WBSCR22/Merm1 is required for late nuclear pre-ribosomal RNA processing and mediates N^7-methylation of G1639 in human 18S rRNA

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ABSTRACT

Ribosomal (r)RNAs are extensively modified during ribosome synthesis and their modification is required for the fidelity and efficiency of translation. Besides numerous small nucleolar RNA-guided 2′-*O* methylations and pseudouridinylations, a number of individual RNA methyltransferases are involved in rRNA modification. WBSCR22/Merm1, which is affected in Williams–Beuren syndrome and has been implicated in tumorigenesis and metastasis formation, was recently shown to be involved in ribosome synthesis, but its molecular functions have remained elusive. Here we show that depletion of WBSCR22 leads to nuclear accumulation of 3′-extended 18SE pre-rRNA intermediates resulting in impaired 18S rRNA maturation. We map the 3′ ends of the 18SE pre-rRNA intermediates accumulating after depletion of WBSCR22 and in control cells using 3′-RACE and deep sequencing. Furthermore, we demonstrate that WBSCR22 is required for N^7-methylation of G1639 in human 18S rRNA in vivo. Interestingly, the catalytic activity of WBSCR22 is not required for 18S pre-rRNA processing, suggesting that the key role of WBSCR22 in 40S subunit biogenesis is independent of its function as an RNA methyltransferase.

Keywords: RNA modification; methyltransferase; ribosome; Williams–Beuren syndrome

INTRODUCTION

Many cellular RNAs require modification of specific residues for their biogenesis, structure, and function. Transfer (t)RNAs and ribosomal (r)RNAs are the most extensively modified RNAs in cells and the introduction of these modifications is a key step in their maturation. Eukaryotic ribosomes consist of ribosomal proteins in complex with the 28S (human)/25S (yeast, plants), 5.8S and 5S rRNAs in the 60S large subunit (LSU), and the 18S rRNA in the 40S small subunit (SSU). The biogenesis of ribosomal subunits is initiated in the nucleolus by RNA polymerase I-mediated transcription of a large primary transcript that contains the sequences of the 18S, 5.8S, and 28S/25S rRNAs and on which the initial ribosomal proteins and biogenesis cofactors assemble (Henras et al. 2008; Woolford and Baserga 2013). Within the primary transcript, mature rRNA sequences are flanked by external transcribed spacers (ETS) and separated by internal transcribed spacers (ITS) that are subsequently removed during ribosomal subunit maturation (Fig. 1A; Henras et al. 2008, 2014; Mullineux and Lafontaine 2012). In humans, early endonucleolytic cleavage at site 2 leads to the separation of the biogenesis pathways of the 60S and 40S ribosomal subunits. Removal of the 5′ETS sequence from the 30S pre-rRNA, which extends from site 01 to site 2, leads to formation of the 21S pre-rRNA. Interestingly, and in contrast to what is known from yeast, the 21S pre-rRNA then undergoes exonucleolytic processing generating 18SE pre-rRNAs (Preti et al. 2013; Sloan et al. 2013). Final 40S subunit maturation steps occur in the cytoplasm, including formation of the mature 3′ end of the 18S rRNA, which has been shown to be performed by the endonuclease Nob1 in yeast, plants, and archaea (Pertschy et al. 2009; Veith et al. 2012; Missbach et al. 2013).

The rRNA modifications cluster in highly conserved areas of the ribosome, such as the peptidyl-transferase center, sites of A- and P-tRNA binding, the peptide exit tunnel and the intersubunit bridge (Piekna-Przybylska et al. 2008). The majority of rRNA modifications are pseudouridylations and 2′-*O* methylations of the ribose that are introduced by small nucleolar RNPs (snoRNPs) guided by box H/ACA or box C/D snoRNAs, respectively (Watkins and Bohnsack 2012). Furthermore, rRNA contains a variety of base methylations catalyzed by stand-alone RNA methyltransferases (Piekna-

