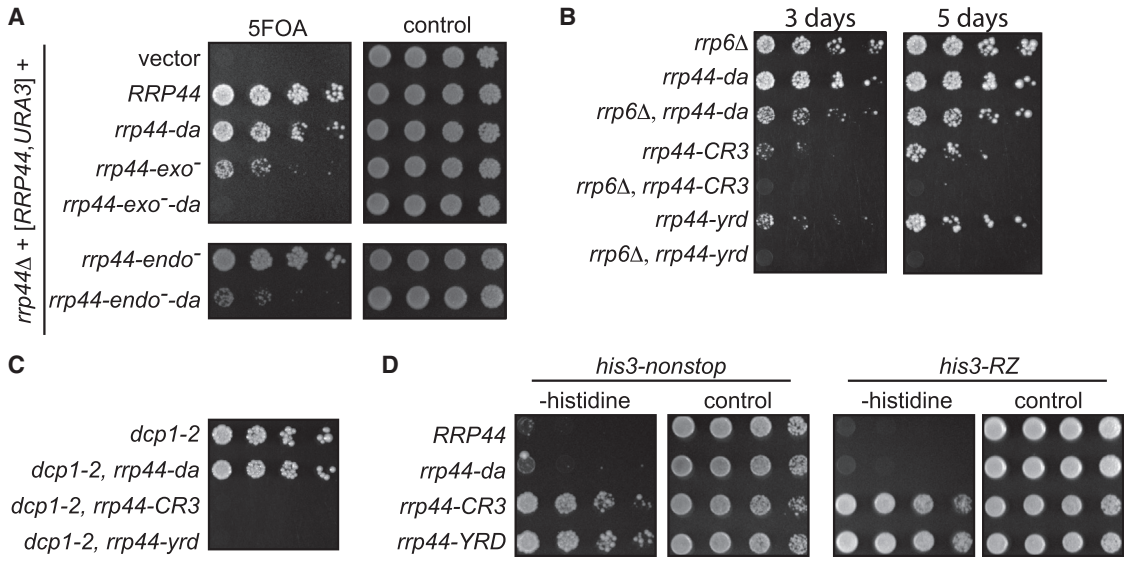

Figure 3. The Rrp44^{da}-Exosome Utilizes Both the Exo- and Endonuclease Activities and Functions in the Nucleus

(A) *rrp44-da* is synthetic lethal with *rrp44-exo*$^{-}$ and *rrp44-endo*$^{-}$. An *rrp44*Δ strain carrying a wild-type *RRP44* allele in a *URA3* plasmid was transformed with *LEU2* plasmids carrying *RRP44* variants. The transformants were serially diluted and spotted on 5FOA and SC-LEU-URA (control) media.

(B and C) Synthetic growth defect of *rrp44-da* with Δ*rrp6* and *dcp1-2*. *rrp44*Δ *rrp6*Δ or *rrp44*Δ *dcp1-2* strains carrying a wild-type *RRP44* allele in a *URA3* plasmid were transformed with *LEU2* plasmids carrying a wild-type *RRP44*, *rrp44-da*, *rrp44-CR3*, or *rrp44-yrd*. The transformants were serially diluted and spotted on 5FOA.

(D) Strains carrying *RRP44* variants were transformed with reporter constructs encoding aberrant *HIS3* mRNAs. The transformants were serially diluted and spotted on media lacking histidine or control plates containing histidine. The *his3-nonstop* reporter is the *HIS3* gene with its stop codon removed. The *his3-RZ* reporter has a hammerhead ribozyme cleavage site immediately upstream of stop codon of the *HIS3* gene.

has the same effect. In contrast, the *rrp44-da* mutation does not affect the *his3-nonstop* reporter mRNA, suggesting that the Rrp44^{da} conformation is not required for nonstop mRNA degradation by the cytoplasmic RNA exosome.

