The C-terminus of Utp4, mutated in childhood cirrhosis, is essential for ribosome biogenesis

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ABSTRACT

The small subunit (SSU) processome is a large ribonucleoprotein that is required for maturation of the 18S rRNA of the ribosome. Recently, a missense mutation in the C-terminus of an SSU processome protein, Utp4/Cirhin, was reported to cause North American Indian childhood cirrhosis (NAIC). In this study, we use Saccharomyces cerevisiae as a model to investigate the role of the NAIC mutation in ribosome biogenesis. While we find that the homologous NAIC mutation does not cause growth defects or aberrant ribosome biogenesis in yeast, we show that an intact C-terminus of Utp4 is required for cell growth and maturation of the 18S and 25S rRNAs. A protein–protein interaction map of the seven-protein t-Utp subcomplex of which Utp4 is a member shows that Utp8 interacts with the C-terminus of Utp4 and that this interaction is essential for assembly of the SSU processome and for the function of Utp4 in ribosome biogenesis. Furthermore, these results allow us to propose that NAIC may be caused by dysfunctional pre-ribosome assembly due to the loss of an interaction between the C-terminus of Utp4/Cirhin and another SSU processome protein.

INTRODUCTION

Ribosome biogenesis in eukaryotes is a complex, highly regulated and energy intensive process. Indeed, over 150 proteins are required, and in a growing Saccharomyces cerevisiae cell nearly 80% of all transcriptional activity is devoted to making ribosomes (1). Ribosome biogenesis begins with the transcription of the 35S polycistronic pre-ribosomal RNA (rRNA) by RNA polymerase I. The pre-rRNA undergoes multiple modification and cleavage events eventually giving rise to the mature 18S, 25S and 5.8S rRNAs (reviewed in 2; Figure 1). The A0, A1 and A2 cleavages that lead to the generation of the 18S rRNA are dependent on the small subunit (SSU) processome, a large ribonucleoprotein associated with the U3 small nucleolar RNA (snoRNA) (3).

Approximately 40 proteins are present in the SSU processome (2), including over 20 proteins termed the U three proteins (Utps) due to their association with the U3 snoRNA. Proteomic (4) as well as biochemical (5) analysis has revealed that these proteins group into subcomplexes that in many cases can form independently of each other (6). One of these subcomplexes, the t-Utp subcomplex, is associated with the ribosomal chromatin (r-chromatin) and is necessary for optimal transcription of the rDNA. The t-Utp subcomplex contains seven proteins: Utp4, Utp5, Utp8, Utp9, Utp10, Utp15 and Utp17. Each of these proteins is essential in yeast and depletion of any of them results in a reduction of the 35S pre-rRNA as well as a complete loss of mature 18S rRNA and a reduction in mature 25S rRNA (3,5). The t-Utps are therefore necessary for both pre-rRNA transcription and 18S rRNA processing. However, it has alternately been proposed that the t-Utp subcomplex plays a role in pre-rRNA stabilization, rather than in rDNA transcription (7). There is evidence that association of the t-Utp subcomplex with the pre-rRNA is one of the earliest steps in ribosome biogenesis (5,6).

The t-Utp subcomplex is conserved in humans and has also been shown to be necessary for both rDNA transcription and pre-rRNA processing (8). Several years ago, a mutation in one of the human t-Utps, Utp4/Cirhin, was reported to cause Native American Indian childhood cirrhosis (NAIC) in Ojibway–Cree children in Quebec (9). NAIC is an autosomal recessive, non-syndromic form of familial cholestasis that presents as neonatal jaundice and progresses to biliary cirrhosis. Liver transplantation is required in childhood or adolescence. The NAIC mutation results in the substitution of tryptophan for arginine at position 565 of Utp4/Cirhin (9). Although

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Utp4/Cirhin has been localized to the nucleolus (8,10) and has been shown to be required for pre-rRNA processing (8), defects in ribosome biogenesis have not been investigated as an etiological agent of the disease.