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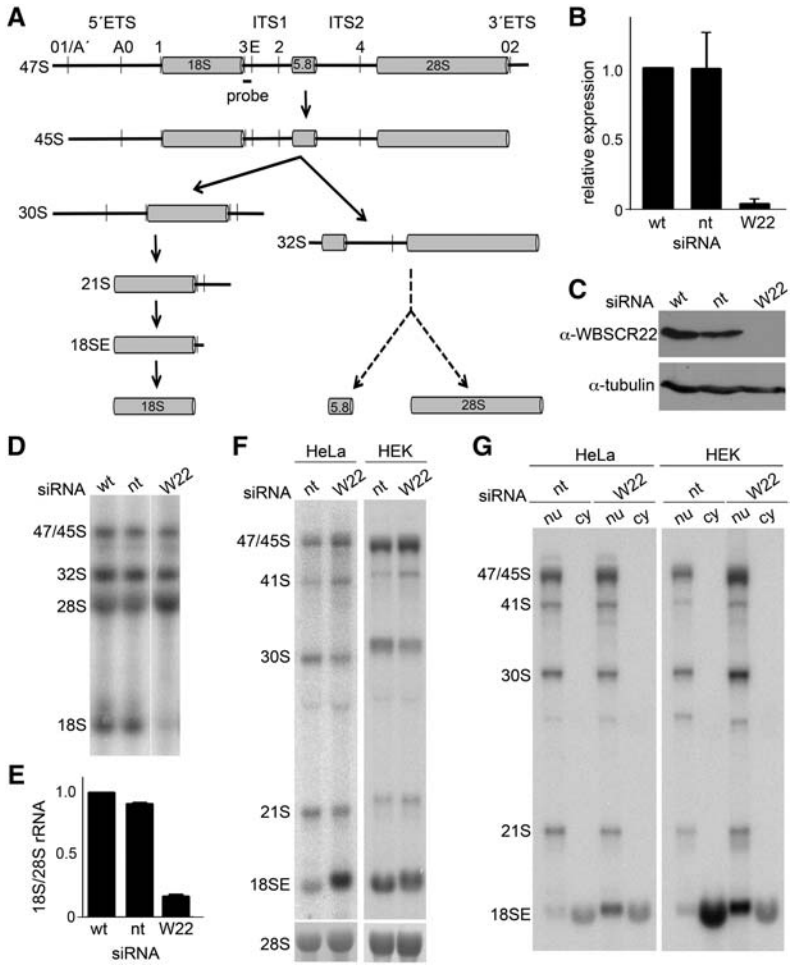

FIGURE 1. Depletion of WBSCR22 strongly reduces de novo 18S rRNA synthesis. (*A*) Simplified schematic overview of the pre-rRNA processing pathway in human cells. Cleavage sites relevant for SSU biogenesis are given *above* the primary transcript. Mature rRNAs are shown as cylinders and spacer sequences as lines. (ETS) External transcribed spacer, (ITS) internal transcribed spacer. (*B*) Levels of WBSCR22 mRNA, normalized to GAPDH mRNA, in wild-type (wt) cells or cells treated with nontarget (nt) siRNAs or those targeting WBSCR22 (W22) were determined by qPCR. (*C*) Proteins from cells as in *B* were analyzed by Western blotting using antibodies against endogenous WBSCR22 or tubulin, as a loading control. (*D*) HeLa cells depleted of WBSCR22 by RNAi (W22), treated with nontarget siRNA (nt) or untreated (wt) were pulse-chase labeled with ^{32}P-orthophosphate. Total RNA was analyzed by agarose–glyoxal gel electrophoresis and visualized using a phosphorimager. Labeled pre-rRNA intermediates and mature pre-rRNAs are indicated on the *left*. (*E*) The ratio of 18S to 28S rRNA in *D* was quantified. Error bars indicate standard deviation. (*F*) RNA was isolated from HeLa and HEK293 cells treated with nontarget siRNAs (nt) or siRNAs against WBSCR22 (W22) and analyzed by Northern blotting using a probe that hybridizes to the 5′ end of ITS1. Pre-rRNAs detected are indicated on the *left*. Methylene blue staining of 28S rRNA was used as a loading control (shown at the *bottom*). (*G*) HeLa and HEK293 cells were fractionated into nucleus and cytoplasm after depletion of WBSCR22 (W22) or treatment with nontarget (nt) siRNAs and RNA was isolated from each fraction. For comparative analysis, RNA extracted from equal numbers of cells was analyzed by agarose–glyoxal gel electrophoresis and Northern blotting using a probe hybridizing to the 5′ end of ITS1.