We repeated the assay for mRNA quality control defects with a different reporter mRNA (Figure 3D, right two images). The *his3-RZ* reporter contains a hammerhead ribozyme and therefore generates a truncated mRNA that lacks a poly(A) tail (Meaux and Van Hoof, 2006). Such mRNA cleavage fragments are also degraded by the cytoplasmic RNA exosome, regardless of whether they contain a stop codon or not. As with *his3-nonstop*, mutations that inactivate the cytoplasmic RNA exosome stabilize the *his3-RZ* mRNA, which allows the cell to synthesize sufficient histidine to grow in the absence of added histidine. As previously reported, the *rrp44-CR3* mutation allows a *his3-RZ* strain to grow in the absence of added histidine and, as expected, the *rrp44-yrd* mutation has the same effect. However, the *rrp44-da* mutation does not have this effect. Together, genetic analyses suggest that the Rrp44^{da}-exosome conformation functions in the nucleus, but is dispensable for cytoplasmic RNA exosome functions.

The RNA Exosome da Conformation Is Required for Specific RNA Degradation Events but Makes Minor Contributions to Others

It has previously been shown that unmodified initiator tRNA (tRNAi^{Met}) binds to the da conformation of the RNA exosome in vitro (Liu et al., 2014), and that the RNA exosome degrades hypomodified tRNAi^{Met} in vivo (Kadaba et al., 2004). We therefore hypothesized that the da conformation of the RNA exosome may be especially important for the degradation of hypomodified tRNAi^{Met} in vivo. Thus, we tested the hypomodified tRNAi^{Met} level by northern blot analysis as previously described. This analysis used a *trm6-504 rrp44Δ* strain transformed with plasmids encoding either wild-type or mutant Rrp44. The *trm6-504* mutation causes a defect in ^{m1}A58 methylation and thus causes the hypomodification that triggers exosome-mediated degradation of tRNAi^{Met} in the strain containing the wild-type *RRP44* gene. In contrast, tRNAi^{Met} accumulates to approximately 2-fold higher levels in the *rrp44-da* strain (Figure 4A). Although the *rrp44-da* mutation only caused a 2-fold increase, this increase was highly reproducible and similar to previous reports (Kadaba et al., 2004; Wang et al., 2008). The *rrp44-exo*$^{-}$ mutation caused similarly high tRNAi^{Met} levels, while the *rrp44-endo*$^{-}$ mutation had no effect, suggesting that the exonuclease is the major activity responsible for hypomodified tRNAi^{Met} degradation. The *rrp44-CR3* and *rrp44-yrd* mutations also increased hypomodified tRNAi^{Met} levels, but this effect was reproducibly smaller than the effect of the *rrp44-da* mutation (see Discussion).

We confirmed the role of the da conformation in pre-tRNAi^{Met} degradation using a growth assay. The temperature sensitive growth of a *trm6-504* strain is caused by reduced tRNAi^{met} level and therefore this temperature sensitivity is suppressed by *rrp44* mutations that affect tRNAi^{Met} degradation. The *trm6-504* strain with a wild-type *RRP44* allele failed to grow at 36 degrees (Figure 4B). This growth phenotype was strongly suppressed by *rrp44-exo*$^{-}$ and *rrp44-da*, while *rrp44-CR3* and *rrp44-yrd* were slightly less effective at restoring growth. This growth phenotype

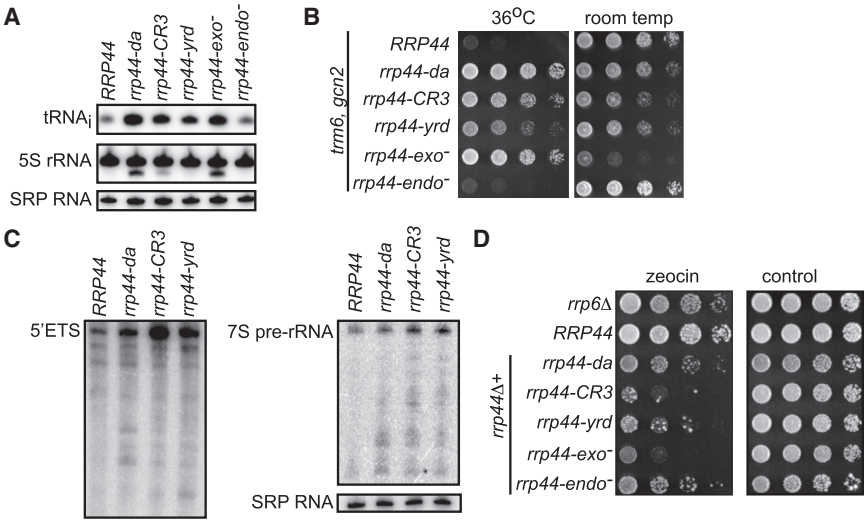

Figure 4. The Rrp44^{da}-Exosome Is Required for Specific Functions of the RNA Exosome

(A) Total RNA isolated from *trm6*, *gcn2*, *rrp44*Δ strains carrying wild-type or mutant alleles of *RRP44* in a *LEU2* plasmid were subjected to northern blot. Shown is a representative result of two independent biological replicates.