In this study, we used the yeast *S. cerevisiae* as a tractable genetic system in which to examine the role of the C-terminus of Utp4 in ribosome biogenesis. While we found that mutations homologous to the NAIC mutation do not cause defects in ribosome biogenesis in yeast, the region surrounding the NAIC mutation is essential for rRNA maturation and cell growth. Furthermore, a protein–protein interaction map of the t-Utp subcomplex indicates that the defects in ribosome biogenesis we observe are likely due to the loss of an essential interaction between Utp4 and Utp8.

**MATERIALS AND METHODS**

**Protein alignments**

Utp4 sequences from the following organisms were obtained by searching BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi): *S. cerevisiae* (NP_010611.1), *Candida glabrata* (XP_449407.1), *Schizosaccharomyces pombe* (NP_596540), *Homo sapiens* (NP_116219.2), *Mus musculus* (NP_035704.2), *Danio rerio* (NP_998447.1) and *Xenopus laevis* (NP_001079449). Sequences were aligned using ClustalX (11) and protein motifs were determined by the SMART database (12).

**Plasmids**

The wild-type *UTP4* gene was cloned into the yeast expression vector, p415GPD (13), and the yeast two-hybrid vectors, pAS2-1 and pACT2 (14) using PCR primers including the desired restriction sites. Site-directed mutagenesis was performed on *UTP4* in the p415GPD and pACT2 vectors using a Change-IT kit (USB Corporation) and oligonucleotides containing the appropriate mutant sequences. Truncations were created by mutating the indicated amino acid to a stop codon. All mutations were confirmed by DNA sequencing. The genes encoding the remaining members of the t-Utp subcomplex (Utp5, Utp8, Utp9, Utp10, Utp15 and Utp17) were cloned into the pAS2-1 and pACT2 vectors using PCR primers. All inserts were fully sequenced by the W. M. Keck Foundation facility at the Yale School of Medicine.

**Yeast growth assays and western blotting**

HA-tagged wild-type or mutant *UTP4* in p415GPD and pACT2 vectors was transformed into YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-A0000 leu2-3200 his3-D200 gal::3HA-UTP4* (3; Figure 3A). For serial dilutions, 0.2 ml of cells at an optical density at 600 nm (OD600) of 1 were resuspended in 1 ml of water, diluted 1/10 and spotted onto medium lacking endogenous Utp4. Cells were incubated at 30 or 37°C for 3 days or at 17°C for 6 days.

For Western blotting, endogenous Utp4 was depleted by first growing yeast cultures to mid-log phase (OD600 = 0.3–0.8) in medium containing 2% galactose and 2% raffinose and lacking leucine and containing 2% glucose (SD-Leu) in order to deplete endogenous Utp4. Cells were incubated at 30 or 37°C for 3 days or at 17°C for 6 days.

For Western blotting, endogenous Utp4 was depleted by first growing yeast cultures to mid-log phase (OD600 = 0.3–0.8) in medium containing 2% galactose and 2% raffinose and lacking leucine (SG/R-Leu), and then by shifting yeast cultures to SD-Leu medium for 20 h at 30°C. Cells (5 ml) at an OD600 of ~0.5 were collected from each culture and protein was extracted by the alkaline lysis method (15). Total protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore). Expression of Utp4 from the plasmids was tested by

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**Figure 1.** Pre-RNA processing in *S. cerevisiae*. The pre-rRNA is transcribed as a 35S polycistronic precursor that undergoes multiple cleavage events to give rise to the mature 18S, 5.8S and 25S rRNAs. The first cleavage can occur at either site A0 (left) or site A3 (right). If cleavage occurs at A0 first, the 33S rRNA is generated and then subsequently cleaved at sites A1 and A2 giving rise to the 20S and 27SA2 rRNAs. If cleavage occurs at A3 first, the 27SA3 pre-rRNA is generated in addition to the 23S rRNA. The 27SA2/27SA3 rRNA is further cleaved at multiple sites, before export to the cytoplasm for final maturation to the 5.8S and 25S rRNAs. The 20S rRNA is also exported to the cytoplasm for final cleavage to give the 18S rRNA.
western blotting with a 1:500 dilution of the anti-HA antibody, 12CA5. Expression of Mpp10 was tested by western blotting with a 1:10,000 dilution of an anti-Mpp10 antibody (16).