Przybylska et al. 2008). While the cellular roles of human RNA methyltransferases have largely remained uncharacterized, the modification of rRNAs is currently best investigated in yeast, where, besides snoRNP-guided modifications, three methyltransferases are known to methylate-specific residues in the 18S rRNA. Firstly, the highly conserved RNA methyltransferase Dim1 dimethylates two adjacent adenosines in the loop of helix 45, close to the 3′ end of the 18S rRNA (Lafontaine et al. 1994). Secondly, the SPOUT-class RNA methyltransferase Emg1 is involved in a unique hypermodification at position 1191 of the yeast 18S rRNA and, interestingly, a mutation in human EMG1 has been shown to cause the Bowen–Conradi syndrome (Armistead et al. 2009; Wurm et al. 2010; Meyer et al. 2011). Finally, the RNA methyltransferase Bud23 was shown to mediate the m^{7}G1575 modification in the yeast 18S rRNA and loss of Bud23 resulted in SSU pre-rRNA processing defects and reduced levels of mature 40S subunits (White et al. 2008).

The human ortholog of Bud23 is the protein WBSCR22/Merm1 (Ebersberger et al. 2014). In Williams–Beuren syndrome, a part of chromosome 7, the Williams–Beuren syndrome critical region (WBSCR), is hemizygously deleted. This region normally contains, among others, the *wbscr22* gene (Doll and Grzeschik 2001; Merla et al. 2002). Based on sequence similarity, WBSCR22 was suggested to contain an *S*-adenosyl-methionine binding site and to belong to the family of Rossman-fold methyltransferases, but no methyltransferase activity has been reported so far. Elevated expression of WBSCR22 was detected in invasive breast cancer and the protein was shown to enhance metastasis formation by suppression of the Zac1/p53-induced apoptosis, accompanied by histone 3 lysine 9 (H3K9) methylation at the Zac1 promoter (Nakazawa et al. 2011). A loss of WBSCR22 was detected in inflammatory and neoplastic lung pathologies and the protein has been linked to glucocorticoid receptor regulation of histone modification (Jangani et al. 2014). Recently, WBSCR22 was shown to be required for the biogenesis of the small ribosomal subunit (Wild et al. 2010; Õunap et al. 2013; Tafforeau et al. 2013), but its molecular function has remained unclear.

Here we demonstrate that impaired 18S rRNA maturation upon depletion of WBSCR22 is caused by the nuclear accumulation of 3′-extended 18SE pre-rRNA intermediates, which we map by deep sequencing. Furthermore, we show that WBSCR22 is an active RNA methyltransferase in vivo

and that it mediates the N^7-methylation of G1639 in the 18S rRNA. Interestingly, the catalytic activity of WBSCR22 is not required for 18S pre-rRNA processing, implying that the key role of WBSCR22 in 40S subunit biogenesis is independent of its function as an RNA methyltransferase.

RESULTS AND DISCUSSION

Depletion of WBSCR22 leads to accumulation of 3′-extended 18SE pre-rRNAs in the nucleus