(B) Strains used in (A) were serially diluted and spotted on SC-LEU.

(C) Total RNAs were isolated from *rrp44*Δ strains carrying a wild-type or mutant *RRP44* allele followed by northern blot probing 5′ETS and 7S pre-rRNA. The SRP RNA serves as a loading control.

(D) *rrp6*Δ and *rrp44*Δ strains carrying wild-type or mutant *RRP44* alleles were serially diluted and spotted on media containing 10 μg/ml zeocin and YPD media as a control.

mirrors the effects seen by northern blot, confirming that the exonuclease activity and da conformation of Rrp44 are required for the rapid degradation of hypomodified tRNAi^{Met}.

The effect of Rrp44 on hypomodified tRNAi^{Met} was initially found in a strain that carries the *rrp44-20* point mutation. This same *rrp44-20* mutation also causes the accumulation of a truncated 5S rRNA (Kadaba et al., 2004). We therefore next analyzed the effect of the same *RRP44* mutations on 5S rRNA, and the results mirrored what we observed for hypomodified tRNAi^{Met}. Specifically, the *rrp44-da* and *rrp44-exo*$^{-}$ strains reproducibly accumulated relatively high amounts of the truncated 5S rRNA, whereas the *rrp44-CR3* and *rrp44-yrd* mutations had a much smaller effect and the *rrp44-endo*$^{-}$ mutation had no effect (Figure 4A).

To investigate the role of the da conformation in other specific nuclear RNA exosome functions, we next tested the effect of *rrp44-da* on the 5′ external transcribed spacer (ETS) and 5.8S rRNA, two prototypical RNA exosome substrates. The RNA exosome degrades the 5′ETS that is generated from 35S pre-rRNA processing events (de la Cruz et al., 1998). The RNA exosome is also involved in the maturation of 5.8S rRNA by processing the 3′-end of 7S pre-rRNA (Allmang et al., 1999). Using northern blot analysis, we reproducibly observed a 2-fold increase of the full-length 5′ETS and an accumulation of its degradation intermediates in *rrp44-da* compared to wild-type (Figure 4C, left image). In addition, *rrp44-da* showed a minor accumulation of the processing intermediates of 7S pre-rRNA (Figure 4C, right image). Importantly, these defects are not as severe as the defects in *rrp44-CR3* or *rrp44-yrd* (e.g., 6-fold increase in 5′ETS), indicating that the Rrp44^{da}-exosome has a minor contribution to the degradation 5′ETS and processing of 7S rRNA to 5.8S rRNA (see below).

Recent studies have implicated the nuclear RNA exosome as important for the DNA damage response both in the budding yeast and HeLa cells (Hieronymus et al., 2004; Manfrini et al., 2015; Marin-Vicente et al., 2015). Specifically, the RNA exosome co-factors Rrp6, Trf4, and the nuclear exosome targeting (NEXT) complex were implicated in the DNA damage response (Gavaldá et al., 2013; Hieronymus et al., 2004; Manfrini et al., 2015). We therefore tested whether mutations in the catalytic subunit of the RNA exosome itself cause sensitivity to zeocin, an agent that induces double-strand breaks (Chankova et al., 2007). As reported previously, *rrp6*Δ strain was sensitive to zeocin (Figure 5D; Manfrini et al., 2015). We found that *rrp44-CR3* and *rrp44-exo*$^{-}$ strains are extremely sensitive to zeocin, while the *rrp44-da* strain showed sensitivity similar to *rrp6*Δ. This shows that the exonuclease activity of Rrp44 is required for the DNA damage response, but the da conformation is less critical.

Taken together, the observations that *rrp44-da* has stronger effects on tRNAi^{Met} and 5S rRNA than the *rrp44-CR3* and *rrp44-yrd* mutations, while *rrp44-CR3* and *rrp44-yrd* has stronger effects of growth, RNA exosome interaction, other RNA degradation reactions, and zeocin sensitivity suggest that the effects on tRNAi^{Met} and 5S rRNA reflect a specific requirement of the da conformation for these two RNA exosome functions (see Discussion).