Ribosome biogenesis analysis

The YPH499 GAL::3HA-UTP4 yeast strain expressing either HA-tagged wild-type or mutant UTP4 from the p415GPD vector was depleted of endogenous Utp4 as described above. Cells (10 ml) at an OD600 of ~0.5 were collected from each culture after 0 and 20 h of growth at 30 or 37°C or after 0 and 72 h of growth at 17°C. Total RNA was extracted from cells by the acid phenol method (17) and 5 µg was run on a 1.25% agarose gel. RNA was visualized by ethidium bromide staining and the bands were quantified by densitometric analysis using an AlphaImager.

Yeast two-hybrid analysis

Bait (pAS2-1, TRP1 marker; Clontech) and prey (pACT2, LEU2 marker; Clontech) vectors containing each member of the t-Utp subcomplex or Utp4 deletion mutants were transformed sequentially into the pJ69-4a strain of yeast (14), which contains HIS3 under the control of the GAL4 promoter and therefore allows protein–protein interactions to be detected by growth on medium lacking histidine. Serial dilutions were performed as above. Cells were spotted or struck out onto medium lacking both leucine and tryptophan to select for the presence of both plasmids and then were spotted or struck out onto medium lacking leucine, tryptophan and histidine to check for interaction between proteins. Growth was examined after 3–5 days of incubation at 30°C.

Coimmunoprecipitations

A C-terminal tandem affinity purification (TAP) tag was added to the chromosomal copy of Utp17 in the YPH499 GAL::3HA-UTP4 yeast strain as previously described (3). YPH499 GAL::3HA-UTP4, UTP17-TAP yeast expressing either HA-tagged wild-type or mutant UTP4 from the p415GPD vector was depleted of endogenous Utp4 as described above. Cells (10 ml) at an OD600 of ~0.5 were collected from each culture after 16–20 h of growth at 30°C. Cells were washed with water, resuspended in 600 µl NET2 (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) with protease inhibitors (Roche mix) and lysed with 0.5 mm glass beads. Lysate was cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Anti-HA antibody (200 µl per sample) was conjugated to protein A-Sepharose CL-4B beads (3 mg per sample) by incubation on a nutator either overnight at 4°C or for 1 h at room temperature. Antibody-bound beads were washed three times in NET2 and then incubated for 1 h with cleared cell lysate at 4°C with mutating. Beads were then washed five times with NET2 to remove unbound protein. Total protein (5%) and immunoprecipitations were separated by 10% SDS–PAGE and then transferred to an Immobilon PVDF membrane. Western blotting of membranes was carried out with a 1:6000 dilution of peroxidase-antiperoxidase antibodies (PAP; Sigma) to detect the protein A portion of the TAP tag or a 1:10,000 dilution of anti-Mpp10 antibodies.

RESULTS

Sequence alignment of human Utp4/Cirhin and yeast Utp4

Prieto and McStay determined that the yeast ortholog of human Cirhin is Utp4 (8), a protein that our laboratory had previously named and studied (3,5). Since Cirhin and Utp4 are orthologs, we attempted to complement depletion of the essential yeast Utp4 with human Utp4/Cirhin. The endogenous UTP4 gene was placed under the control of a galactose-inducible, glucose-repressible promoter in yeast and endogenous Utp4 was depleted by growth on solid medium containing glucose. When human Utp4/Cirhin was constitutively expressed from the p415GPD plasmid, no growth was observed (data not shown). Therefore, human Utp4/Cirhin was not able to complement yeast Utp4. Because we were not able to study human Utp4/Cirhin in yeast, we instead generated the orthologous NAIC mutation in yeast Utp4.

