Implication of Ccr4-Not complex function in mRNA quality control in *Saccharomyces cerevisiae*

JANNIE ASSENHOLT,¹ JOHN MOUAIKEL,^{1,2} CYRIL SAGUEZ,^{1,3} MATHIEU ROUGEMAILLE,^{1,2} DOMENICO LIBRI,^{1,2} and TORBEN HEICK JENSEN^{1,4}

¹Centre for mRNP Biogenesis and Metabolism, Department of Molecular Biology, Aarhus University, 8000 Aarhus C., Denmark

²Centre National de la Recherche Scientifique, Centre de Genetique Moleculaire, 91190 Gif sur Yvette, France

ABSTRACT

Production of messenger ribonucleoprotein particles (mRNPs) is subjected to quality control (QC). In *Saccharomyces cerevisiae*, the RNA exosome and its cofactors are part of the nuclear QC machinery that removes, or stalls, aberrant molecules, thereby ensuring that only correctly formed mRNPs are exported to the cytoplasm. The Ccr4-Not complex, which constitutes the major *S. cerevisiae* cytoplasmic deadenylase, has recently been implied in nuclear exosome-related processes. Consistent with a possible nuclear function of the complex, the deletion or mutation of Ccr4-Not factors also elicits transcription phenotypes. Here we use genetic depletion of the Mft1p protein of the THO transcription/mRNP packaging complex as a model system to link the Ccr4-Not complex to nuclear mRNP QC. We reveal strong genetic interactions between alleles of the Ccr4-Not complex with both the exosomal *RRP6* and *MFT1* genes. Moreover, Rrp6p-dependent in vivo QC phenotypes of $\Delta mft1$ cells can be rescued by codeletion of several Ccr4-Not components. We discuss how the Ccr4-Not complex may connect with the mRNP QC pathway.

Keywords: Ccr4-Not complex; RNA exosome; RNA quality control

INTRODUCTION

In *Saccharomyces cerevisiae*, cotranscriptional formation and release of messenger ribonucleoprotein particles (mRNPs) rely on a functional THO complex (for reviews, see Schmid and Jensen 2008; Rougemaille et al. 2008b; Rondon et al. 2010). Deletion of one of the individually inessential subunits of this tetrameric assembly (Chavez et al. 2000) leads to several defects (Rougemaille et al. 2007, 2008a; Saguez et al. 2008), among which is a decreased recruitment of the essential mRNP maturation and export factor Sub2p (Strasser et al. 2002; Zenklusen et al. 2002). As a consequence, mRNA biogenesis is severely compromised, leaving a fraction of nascent mRNA retained in nuclear “dots” at or near the site of transcription (Jensen et al. 2001a, 2004; Libri et al. 2002; Zenklusen et al. 2002; Thomsen et al. 2003; Rougemaille et al. 2007, 2008a). Whereas retained RNA is remarkably stable (Rougemaille

et al. 2007), another pronounced phenotype of THO/Sub2 mutants is that of decreased mRNA levels and transcript 3' end truncation (Libri et al. 2002; Rougemaille et al. 2007). This presumably occurs due to the combined effects of 3'-5' RNA degradation and defects in transcription elongation/termination (Jensen et al. 2001b, 2004; Libri et al. 2002; Saguez et al. 2008). Finally, in THO-deletion strains, several gene 3' ends become trapped in a complex containing at least the transcribed locus itself, its nascent RNA, 3' end processing factors, and nuclear pore complex (NPC) components (Rougemaille et al. 2008a). This complex has been suggested to contain a mRNP intermediate stalled in the process of acquiring nuclear export competence. Its formation leads to the differential partitioning of multiple genomic loci in standard chromatin preparations, a phenomenon referred to as differential chromatin fractionation (DCF).

Given the strong phenotypes at the nascent mRNA/mRNP level, THO-deletion mutants have provided a popular test system for mRNA quality control (QC). Although the precise flaw inflicted to mRNP production is not delineated, data suggest that malfunctioning of the 3' end cleavage/polyadenylation factor, CPF, resulting in inefficient RNA 3' end polyadenylation, may be the direct cause of several of the observed defects (Saguez et al. 2008).

³Present address: Commissariat à l'Energie Atomique, Institut de Biologie et de Technologies de Saclay, 91101 Gif sur Yvette, France.

⁴Corresponding author.

E-mail thj@mb.au.dk.