The Balance between the Two RNA Exosome Conformations Is Required for Growth

Next, we sought to identify the relationship between the two conformations, Rrp44^{da}- and Rrp44^{ch}-exosome, since the EM studies suggest dynamic conformational change between them (Liu et al., 2014, 2016). Instead of being maintained by protein contacts, the Rrp44^{ch} conformation is thought to be stabilized by simultaneous interactions of long RNAs with the channel and exonuclease domain (Liu et al., 2016). Specifically, the interaction surface between the exonuclease domain and the RNA exosome core is much larger and electrostatically more favorable in the da conformation than in the ch conformation (Liu et al., 2016). Because of this reliance on RNA to stabilize the ch conformation, we could not identify specific Rrp44 residues required for the ch conformation. As an alternative way to disrupt channeling through the exosome core, we took advantage of the previously reported and characterized channel occluding mutations of Rrp41 and Rrp45 (*rrp41-L* and *rrp45-L*), in which an 11 amino acid residue insertion physically and electrostatically

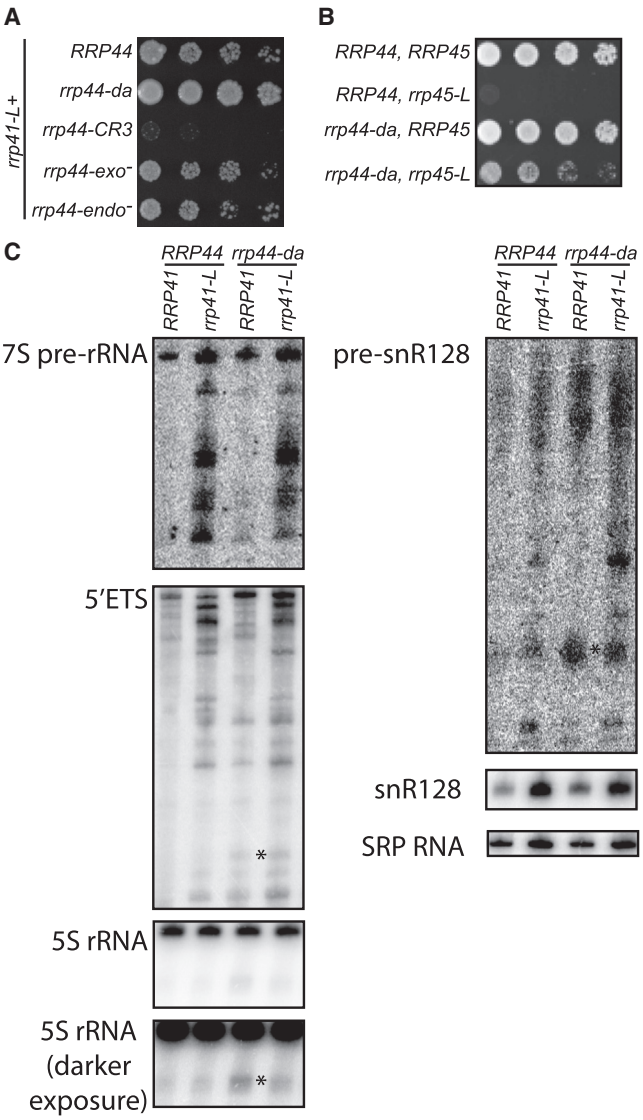

Figure 5. The Balance between Two RNA Exosome Conformations Is Required for Growth

(A and B) *rrp44-da* suppresses the growth defect of *rrp41-L* and *rrp45-L*. *rrp44*Δ *rrp41*Δ or *rrp44*Δ *rrp45*Δ strains carrying *RRP44* variants in a *LEU2* plasmid and a wild-type *RRP41* or *RRP45* in a *URA3* plasmid were transformed with a *TRP1* plasmid carrying the *rrp41-L* or *rrp45-L* allele. The transformants were serially diluted and spotted on 5FOA media.