In order to be able to use the baker’s yeast, S. cerevisiae, as a model system to test whether the NAIC mutation causes defective ribosome biogenesis, we had to first identify the orthologous mutation in yeast. To find the NAIC mutation in yeast, we generated a ClustalX alignment of several yeast and vertebrate species, indicating extensive overall protein conservation (Figure 2A). The overall identity/similarity between yeast and human is 20%/45% over the entire length of the protein. The region of Utp4 containing the NAIC mutation is shown in Figure 2B. Protein motif prediction algorithms also predict that both the yeast and the human protein have numerous N-terminal WD40 repeats (Figure 2C), which are known protein–protein interaction domains. In addition to this high degree of sequence conservation, both human and yeast proteins have been demonstrated to reside in the nucleoli of their respective organisms (3,8,10). Thus, in agreement with previously published results (8), conservation of primary sequence, conservation of predicted protein secondary structure and subcellular localization are all consistent with yeast Utp4 being the ortholog of human Utp4/Cirhin.

In NAIC, Utp4/Cirhin is mutated at arginine 565 to tryptophan (9). However, inspection of yeast Utp4 indicates that there is a lysine, not an arginine, at the analogous position (Figure 2B; S. cerevisiae amino acid 616). Furthermore, because the sequences can be aligned in at least two ways in this area the lysine at S. cerevisiae amino acid 627 could also be the analogous amino acid (Figure 2B). We thus mutated both K616 and K627 to amino acid 616. Despite the two possible alignments, the region surrounding the NAIC mutation is still fairly well conserved with 14.3% identity and 36.4% similarity. In order to examine whether the region surrounding the NAIC mutation is required for function, we additionally made C-terminal truncations at positions E601, E691, D745 and L760 (Figure 2B and C).
Figure 2. Alignment of yeast Utp4 and homologs. (A) Alignment of several yeast and vertebrate Utp4 sequences created by ClustalX (11). (B) Close-up of the alignment in part (A) showing the C-terminus of Utp4. The K616W and K627W point mutations are marked by lightning bolts and the E601X, E691X, D745X and L760X truncations are indicated with arrowheads. (C) Diagram of motifs predicted by the SMART database (12). Mutations indicated as in (B).
Mutations in the C-terminus of yeast Utp4 cause defects in ribosome biogenesis

Since the vast majority of ribosome biogenesis genes are essential for viability, defects in ribosome biogenesis cause growth defects in yeast. Therefore, to test whether any of the Utp4 mutants that we generated (K616W, K627W, E601X, E691X, D745X and L760X) cause a defect in ribosome biogenesis, several growth assays were performed. The endogenous UTP4 gene was placed under the control of a galactose-inducible, glucose-repressible promoter and HA-tagged Utp4 proteins were constitutively expressed from a plasmid (Figure 3A). Expression of all constructs was confirmed by Western blot analysis using an anti-HA antibody after 20 h of growth in glucose (Figure 3B). All mutated proteins were expressed at comparable levels to the wild-type protein, except for Utp4 truncated at D745. The decrease in expression of this mutant protein is likely due to misfolding of the protein, followed by degradation.

Serial dilutions of mutant and control strains were spotted onto plates containing glucose. These plates were incubated at the normal temperature of 30°C, as well as at 17 and 37°C (Figure 3C, summarized in Figure 3D). The truncations at positions E601 and D745 resulted in a severe growth defect at all temperatures, while the truncation at position E691 showed a slight growth defect at 30 and 17°C and a more severe defect at 37°C. No growth defect was observed with the L760 truncation or with either of the NAIC point mutations at any temperature. The K616W and K627W NAIC point mutant strains were further tested for defects in ribosome biogenesis using more sensitive assays including growth curves in liquid medium and growth on solid medium in the presence of antibiotics as described (18). No defects were observed in any of these assays (data not shown).