WBSCR22/Merm1 was recently shown to be involved in the biogenesis of the 40S ribosomal subunit (Õunap et al. 2013; Tafforeau et al. 2013). To get further insight into the molecular function of WBSCR22 in ribosome biogenesis we investigated the pre-rRNA processing defect in more detail. We performed pulse-chase labeling experiments to monitor the production of newly synthesized mature rRNAs in control cells and in cells depleted of WBSCR22 (Fig. 1B,C). Knockdown of WBSCR22 led to a strong (~80%) reduction in the levels of the mature 18S rRNA (Fig. 1D,E). Consistent with this, Northern blot analysis of total RNA from HeLa cells using a probe hybridizing at the 5′ end of the internal transcribed spacer 1 (ITS1) revealed a strong accumulation of the 18SE pre-rRNA, the immediate precursor of the 18S rRNA. In cells depleted of WBSCR22, a slight reduction in the levels of the 30S and 21S pre-rRNAs was also observed (Fig. 1A,F, left panel). The 18SE pre-rRNA is produced from the 21S pre-rRNA by exonucleolytic processing, which has been proposed to occur during pre-ribosome export (Preti et al. 2013). We therefore analyzed the subcellular distribution of the 18SE pre-rRNA in control cells and those depleted of WBSCR22 by fractionating cells into nucleus and cytoplasm. In HeLa cells treated with nontarget siRNAs, 18SE pre-rRNA could be detected in the nucleus but was primarily found in the cytoplasm. However, knockdown of WBSCR22 resulted in a significant accumulation of 18SE pre-rRNAs in the nucleus (Fig. 1G). Surprisingly, and in contrast to our observations from HeLa cells, depletion of WBSCR22 from HEK293 cells caused a slight reduction in the total 18SE pre-rRNA levels in these cells (Fig. 1F, right panel). However, the HEK293 cells used have significantly higher levels of the 18SE pre-rRNA (Fig. 1F; compare HeLa and HEK293 lanes with RNA from control [nt] cells) and cellular fractionation demonstrated that this additional 18SE pre-rRNA is present in the cytoplasm (Fig. 1G). Therefore, although as in HeLa cells, knockdown of WBSCR22 increased the levels of nuclear 18SE pre-rRNAs, this was accompanied in HEK293 cells by a significant decrease in the large cytoplasmic pool, leading to a slight overall reduction in 18SE pre-rRNA levels (Fig. 1G). Taken together, our data show that depletion of WBSCR22 results in nuclear accumulation of 18SE pre-rRNAs in both HeLa and HEK293 cells and to depletion of the large cytoplasmic pool of 18SE pre-rRNA normally found in the HEK293 cells. These findings further suggest that the cytoplasmic processing of the 18SE pre-rRNA might be more efficient in HeLa cells, preventing the accumulation of a cytoplasmic pool of 18SE pre-rRNA as observed in the HEK293 cells.

At closer inspection, we observed that the band corresponding to the 18SE pre-rRNA migrates slightly slower after WBSCR22 depletion than in control cells (Fig. 1F). An RNase H-based assay was therefore used to analyze the length of 18SE pre-rRNAs in cells depleted of WBSCR22 compared with control cells (Fig. 2A). Hybridization of a DNA oligonucleotide close to the 3′ end of mature 18S rRNA enabled truncation of 18SE pre-rRNAs by cleavage with RNase H. The resulting small fragments (<100 nt) could then be separated by polyacrylamide gel electrophoresis, allowing short extensions to be readily visualized by Northern blotting. Using an oligonucleotide that hybridizes 75–45 nt upstream of the mature 3′ end of the 18S rRNA (oligo 1) on pre-rRNAs from control cells, revealed two fragments (Fig. 2B, lane 1), most likely corresponding to the predominant nuclear and cytoplasmic forms of 18SE pre-rRNAs. 3′ end exonucleolytic trimming of human 18SE pre-rRNA has previously been suggested to involve an asymmetric distribution of longer forms (mostly up to 45 nt) in the nucleus and shorter forms (+14–24 nt) in the cytoplasm (Preti et al. 2013). This is supported by our finding of a size difference of ~25 nt in the two populations of 18SE pre-rRNA fragments (Fig. 2B, lane 1). To test the specificity of the RNase H assay we used a second oligonucleotide to direct cleavage 10 nt toward the 3′ end of 18S rRNA (65–35 from the 3′ end; oligo 2) (Fig. 2A). Here, RNase H digestion resulted in shorter pre-rRNA fragments of the same pattern as for oligo 1, confirming that the detected fragments were indeed derived from the 3′ end of 18SE pre-rRNAs (Fig. 2B, lane 3). Interestingly, knockdown of WBSCR22 and RNase H cleavage of the RNA resulted in the accumulation of additional pre-rRNA fragments ranging in size between the low and the high molecular weight fractions observed in wild-type cells and, in addition, a significant increase in higher molecular weight intermediates (Fig. 2B, lanes 2,4). These data suggest that depletion of WBSCR22 leads to inhibition of the 3′ end trimming of 18SE pre-rRNAs and to nuclear accumulation of the 3′-extended 18SE intermediates.