(C) *rrp44*Δ *rrp41*Δ strains carrying *RRP44* and *RRP41* variants were subjected to total RNA isolation followed by northern blot analysis probing 7S pre-rRNA, 5′ETS, 5S rRNA, snR128 snoRNA, and SRP RNA. The asterisk (*) indicates 5′ETS, 5S, and snR128 RNA species specifically and reproducibly detected in the *rrp44-da* strain.

blocks the central channel of the RNA exosome (Wasmuth and Lima, 2012). *rrp41-L* and *rrp45-L* have slow growing and lethal phenotypes, respectively, which suggests that the central channel is essential. To test the relationship between the two RNA exosome conformations, we tested the genetic interaction between the *rrp44-da* and *rrp41-L* or *rrp45-L* mutations (Figure 5A). Strikingly, *rrp44-da* suppressed the slow growing phenotype of *rrp41-L*. This suppression is specific for the *rrp44-da* allele as *rrp44-exo*$^{-}$ and *rrp44-endo*$^{-}$ do not have a significant effect and *rrp44-CR3* is synthetic lethal with *rrp41-L* (and thus has the opposite effect of the *rrp44-da* allele). Similarly, the *rrp44-da* mutation suppressed the lethality of the *rrp45-L* channel occluding mutation (Figure 5B). We conclude that a proper balance between two conformations is important for the essential function of the RNA exosome (see Discussion).

Having generated strains with either the da conformation or the ch disrupted, we further compared the role of the two conformations in specific RNA exosome functions by northern blotting for known RNA exosome substrates. In addition, we tested whether the suppression of *rrp41-L* growth phenotype by *rrp44-da* was accompanied by restoration of RNA processing and degradation defects. For 7S pre-rRNA to 5.8S rRNA processing, we detected intermediates in the *rrp41-L* strain and lower levels of the same intermediates in the *rrp44-da* strain (Figure 5C). This confirms the conclusion that the direct-access route makes a much smaller contribution to 5.8S processing than the channel route. Furthermore, the processing defect seen in the *rrp41-L rrp44-da* double mutant closely resembled that seen in *rrp41-L*, indicating that suppression of the *rrp41-L* growth phenotype is not accompanied by suppression of this rRNA processing defect.

As described above, the *rrp44-da* strain accumulated full-length 5′ETS as well as some degradation intermediates. The *rrp41-L* strain also accumulated 5′ETS degradation intermediates, but not the full-length 5′ETS. The *rrp41-L* strain accumulated much higher levels of degradation intermediates than the *rrp44-da* strain, again, confirming that the channel route is the major degradation route for 5′ETS. Several intermediates were specific for *rrp41-L*, whereas one specific intermediate was reproducibly only detected in *rrp44*-da, although at low levels (Figure 5C, asterisk). Rather than suppressing the *rrp41-L* phenotype, the effect of combining *rrp41-L* with *rrp44-da* appeared additive, such that both sets of intermediates from the single mutants and the accumulation of full-length 5′ETS were seen in the double mutant.

Although, as pointed out above, for some RNA exosome substrates we saw no suppression of the *rrp41-L* phenotype by *rrp44-da*, for other substrates we did see a suppression that correlates with the suppression of the growth phenotype. Specifically, the RNA subunit of the signal recognition particle (SRP) is commonly used as a loading control, but we noted that it was reproducibly 3-fold more abundant in the *rrp41-L* mutant than in the *RRP41* control strain, consistent with a recent report that this RNA is also a substrate for the RNA exosome (Leung et al., 2014). The *rrp41-L rrp44-da* double mutant strain accumulated only 2-fold more SRP than the *RRP41*, *RRP44* control strain (Figure 5C). Thus, the increased growth rate of this double mutant correlates with a smaller defect in the processing of this particular RNA. We noticed a similar trend with the *snR128* small nucleolar RNA (snoRNA). 3′ extended species of this snoRNA accumulate in RNA exosome mutants, and we observed this phenotype for the *rrp41-L* strain as well. Strikingly, however, the mature *snR128* also over accumulated in the *rrp41-L* strain, and this over accumulation was slightly, but reproducibly, less severe in the *rrp41-L rrp44-da* double mutant

(Figure 5C). Overall, these data indicate that although the *rrp44-da* mutation suppresses the growth phenotype of the blocked RNA exosome channel in *rrp41-L*, most of the RNA processing defects in *rrp41-L* are not suppressed. We did see some minor suppression of SRP and *snR128* defects, but whether this suppression is cause or effect of the suppression of the growth defect is not yet clear. In addition, the comparison of the *rrp41-L* and *rrp44-da* strains confirmed the above conclusion that the da conformation of the RNA exosome is required for a few specific functions, while the ch conformation is required for many other functions.