To confirm that growth defects were caused by disruption of ribosome biogenesis, 25S and 18S rRNA from strains expressing mutant Utp4 proteins were analysed and good correlation was seen between growth and rRNA maturation (Figure 4). rRNA was visualized by ethidium bromide staining (Figure 4, left) and the intensity of each band was quantified with each depleted value graphed as a percentage of the undepleted value for the same strain (Figure 4, right). The truncations at E601 and D745 cause a substantial decrease in the level of 18S rRNA after 20 h at 30 and 37°C, as is expected for mutations that disrupt the function of SSU processome proteins. Since cells grow more slowly at colder temperatures, RNA was extracted after 72 h at 17°C; smaller reductions in 18S rRNA were observed. The truncation at E691 results in an intermediate decrease in mature rRNA at 37°C, but little effect was seen at either 30 or 17°C. Disruption of the function of a t-Utp protein has additionally been reported to cause a decrease in 25S rRNA since this complex is required for optimal transcription of the pre-rRNA (3,5). Therefore, as expected, a concomitant

**Figure 3.** Utp4 mutations cause growth defects in yeast. (A) Schematic of the yeast strain used for testing Utp4 mutants. Endogenous Utp4 was placed under the control of the inducible GAL4 promoter. HA-tagged wild-type or mutant Utp4 constructs were constitutively expressed from the p415GPD plasmid. (B) Western blot confirming that endogenous Utp4 was not expressed when yeast are grown in glucose and that plasmid-encoded Utp4 mutants expressed Utp4-WT expressed Utp4 mutants expressed. Blotting with an anti-Mpp10 antibody was used to assess gel loading. (C) Serial dilutions of yeast expressing Utp4 constructs were grown on solid medium at different temperatures for 3 days (30 and 37°C) or 6 days (17°C). (D) Table summarizing the results in (C).
decrease in 25S rRNA was also observed in strains with growth defects. Overall, these results indicate that the C-terminus of Utp4 is important for the function of the protein in ribosome biogenesis.

**Subunit architecture of the t-Utp subcomplex**

Because truncations of the C-terminus of Utp4 led to a defect in ribosome biogenesis, we hypothesized that such truncations abolish the interaction between Utp4 and another member of its protein subcomplex. Therefore, we derived a protein interaction map of the yeast t-Utp subcomplex using a directed yeast two-hybrid approach. The bait vector containing each t-Utp (Utp4, Utp5, Utp8, Utp9, Utp10, Utp15 or Utp17) was transformed individually into the pJ69-4a yeast strain. Prey vectors containing a t-Utp were then transformed into yeast carrying the bait vectors in a pairwise fashion. Interactions between proteins were checked for by growth on medium lacking histidine (Figure 5A, selective).

The results indicate that Utp4 interacts with Utp5 and Utp8. Utp5 and Utp8 both also interact with Utp10. Utp5 additionally interacts with Utp15, and Utp8 additionally interacts with Utp9 and Utp17. Utp10 and Utp17 interact with each other. Utp15 interacts with Utp9 and Utp10. Utp17 is the only t-Utp that interacts with itself. The network of interactions is shown in Figure 5B.

**The C-terminus of Utp4 is necessary for interaction with Utp8**

Since Utp4 interacts with Utp5 and Utp8, we used the yeast two-hybrid system to test whether the C-terminus of Utp4 is required for either of these interactions. Each of the HA-tagged Utp4 truncations (E601X, E691X, D745X or L760X) was cloned into the bait vector and either Utp5 or Utp8 was used as prey. Interactions were determined by growth on medium lacking histidine (Figure 6). Although Utp5 was able to interact with all Utp4 truncations, Utp8 only interacted with the truncation at L760. These results indicate that residues 1–601 are sufficient for interaction with Utp5 while residues 1–745 are not sufficient for interaction with Utp8.

**The C-terminus of Utp4 is necessary for efficient formation of the SSU processome**

We next asked whether the loss of interaction between Utp4 and Utp8 interfered with formation of either the t-Utp subcomplex or the SSU processome. A TAP tag was added to endogenous Utp17, a t-Utp, in a
Each of the Utp4 truncations (E601X, E691X, D745X or L760X) was constitutively expressed from a plasmid and endogenous Utp4 was depleted by 16–20 h growth in glucose. Coimmunoprecipitations (co-IPs) were performed to determine whether the truncated Utp4 proteins were able to associate with the t-Utp subcomplex (co-IP with Utp17) or with the SSU processome (co-IP with Mpp10). The results indicate that truncations at E601, E691 and D745 reduce or completely abolish association with both the t-Utp subcomplex and SSU processome (Figure 7A, compare lanes 4 and 12 to lanes 6, 8 and 10). Furthermore, in the case of the truncations at E601 and D745, the cells’ ability to grow when expressing truncated Utp4 (Figure 3C) is directly correlated with whether Utp4 and Utp8 can interact (Figure 6B) and whether the SSU processome can form (Figure 7A) suggesting that the interaction between Utp4 and Utp8 is necessary for ribosome biogenesis (Figure 7B).