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Interestingly, all of the mentioned phenotypes depend on the nuclear-specific 3'-5' exonuclease Rrp6p of the *S. cerevisiae* nuclear exosome (Libri et al. 2002; Rougemaille et al. 2008a). Rrp6p is one of two active subunits of the nuclear exosome, which functions in a variety of exo- and endonucleolytic activities, e.g., processing of stable nuclear and nucleolar RNAs as well as degradation of malformed nuclear transcripts (for reviews, see Houseley and Tollervey 2009; Lykke-Andersen et al. 2009; Tomecki et al. 2010). The role of Rrp6p in mRNA QC is apparent when examining *HSP104* RNA in THO mutants codeleted for Rrp6p: Lowered RNA levels, the 3'-5' end truncation bias, DCF formation, and transcription site-linked RNA dots are all phenotypes abolished by *RRP6* deletion (Libri et al. 2002; Rougemaille et al. 2007, 2008a). A current model for Rrp6p-dependent QC posits that 3' end polyadenylation is constantly challenged by nuclear exonucleolysis (Saguez et al. 2008). In a THO-deletion background, polyadenylation efficiency is decreased, thereby creating an opportunity for Rrp6p to elicit 3'-5' degradation of the nascent RNA. Consistent with this idea, the catalytic activity of Rrp6p is essential for triggering RNA QC phenotypes in THO mutants (Assenholt et al. 2008). Rrp6p-challenged mRNA biogenesis may then in turn also lead to the slow release of nascent RNA, triggering DCF formation.

Recently, a functional link between the RNA exosome and the Ccr4-Not complex was established, suggesting that Ccr4-Not might be connecting the exosome to its coactivator TRAMP (Azzouz et al. 2009), a complex that stimulates many exosomal activities, including RNA QC (LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005; Rougemaille et al. 2007). The Ccr4-Not complex consists of at least nine subunits, including the major cytoplasmic deadenylase activity in *S. cerevisiae*, Ccr4p, and Pop2p/Caf1p (Liu et al. 1998; Chen et al. 2001; Tucker et al. 2001). Due to its role in bulk mRNA deadenylation, the cytoplasmic localization of Ccr4-Not is not unexpected (Tucker et al. 2001, 2002); however, the complex has also been implicated in transcription regulation (Denis 1984; Sakai et al. 1992; Collart and Struhl 1994; Deluen et al. 2002; Lenssen et al. 2002) and shown to physically associate with transcription-related factors and complexes such as the Mediator, TBP, TFIID, Paf1p, SAGA (Benson et al. 1998; Chang et al. 1999; Badarinarayana et al. 2000; Liu et al. 2001), and, recently, RNA polymerase II (RNAPII) (Kruk et al. 2011). Thus, a nuclear localization is demonstrated, which has been suggested to occur particularly under stress conditions (Collart and Timmers 2004). In line with this, a human homolog of Pop2p/Caf1p, hCaf1, shows differential subcellular localization during cell cycle progression in HeLa cells (Morel et al. 2003).

FIGURE 1. Genetic interactions between *RRP6* and Ccr4-Not complex genes. (A) The indicated single- and double-deletion strains were spotted in 10-fold dilutions onto YEA plates and incubated for 3 d at the indicated temperatures. (B) Northern blotting analysis of total RNA purified from the indicated strains grown at 25°C. Membranes were probed for established Rrp6p substrates, snR38 and 5.8S rRNA (top) as well as snR71 (bottom), as indicated. A *SCR1* RNA probe was used as a loading control.

By using THO-defective cells as a model system for mRNP deficiency, we report genetic and biochemical evidence for a novel role of the Ccr4-Not complex in nuclear mRNP QC.

RESULTS AND DISCUSSION

Strong genetic interactions between subunits of the Ccr4-Not complex and RRP6

To initiate our investigation into a possible functional connection between the Ccr4-Not complex and Rrp6p, we combined the $\Delta rrp6$ mutant of the W303 strain background with individual viable deletions of the Ccr4-Not complex genes and compared the growth capabilities of the resulting double mutants at different temperatures with that of their parental single deletions. Codeletion of *RRP6* with *CCR4*, *POP2*, or

NOT2 genes robustly reduced growth compared to the single deletions at 30°C and 34°C (Fig. 1A). A more subtle interaction was observed with *NOT4* and *CAF130*, and no genetic interactions were found between *RRP6* and *NOT3* or *CAF40*. This overall confirmed and extended previous genetic data (Azzouz et al. 2009), although strain background differences were also noticeable; e.g., we were unable to obtain viable spores from a cross between $\Delta rrp6$ and $\Delta not5$ (data not shown). The observed genetic interaction may be based on the previously reported physical interactions between the nuclear exosome and Ccr4-Not components (Azzouz et al. 2009).