To map the 3′ ends of the extended 18SE pre-rRNA intermediates we performed 3′-RACE (rapid amplification of cDNA ends) experiments followed by Illumina deep sequencing. After polyadenylation and RT-PCR of total RNA, 18SE pre-rRNA 3′ ends were specifically amplified using a forward primer that extends 13 nt into the ITS1 region (Preti et al. 2013). Illumina deep sequencing of the amplicons from control cells revealed a continuum of 18S rRNA precursors, consistent with progressive exonucleolytic processing. Compared with nontarget siRNA-treated cells, depletion of WBSCR22 resulted in a clear shift to 3′-extended 18SE pre-rRNA (Fig. 2C). Interestingly, we observed that in knockdown cells >60% of the reads extended to at least nucleotide

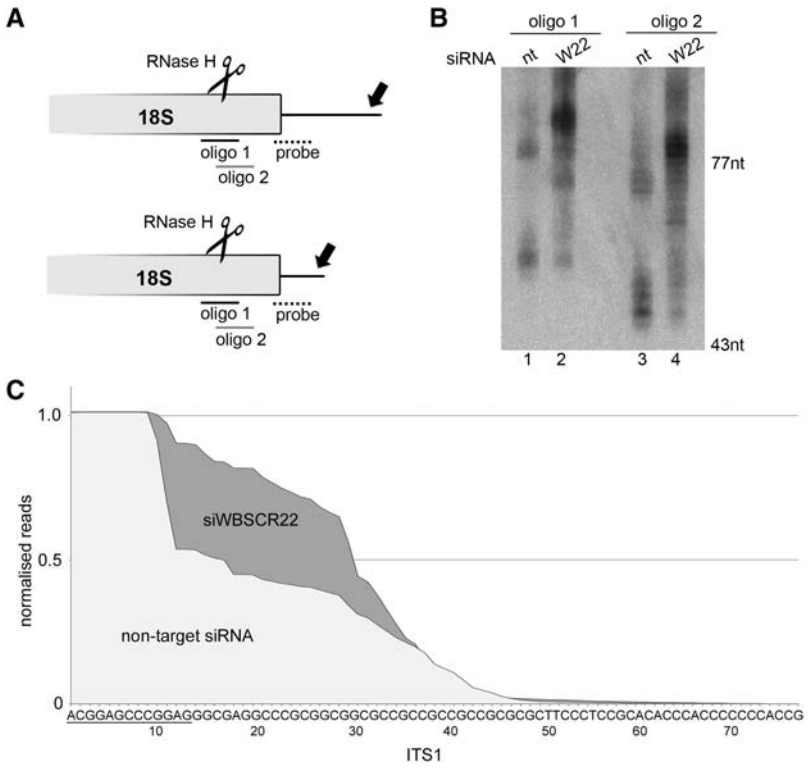

FIGURE 2. Depletion of WBSCR22 leads to accumulation of 3′-extended 18SE pre-rRNAs. (*A*) Schematic view of RNase H assays. The 3′ end of the 18S rRNA sequence is shown as a box and ITS1 fragments as thin lines. DNA oligonucleotide base-pairing sites (oligo 1, black line; oligo 2, gray line) for RNase H treatment are indicated *below* and the hybridization site of the Northern probe as a dotted line. Arrows indicate that the extensions of the 18SE pre-rRNAs into ITS1 are of different length. (*B*) Total RNA from HeLa cells treated with nontarget (nt) siRNAs or siRNAs directed against WBSCR22 (W22) was hybridized with a DNA oligonucleotide spanning 75–45 (oligo 1) or 65–35 nt (oligo 2) upstream of the mature 3′ end of 18S rRNA. The RNA/DNA hybrid was digested with RNase H and the resulting fragments were separated on a polyacrylamide gel and analyzed by Northern blotting using a probe that hybridizes at the 5′ end of ITS1. Two ^{32}P-labeled DNA oligonucleotides of 43 and 77 nt were used as approximate size markers. (*C*) 3′-RACE experiment of 18SE pre-rRNA followed by Illumina next-generation sequencing and mapping of reads on the human 47S pre-rRNA sequence. The graph shows normalized sequence reads from nontarget siRNA-treated cells and WBSCR22 depleted cells (siWBSCR22) aligned to the ITS1 region; numbers *below* indicate nucleotide positions in ITS1. A step in the graph defines the relative number of reads that end at this position. The primer binding site is underlined.