DISCUSSION

Structural studies have captured the RNA exosome in two conformations in vitro (Liu et al., 2014; Makino et al., 2015). One conformation is consistent with RNA threading through the central channel of the exo-9 core to access the exonuclease active site, while in the other conformation, RNA substrates directly access the active site, bypassing the channel. Here, we provide evidence that the Rrp44^{da}-exosome is present in vivo, and that it has specific functions. We identified and mutated five residues in the exonuclease domain that interact with exo-9 in the da conformation, but are facing the solvent in the ch conformation. We show that mutation of these five residues reduces the co-immunoprecipitation of Rrp44 with Rrp43 and causes a slow growth phenotype. We conclude that the da conformation of the RNA exosome exists in vivo and contributes to RNA exosome function.

Several observations suggest that the da conformation of the exosome has specific, but limited, functions. Most importantly, the severity of RNA exosome defects seen in different *RRP44* alleles cannot be explained by quantitative differences in Rrp44 activity, with some alleles more severely affecting overall exosome activity and others having a smaller effect. For example, the *rrp44-da* mutation has a smaller effect than *rrp44-yrd* on growth, cytoplasmic RNA exosome functions, and most nuclear RNA exosome functions. In contrast, the *rrp44-da* mutation has a larger effect than *rrp44-yrd* on degradation of hypomodified tRNAi^{Met} and truncated 5S rRNA. In fact, for these latter two functions, the severity of the defect in *rrp44-da* is similar to that seen in the catalytically inactive *rrp44-exo*$^{-}$ mutant. Second, while the *rrp44-da*, *rrp44-CR3*, and *rrp44-yrd* alleles all affect Rrp43 co-immunoprecipitation, the severity of these defects does not correlate well with growth and RNA degradation defects. Specifically, the *rrp44-da* and *rrp44-CR3* mutations have similar effects on RNA exosome core interactions and *rrp44-yrd* has a larger effect. This is in contrast to the growth defects that are milder for *rrp44-da* and more severe for *rrp44-CR3* and *rrp44-yrd*. Because of this disconnect between the effect on RNA-exosome binding and growth, we conclude that the effects seen for *rrp44-da* are not simply due to reduced interaction with the RNA exosome core. Third, there is an allele specific suppression of the *rrp41-L* growth phenotype. Specifically, the slow growth phenotype of the *rrp41-L* mutant is suppressed by the *rrp44-da* mutation, but enhanced by the *rrp44-CR3* mutation. Such an allele-specific interaction is difficult to explain by both *rrp44* alleles reducing overall RNA exosome function, but is readily explained by one of the alleles disrupting a specific function. While we have not directly measured the effect of the *rrp44-da* mutation on in vitro catalytic activity, such an effect is unlikely given the location of the mutations. More importantly, even if the mutations reduced the overall catalytic activity, such an effect could not explain the specific in vivo phenotypes. Based on all of these data, we conclude that the *rrp44-da* allele disrupts a specific aspect of RNA exosome function. Based on the structural studies and the effect on RNA exosome core co-immunoprecipitation, the most likely explanation is that the *rrp44-da* allele specifically disrupts the da conformation of the RNA exosome.

By analyzing a variety of previously characterized RNA exosome functions either by northern blot or growth phenotypes, we show that the ch and da conformations of the RNA exosome have distinct functions. Using channel-occluding mutations and qRT-PCR, it has previously been shown that the ch conformation is required for the degradation of cryptic unstable transcripts (CUTs) and 5′ETS and processing of 5.8S rRNA and U4 small nuclear RNA (snRNA) by the nuclear exosome and mRNAs by the cytoplasmic exosome (Drazkowska et al., 2013; Wasmuth and Lima, 2012). We confirm the 5.8S rRNA processing and 5′ETS degradation defects and show that channel-occluding mutations also lead to defects in snoRNA processing. We also noted that the RNA subunit of SRP was more abundant in the channel-occluding mutant, consistent with a recently described role of the RNA exosome in SRP quality control (Leung et al., 2014). Most of the substrates affected by the channel-occluding mutations were affected much less strongly by the da mutation. Conversely, the da mutant accumulated a truncated 5S rRNA form, while the channel-occluding mutation had no effect on 5S rRNA. We also found that the da mutant of Rrp44 was completely inactive in degrading hypomodified tRNAi^{Met}.