It was proposed that the Ccr4-Not complex participates with the exosome in the processing of stable RNAs, e.g., snoRNAs (Azzouz et al. 2009). We therefore tested whether the deletion of Ccr4-Not complex subunits interfered with the 3' end processing of known Rrp6p targets such as

FIGURE 2. Ccr4-Not complex components are implicated in mRNA QC. (A) Genetic interactions between *MFT1* and Ccr4-Not complex genes. The indicated strains were spotted onto YEM1 plates in 10-fold dilutions and incubated for 3 d at the indicated temperatures. Note that a growth defect of the $\Delta mft1 \Delta not2$ strain over the respective single deletions is undetectable after only 2 d of growth (Saguez et al. 2008). (B) *HSP104* 3' end RNA FISH analysis of the indicated strains. Exponentially growing cells were incubated for 15 min at 37°C, fixed, and subjected to *HSP104* RNA-FISH visualization using Cy3-labeled probes targeted toward the 3' end of the transcript (top). The fraction of cells with the FISH signal in a given experiment was quantified from three independent experiments and normalized to the result of the $\Delta mft1$ strain (bottom), which in these experiments had 51% $\pm$ 6% of cells with positive *HSP104* 3' end signal. (C) poly(A)⁺ RNA FISH analysis of the indicated strains performed as in B. A Cy3-labeled, LNA-modified dT₂₀ probe was used for polyA targeting, and DAPI staining was used to visualize the chromatin-rich part of the nucleus. The fractions of cells from a representative experiment harboring strong nuclear poly(A)⁺ RNA accumulation are indicated.

snoRNA38 (snR38), snoRNA71 (snR71), or 5.8S rRNA (Allmang et al. 1999; van Hoof et al. 2000; Peng et al. 2003). As shown in Figure 1B, no major processing defects or RNA level defects were evident in the Ccr4-Not deletions. Only RNA purified from $\Delta rrp6$ cells exhibited the typical unprocessed 5.8S + 30 nt rRNA species as well as 3' end extended snR38 and snR71. Although other RNA substrates (e.g., U14) were reportedly 3' end extended in strains deleted of selected Ccr4-Not complex subunits, these effects were moderate as compared to the defects in $\Delta rrp6$ cells (Azzouz et al. 2009) and were not readily visible in our hands (data not shown). Thus, the basis for the genetic interactions between *RRP6* and Ccr4-Not subunit genes is likely to also involve other substrates.

Functional links between the Ccr4-Not complex and mRNA QC

Next we turned our focus to mRNA QC. To this end, we analyzed the growth capabilities of double-deletion mutants of the $\Delta mft1$ allele combined with individual Ccr4-Not gene deletions (Fig. 2A). Of the tested genes, *CCR4*, *POP2*, *NOT2*, and *NOT4* interact with *MFT1*. Consistently, a synthetic lethal interaction between $\Delta ccr4$ and the deletion of the THO complex component *HPR1* gene was reported (Chavez et al. 2000). No genetic interactions could be seen for *NOT3*, *CAF40*, and *CAF130*. These results suggest a functional relationship between the Ccr4-Not and THO complexes, possibly associated with mRNA QC.

To assay for RNA QC phenotypes, we first employed *HSP104* RNA FISH analysis. Exponentially growing cells were exposed to a 15-min, 37°C heat shift to induce transcription of the *HSP104* gene. Subsequently, the cells were fixed, and *HSP104* RNA was visualized employing fluorescently labeled oligonucleotide probes directed toward the 3' end of the transcript. As previously reported, the deletion of the *MFT1* gene led to the appearance of one intense *HSP104* dot signal per cell nucleus (Fig. 2B, top), overlapping the chromatin-associated DAPI signal (data not shown) and thus indicating retention at or near the transcription site. However, combining $\Delta mft1$ with $\Delta ccr4$, $\Delta pop2$, $\Delta not2$, or $\Delta not4$ all caused a decrease in the number of cells harboring the *HSP104* RNA dot (Fig. 2B, top and bottom). The *NOT3*, *CAF40*, and *CAF130* deletions all left the intensity of the $\Delta mft1$ dot unchanged, much like the negative control, a deletion of the *PAN2* deadenylase gene. We note a correlation between the Ccr4-Not subunits required for *HSP104* RNA dot formation (Ccr4p, Pop2p, Not2p, and Not4p) and the strengths of the synthetic negative interactions of their deletions with $\Delta mft1$ (Fig. 2A) and $\Delta rrp6$ (Fig. 1A). The generality of relief of $\Delta mft1$ -induced RNA retention by the *CCR4* deletion was tested using a fluorescently labeled oligo-dT₂₀ LNA-modified probe and resulted in a similar conclusion (Fig. 2C).