+31 of ITS1, while in cells treated with nontarget siRNAs ~50% of the reads did not even contain nucleotide +15 and only 35% of the 18SE pre-rRNA sequences extended to nucleotide +31 (Fig. 2C). Reads extending beyond position +46 of the ITS1 region represented <2% of all reads in control cells, but such long precursors were enriched after depletion of WBSCR22. It is likely that short fragments are preferentially amplified in 3′-RACE analyses (see also Preti et al. 2013) implying that longer intermediates such as those accumulated following WBSCR22 depletion, which are readily detectable by Northern blotting (Fig. 2B), are underrepresented here. In addition, it was shown that poly(A) polymerases exhibit a slight preference toward purines (Munafó et al 2010), which might affect polyadenylation of sequences ending with pyrimidines. Our detection of the diverse set of 18S rRNA precursors further underlines the importance of exonucleases in the processing of human ITS1. While exosome-associated Rrp6 has been shown to mediate 3′–5′-processing of ITS1 in early pre-40S complexes (Sloan et al. 2013), other currently unidentified 3′–5′-exonucleases, which may be regulated by or require the previous action of WBSCR22, are likely also required for later stages of 18S pre-rRNA processing. Interestingly, the accumulation of nuclear 3′-extended 18SE pre-rRNA intermediates is not a general effect observed upon depletion of proteins required for export competence of pre-40S particles. Depletion of the small subunit ribosomal protein RPS15 abolished pre-40S nuclear export but did not result in a block of 18SE pre-rRNA processing (Preti et al. 2013), indicating that WBSCR22 might act earlier than RPS15 during pre-40S maturation and that the 3′–5′-processing events might largely occur in the nucleus. Impaired nuclear export of pre-40S particles in cells depleted of WBSCR22 (Wild et al. 2010) is probably due to incomplete trimming of nuclear 18SE pre-rRNAs, rather than a direct involvement of WBSCR22 in mediating nuclear export of pre-40S particles.

WBSCR22 is required for N^7-methylation of G1639 in the 18S rRNA

While most of the proteins that mediate modifications in rRNAs in yeast have been identified, the activity of many RNA methyltransferases in human cells has not been analyzed. Based on sequence similarity, WBSCR22 was identified as the human ortholog of yeast Bud23 (Ebersberger et al. 2014). Bud23 mediates the N^7-methylation of G1575 in the yeast 18S rRNA (White et al. 2008) and this modification is also conserved in human 18S rRNA (m^7G1639) (Fig. 3A; Choi and Busch 1978; Piekna-Przybylska et al. 2008). We therefore analyzed whether WBSCR22 is required for this modification, which is the only known m^7G residue in human 18S rRNA (Choi and Busch 1978; Piekna-Przybylska et al. 2008). To detect the methylation status of the rRNA in the presence and after depletion of WBSCR22, we took advantage of the specific reactivity of the m^7G modification to aniline. An m^7G containing RNA can be specifically cleaved at the modification site by aniline treatment after reduction of the RNA with sodium borohydride (Wintermeyer et al. 1972; Behm-Ansmant et al. 2011). Aniline-treated total RNA was separated on an agarose