**DISCUSSION**

A missense mutation in the C-terminus of human Utp4/Cirhin has been reported to cause North American Indian childhood cirrhosis (9). In this study we show that, unexpectedly, the homologous mutation does not affect ribosome biogenesis in the baker’s yeast, *S. cerevisiae*. However, we have also shown that truncation of Utp4 by as few as 31 amino acids leads to reduced growth and reduced levels of mature rRNAs likely due to the loss of an interaction with Utp8. Moreover, truncation of Utp4 prevents the formation of both the t-Utp subcomplex and the SSU processome. These findings indicate that the region including the NAIC mutation is important for the function of Utp4 in ribosome biogenesis, and our results are therefore important for understanding the protein contacts that make the subcomplexes that compose the SSU processome.
Although the NAIC mutation does not result in a mutant phenotype in yeast, *S. cerevisiae* still provides a good model for studying eukaryotic ribosome biogenesis. Overall rRNA processing is well conserved from yeast to humans (19). Furthermore, approximately 90% of ribosome biogenesis genes are conserved from yeast to humans, and recent studies have shown that ribosome biogenesis proteins that are essential in yeast, including Utp4, are also essential in mice (20–22). Interestingly, Utp8, the protein that interacts with the C-terminus of Utp4, is one of the few proteins in the SSU processome that is not conserved in mammals. The lack of an Utp8 ortholog in humans is one possible reason why human Utp4/Cirhin does not complement yeast Utp4. In humans, Utp8 is likely replaced by a functional analogue that has yet to be discovered, although one candidate, Cirip, was identified in a yeast two-hybrid screen using human Utp4/Cirhin as a bait (23). Cirip increases the activity of the HIV-1 LTR enhancer element, an NF-κB responsive gene. However, since Cirip has not been detected in the nucleolus, it is unlikely to replace the role of Utp8 in ribosome biogenesis.

In order to more completely understand the assembly and composition of large pre-ribosome complexes, we carried out a directed one-by-one yeast two-hybrid analysis of the seven member t-Utp subcomplex. This enabled us to determine binary protein–protein interactions of this subcomplex and gives us insight into how the individual components come together to function as a single macromolecule. Previous high-throughput yeast two-hybrid studies largely failed to include the t-Utps and have only identified the interaction between Utp5 and Utp15 (24–26). Recently, however, a new method using a protein-fragment complementation assay (PCA) reported interactions involving six of the seven t-Utp proteins (27). While many of the detected interactions were the same between that study and this one, Tarassov *et al.* found several interactions that we did not and vice versa (summarized in Figure 5C). While it is difficult to determine exactly why these differences exist, it is clear that the two methods have different physiochemical limitations: yeast two-hybrid is biased toward nuclear interactions while PCA is biased towards proteins with transmembrane domains (28). Since the t-Utps are nucleolar proteins, the yeast two-hybrid system is an appropriate method for determining interactions between subcomplex members. Furthermore, yeast two-hybrid analysis can detect weak interactions with $K_d$ values in...
the range of 10–100 μM (29). Importantly, both yeast two-hybrid and PCA detect an interaction between Utp4 and Utp8, strengthening the validity of this interaction.

Although we were not able to use S. cerevisiae as a model for NAIC, we were able to determine that an intact C-terminus of Utp4 is required for ribosome biogenesis and that this role is mediated by its interaction with Utp8. Future work should be carried out to determine whether the NAIC mutation abrogates the interaction between human Utp4/Cirhin and the as-yet-to-be discovered human functional analogue of Utp8, disrupting ribosome biogenesis and leading to disease.

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