Loss of HSP104 dots is not based on decreased transcription

Given the reported role of Ccr4-Not in transcription regulation (Denis and Malvar 1990; Collart and Struhl 1994; Benson et al. 1998; Liu et al. 1998; Deluen et al. 2002; Lenssen et al. 2002, 2005; Kruk et al. 2011), it was essential to investigate the possibility that the disappearance of the *HSP104* RNA FISH signal in the affected double deletions was not simply due to the reduced transcription levels imposed by the added Ccr4-Not gene deletion. We therefore tested *HSP104* gene transcription activity by two different approaches. First, we measured the levels of nascent *HSP104* RNA by utilizing 5' end RNA-FISH probes. Since none of the tested single-deletion mutants exhibited *HSP104* RNA retention as evaluated by *HSP104* RNA 3' end probes (data not shown), this analysis provided a simple surrogate transcription assay. As controls, we included the *rpb1-1* and *rad3-7.7* mutant strains, which were previously shown to be

FIGURE 3. *HSP104* gene transcription levels are not affected by Ccr4-Not gene deletion. (A) *HSP104* 5' end RNA FISH analysis of the indicated strains. Exponentially growing cells were incubated for 15 min at 37°C, fixed, and subjected to *HSP104* RNA visualization using Cy3-labeled probes targeted toward the 5' end of the transcript. Wild-type (WT), as well as *rpb1-1* and *rad3-7.7*, cells served as positive and negative controls, respectively. The fractions of cells from a representative experiment, harboring a positive *HSP104* 5' end signal, are indicated. (B) ChIP analysis of RNAPII (Rpb1p). Y80 antibody recognizing the N terminus of Rpb1p was used to precipitate chromatin from the indicated cells grown exponentially and heat-shifted for 15 min to 37°C before formaldehyde cross-linking. *HSP104* gene 5' end levels were measured by qPCR (output to input ratio) and normalized to Rpb1p ChIP signal for the *ACT1* gene. Average signals and SDs were derived from three independent IP experiments.

defective in transcription initiation at elevated temperatures (Nonet et al. 1987; Jensen et al. 2004). Apart from these negative controls, all the strains displayed a distinct nuclear *HSP104* RNA dot signal (Fig. 3A). Second, we subjected $\Delta ccr4$ and $\Delta pop2$ cells, as well as their respective double deletions with $\Delta mft1$, to RNAPII chromatin immunoprecipitation (ChIP) assays using an antibody directed toward the N terminus of Rpb1p, the largest subunit of RNAPII. As shown in Figure 3B, RNAPII levels at the *HSP104* gene 5' end in the $\Delta mft1$, $\Delta pop2$, $\Delta mft1\Delta pop2$, $\Delta ccr4$, and $\Delta mft1\Delta ccr4$ cells were not, or were only mildly, affected compared with a wild-type (WT) control background. We therefore conclude that *HSP104* RNA retention in $\Delta mft1$ is not abolished by these gene deletions because of decreased transcription activity but rather because of a defective mRNP QC system (see also below).

DCF and *HSP104* RNA 3' end truncation in $\Delta mft1$ are suppressed by deletion of *CCR4* or *POP2*

We next turned to two other assays linked to aberrant *HSP104* RNA biogenesis in $\Delta mft1$ cells: DCF formation and RNA degradation. These analyses were focused on the

$\Delta pop2$ and $\Delta ccr4$ gene deletions. DCF formation was assessed in cells grown for 15 min at 37°C and formaldehyde cross-linked as previously described (Rougemaille et al. 2008a). Since DCF is preferentially localized at the 3' end of genes, its formation was interrogated by qPCR using amplicons specific for the 5' and 3' ends of the *HSP104* gene. The approximately 10-fold increase in *HSP104* DNA 3' ends pelleted from $\Delta mft1$ compared with WT cells was strongly decreased by both *POP2* and *CCR4* codeletion (Fig. 4A). The $\Delta pop2$ and $\Delta ccr4$ single deletions showed only marginal DCF formation. Consistently, the *HSP104* RNA 3'-5' end truncation bias measured in the total RNA preparation from $\Delta mft1$ cells was also fully restored by the codeletion of *POP2* or *CCR4* (Fig. 4B,C). The generally low level of *HSP104* RNA in the $\Delta mft1$ background was not restored by $\Delta pop2$ or $\Delta ccr4$. We conclude that Ccr4p and Pop2p help induce mRNA degradation and DCF formation in a THO gene-deletion background.