Although we identified distinct functions for the da and ch conformations, both appear to be important for degradation of some substrates, such as 5′ETS, although the pattern of intermediates that accumulate in the two mutants is distinct. This may be because the 5′ETS can be degraded by either pathway or because 5′ETS degradation is initiated by the da conformation and then finished by channeling through exo-9. This switch between access routes would require that the 3′ end of 5′ETS dissociates from the Rrp44 catalytic site, a possibility consistent with oligo-adenylation by TRAMP at internal sites (Schneider et al., 2012).

Surprisingly, the growth defect of channel-occluding mutations, *rrp41-L* and *rrp45-L*, is suppressed by disruption of the direct-access route (Figures 5A and 5B). Although many of the defects seen in the single mutants are not reversed in the double mutant, accumulation of full-length *snR128* snoRNA and SRP RNA in *rrp41-L* is decreased in the *rrp41-L rrp44-da* double mutant. A possible explanation is that when one conformation of the RNA exosome is inhibited, the other conformation inappropriately acts on these RNAs. Partially disrupting both conformations could suppress phenotypes by interfering with the inappropriate action of the alternative conformation.

Strikingly, the defects (in initiator tRNA and 5S rRNA) we describe for *rrp44-da* closely resemble those described previously for the *rrp44-20* allele (Kadaba et al., 2004). The single

amino acid substitution in *rrp44-20* (Gly_{833}-Asp) is positioned within the RNA binding channel of the exonuclease domain near the −5 nucleotide (numbering from the active site). Although this part of the RNA binding channel is shared between the direct-access route and the channel route, the mutation appears to have a larger effect on direct access-dependent substrates. We suggest that introduction of a bulky, negative charged residue at this position has a more disruptive effect on the short (12 nt) RNA path of the direct-access route than the much longer (30 nt) path through the channel. Our results raise the possibility that defects in the two different conformations cause different human diseases. Specifically, multiple myeloma genomes often contain mutations in the Rrp44 exonuclease domain, but not in other RNA exosome subunits (Weißbach et al., 2015). In contrast, pontocerebellar hypoplasia is caused by point mutations in the exo-9 core (EXOSC3 and EXOSC8; Boczonadi et al., 2014; Wan et al., 2012). The residues mutated in *rrp44-da* are highly conserved in the human ortholog (Figure S2), suggesting that the da conformation is also important in humans. Thus, we speculate that defects in the direct-access function of DIS3 might contribute to the development of multiple myeloma, while defects in the channel-dependent functions may lead to pontocerebellar hypoplasia. The specific mutations in multiple myeloma may either directly affect the ability to adopt the da conformation, analogous to *rrp44-da*, or affect RNA interactions more severely in the short direct-access route than in the longer channel route, as we propose for *rrp44-20*.

Genetic analyses suggest that the da conformation of the RNA exosome is important for both exo- and endoribonuclease activities. How RNA substrates access the endonuclease active site in general is unknown. Multiple crystal structures indicate the endonuclease domain is static, with the active site readily accessible from the solvent (Bonneau et al., 2009; Makino et al., 2013a, 2015). However, biochemical analyses indicate that channel-occluding mutations also affect the endonuclease activity of the RNA exosome (Drazkowska et al., 2013; Wasmuth and Lima, 2012). This suggests that there may be an additional uncharacterized conformation of Rrp44 that orients the endonuclease site toward the channel. One explanation why the *rrp44-da* mutation inhibits endonuclease activity is that this hypothetical conformational change of the endonuclease domain likely breaks the contact of exo-9 with the YRD motif of Rrp44 and therefore integrity of the RNA exosome in this hypothetical conformation would depend more heavily on interactions between exo-9 and the exonuclease domain of Rrp44.

Regardless of whether substrates utilize the direct-access route or the channel route, the RNA exosome function requires additional proteins that are thought to mediate substrate specificity. Mutations in the TRAMP subunit Trf4 also affect both the degradation of hypomethylated $tRNAi^{Met}$ and the accumulation of truncated 5S rRNA (Kadaba et al., 2004, 2006), suggesting that TRAMP is capable of delivering RNA substrates to the direct-access route, in addition to its better characterized ability to deliver channel-dependent substrates. Thus, the route the RNA takes in the RNA exosome may not be dictated by the co-factor that delivers it to the substrate.