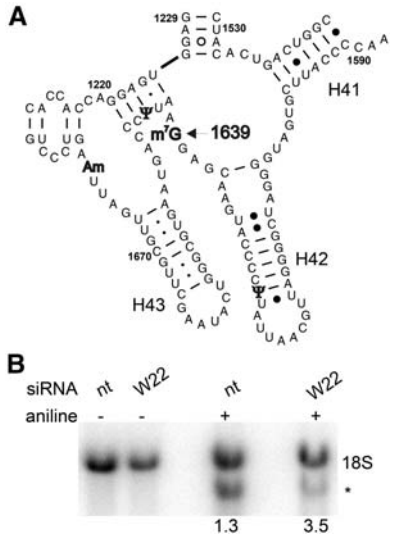

FIGURE 3. WBSCR22 is required for m^7G-methylation of G1639 in the 18S rRNA. (*A*) 2D structure scheme of helices (H) 41, 42, and 43 of human 18S rRNA showing the conserved m^7G1639 modification and other snoRNP-mediated modifications. (*B*) RNA was isolated from WBSCR22 knockdown (W22) and control (nt) cells, reduced with sodium borohydride and aniline treated. The resulting cleavage products were separated on an agarose–glyoxal gel, transferred to a membrane, and probed with an oligonucleotide hybridizing upstream of G1639 in the 18S rRNA sequence. Numbers give the ratio of full-length 18S RNA to the 5′-cleavage product (*) after aniline treatment.

gel and analyzed by Northern blotting using a probe hybridizing in the 18S rRNA sequence upstream of the putative modification site. As expected for a single m^7G methylation in the 3′-part of the 18S rRNA, aniline-induced chain scission resulted in a specific cleavage product (5′-fragment) of 18S rRNA, which was ~250 nt shorter than the full-length 18S rRNA (Fig. 3B). Interestingly, comparison of RNA from cells treated with nontarget siRNAs and cells depleted of WBSCR22 indicated reduced cleavage of mature 18S rRNA in the absence of WBSCR22, demonstrating decreased m^7G modification of 18S rRNA molecules. The residual modification observed in siRNA-treated cells is likely to reflect the detection of rRNA modifications introduced before WBSCR22 depletion. This is supported by ^3H-labeling experiments that have revealed a half-life of 72 h for 18S rRNA in mouse fibroblasts (Abelson et al. 1974). Interestingly, despite the high efficiency of such chemical treatments (Alexandrov et al. 2002), only partial cleavage (~60%) of the 18S rRNA from control cells was observed, perhaps suggesting that not all 18S rRNA molecules contain the m^7G modification, even in wild-type cells.

The catalytic activity of WBSCR22 is not required for pre-rRNA processing

Our data therefore indicate that the WBSCR22 protein is required for both 18S rRNA maturation and G1639 modification raising the question of whether the catalytic activity of WBSCR22 and the modification are required for 40S biogenesis, or whether the presence of the protein is sufficient. We therefore established an RNAi-rescue system by generating HEK293 stable cell lines, which can express Flag-tagged, siRNA-insensitive wild-type WBSCR22 or mutants in a tetracycline-inducible manner. Based on published SAM binding mutants of the yeast homolog Bud23 (White et al. 2008), G63E or D82K mutations were introduced into the highly conserved regions of WBSCR22. To analyze the catalytic inactivity of the two WBSCR22 mutants in vivo, we subjected RNA from cells depleted of endogenous WBSCR22 and either expressing siRNA-insensitive wild-type (wt) or mutant (G63E; D82K) WBSCR22 to sodium borohydride reduction and aniline cleavage. As expected, we detected the cleavage product generated by aniline treatment of m^7G-modified 18S rRNA in RNA from cells expressing the siRNA-insensitive wild-type WBSCR22 (Fig. 4A). Interestingly, hardly any aniline cleavage product was present in cells that express the WBSCR22 G63E mutant, confirming that this mutant lacks catalytic activity, while cells expressing the D82K mutant contained a slightly reduced amount of m^7G1639-modified 18S rRNA (Fig. 4A). To investigate the potential of the two mutants to complement the pre-rRNA processing defects caused by depletion of endogenous WBSCR22, expression of wild-type or mutant siRNA-insensitive WBSCR22 was induced in siRNA-treated cells before fractionation, RNA isolation, and Northern blot analysis. Interestingly, expression of wild-type or either WBSCR22 mutant restored normal cytoplasmic levels of 18SE pre-rRNA (Figs. 1G, 4B), indicating that the catalytic activity of WBSCR22 is dispensable for pre-rRNA processing. We further observed that the loss of the m^7G modification after depletion of endogenous WBSCR22 seemed more severe in the rescue with the G63E mutant than without expression of ectopic WBSCR22 (Figs. 3B, 4A). This observation can be explained by the impairment of 18S rRNA synthesis upon depletion of WBSCR22 causing a strong overrepresentation of 18S rRNA molecules that were modified before WBSCR22 depletion, compared with the WBSCR22 G63E mutant where 18S rRNA production is similar to wild-type (Fig. 4B) and pre-rRNAs not modified at G1639 are likely synthesized.