CONCLUSIONS

We report that four subunits of the Ccr4-Not complex—Ccr4p, Pop2p, Not2p, and Not4p—severely impact *HSP104* RNA

FIGURE 4. DCF and *HSP104* 3' end truncation-phenotypes of $\Delta mft1$ cells are restored upon *POP2* or *CCR4* codeletion. (A) DCF (DNA content in the “P18k” pellet obtained after a 18000g centrifugation step) was measured using qPCR amplicons directed toward the 5' or 3' ends of the *HSP104* gene. DNA amounts were normalized to an intergenic region in chromosome V and plotted as arbitrary units relative to the wild-type (WT) sample. Average signals and SDs reflect DCF enrichment from three independent experiments. (B) *HSP104* RNA 5' and 3' end levels were quantified by RT-qPCR analysis of total RNA preparations from the same cell cultures and using the same amplicons as in A. *HSP104* RNA amounts in different mutant backgrounds are plotted relative to the WT after normalization to *ACT1* RNA. (C) *HSP104* 3' end/5' end ratios plotted relative to the WT sample. In B and C, the average signals and SDs were derived from three independent experiments.

transcription site-associated retention induced by *MFT1* gene deletion. Moreover, the removal of these genes results in synthetic growth phenotypes when combined with both the $\Delta mft1$ or $\Delta rrp6$ deletions. The latter result implies that the need for Ccr4-Not function in an $\Delta mft1$ context may go through Rrp6p. Although the data link Ccr4-Not to nuclear mRNP QC in *S. cerevisiae*, they do not allow us to conclude whether one component is central or whether the entire complex is involved. This is because individual subunits affect the integrity of the complex; e.g., the interaction between Ccr4p and the rest of the complex is lost in a $\Delta pop2$ background (Bai et al. 1999), and complex formation is dependent on Not2p (Russell et al. 2002).

Given its intimate connection to transcription, and possibly transcription-coupled processes, Ccr4-Not may generally challenge mRNP quality in THO mutants in a way that is eventually detected by an Rrp6p-dependent mechanism. The Ccr4-Not complex could, e.g., be required as a structural component of the QC machinery, perhaps mediating the assembly of a functional Rrp6p-containing complex. This is consistent with the physical interactions reported between several Ccr4-Not components and the RNA exosome (Azzouz et al. 2009). Another possibility is that Ccr4p deadenylase activity is carried out successively with Rrp6p function and that the combination of both activities results in RNA retention. Finally, as the presence of RNA appears to prevent exosome-Ccr4-Not complex interaction (Azzouz et al. 2009), the lack of Ccr4p deadenylation activity may alter the nature of its exosome interaction. Regardless of the mechanistic details, the evidence presented here should inspire further analysis into the functional relationships among Ccr4-Not, TRAMP, and the RNA exosome in nuclear mRNP QC.

MATERIALS AND METHODS

Plasmids, strains, and growth assays

All strains were derived from the *S. cerevisiae* W303 background, and gene deletions as well as strain crosses were done using standard procedures. For yeast plating assays, exponentially growing cells were spotted onto YM1 plates in 10-fold dilutions starting at OD = 0.3 and incubated for 3 d at 30°C, 34°C, or 37°C.

RNA-FISH analysis

HSP104 RNA localization was probed on fixed cells grown for 15 min at 37°C as previously described (Jensen et al. 2001b; Thomsen et al. 2003). Cy3-labeled FISH probes were as follows: *HSP104* RNA 3' end (THJ203-206) (Jensen et al. 2001b), *HSP104* RNA 5' end (THJ361-364) (Thomsen et al. 2008), or an LNA-modified dT₂₀ probe (Thomsen et al. 2005). Slide processing and image preparation were done as previously described (Thomsen et al. 2005).

DNA and RNA preparations

Northern blotting, heavy chromatin, RT-qPCR, and Rpb1p ChIP analyses were performed as previously described (Midtgaard et al.

2006; Rougemaille et al. 2007, 2008a; Assenholt et al. 2008), except that chromatin extracts were immunoprecipitated using an anti-Rpb1p polyclonal antibody (Y-80, Santa-Cruz) pre-conjugated to Dynabeads-protein A (Invitrogen). A 5' end radiolabeled probe (5'-AGATCTGAGTGAGCTGAGAAGG-3') was used for snR71 Northern hybridization.

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Jannie Assenholt, John Mouaikel, Cyril Saguez, et al.

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