In summary, we show that the da conformation of the RNA exosome is present in vivo and functions on specific substrates in the nucleus. A major difference between the two conformations is the length of the paths, which is ~30 nt and 12 nt, respectively (Bonneau et al., 2009; Liu et al., 2014; Makino et al., 2015; Malet et al., 2010). The longer RNA binding path through a channel is likely to increase continuous binding to long single-stranded RNAs and thus processivity of the RNA exosome, while direct access may be more suitable for structured RNAs or RNAs that are part of large RNPs that don't fit through the central channel (such as $tRNAi^{Met}$ and 5S rRNA). The access point utilized by a particular substrate could therefore result in distinct outcomes in the processing or degradation reactions. Taken together, we propose that the RNA exosome adopts different conformations to accommodate RNA substrates with vastly different characteristics. This resembles the allosteric activation model that was proposed soon after the discovery of the RNA exosome, but has since lost favor (Mitchell and Tollervey, 2000).

EXPERIMENTAL PROCEDURES

Plasmids and Strains

Yeast strains were generated using standard genetic techniques and are listed in Table S1. The *leu2-Δ0* and *trp1Δ::hisG* alleles were introduced as described (Alani et al., 1987; Brachmann et al., 1998). Plasmids were generated using standard molecular biology techniques and are described in Table S2. Rrp44 was expressed from low copy plasmids and driven by the *RRP44* promoter. The *rrp44-da* allele was generated by gene synthesis (GENEWIZ).

Co-immunoprecipitation Assay and Western Blotting

Co-immunoprecipitation (coIP) was performed as previously described with some modifications (Schaeffer et al., 2012). In brief, a yeast strain (yAV1117) that carries Myc-tagged Rrp43 was transformed with TAP-tagged *RRP44* alleles. Exponentially growing transformants in 50 ml media were resuspended in IP0 for low stringency (50 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM β-mercaptoethanol, and 0.1 mM PMSF, with complete EDTA-free protease inhibitors [Roche]) or IP50 (IP0 with 50 mM NaCl) for medium and high stringency. Cells were lysed by vortexing with acid washed glass beads. 200 μl lysate was incubated with 12 μl IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) for 2 hr at 4°C. The beads were washed four times with 1 ml IP0 (low stringency), IP50 (medium stringency), or IP1000 buffer (IP0 with 1 M NaCl; high stringency). The high stringency IP was washed once more with 1 ml IP50 buffer to remove residual salts. Washed beads were mixed with SDS-PAGE sample buffer and heated to 95°C for 5 min to elute bound proteins. Eluted proteins were analyzed by western blot with anti-protein A antibodies (Sigma-Aldrich), anti-Myc antibodies (a generous donation from Dr. Eric Wagner), anti-Rrp44 (NeoBioLab), and anti-Pgk1 antibodies (Invitrogen).

Growth Assays

For growth assays, strains were serially diluted and spotted on the indicated media. Zeocin sensitivity was tested on YPD plates containing 10 μg/ml zeocin.

Northern Blot Analysis

RNA was isolated from cells growing exponentially at 30°C, with the exception of the experiment described in Figure 5A, where cells were grown at 30°C and incubated at 37°C for 4 hr to reveal the *trm6* effect before harvesting as previously described (Kadaba et al., 2004). 10 μg RNA was subjected to 6% polyacrylamide (19:1) 8 M urea gel electrophoresis and transferred to Zeta-Probe GT Blotting membranes (Bio-Rad). Blots were probed using ^{32}P-radiolabeled oligonucleotides listed in Table S3.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, three tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.059.

AUTHOR CONTRIBUTIONS

J.H. conducted all experiments. J.H. and A.v.H. designed and analyzed experiments and wrote the paper.

ACKNOWLEDGMENTS

We thank members of the A.v.H. laboratory for critical discussions, Jim Anderson and Chris Lima for providing strains, Eric Wagner for providing antibodies, and Nayun Kim for help with the DNA damage response assays. This work was supported by NIH R01 grant GM099790 to A.v.H.

Received: December 21, 2015
Revised: June 6, 2016
Accepted: August 18, 2016
Published: September 20, 2016

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