In yeast, several rRNA methyltransferases like Bud23, Emg1, and the dimethylase Dim1 support pre-rRNA processing independent of their catalytic activity (Lafontaine et al. 1995; Leulliot et al. 2008; White et al. 2008; Meyer et al. 2011), while loss of other methyltransferases, such as Bmt5 and Bmt6, and their modification does not show any phenotype (Sharma et al. 2014). However, a catalytically inactive mutant of yeast Nop2, an m^5C rRNA methyltransferase, fails to rescue the pre-60S ribosome biogenesis defect caused by deletion of endogenous Nop2 (Sharma et al. 2013). Further, dimethylation of yeast 18S rRNA by Dim1 has been shown to be important for translation fidelity and a quality control pathway in which pre-rRNAs are degraded in the absence of Dim1 has been proposed (Lafontaine et al. 1998). In

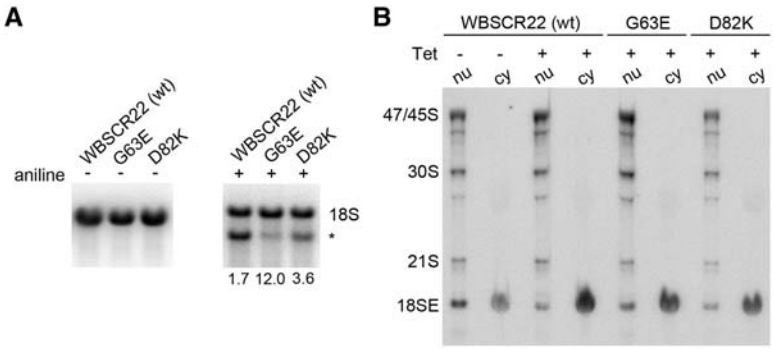

FIGURE 4. The catalytic activity of WBSCR22 is not required for pre-rRNA processing. (*A*) RNA isolated from HEK293 cells depleted of endogenous WBSCR22 and either expressing siRNA-insensitive wild-type (wt) or mutant (G63E; D82K) WBSCR22 was reduced with sodium borohydride and aniline treated. The resulting cleavage products were separated on an agarose–glyoxal gel, transferred to a membrane, and probed with an oligonucleotide hybridizing upstream of G1639 in the 18S rRNA sequence. Numbers give the ratio of full-length 18S RNA to the 5′-cleavage product (*) after aniline treatment. (*B*) Following RNAi targeting WBSCR22, protein extract from stable HEK293 cell lines with or without tetracycline (tet)-induced expression of siRNA-insensitive WBSCR22 wild-type (wt) or catalytic inactive mutants (G63E or D82K) was fractionated in nucleus and cytoplasm, RNA was isolated, separated by agarose–glyoxal gel electrophoresis, and analyzed by Northern blotting using a probe hybridizing at the 5′ end of ITS1. Pre-rRNA intermediates are indicated on the *left*.

contrast, depletion of WBSCR22 from human cells does not affect the stability of early pre-rRNAs and our data question the extent of G1639 modification of the mature 18S rRNA. Consistent with recent findings from yeast on the heterogeneity of snoRNA-guided modifications, our data suggest that in human cells many rRNA modifications might also individually not be essential (see for example, Decatur et al. 2007; Liang et al. 2007; Buchhaupt et al. 2014). We also provide further evidence that RNA methyltransferases often perform additional, essential functions in ribosome biogenesis, besides their role in rRNA modification. In the case of WBSCR22, it will be interesting to investigate its interaction network and its essential role in SSU biogenesis. Bud23 was shown to interact with components of the SSU processome and the general methyltransferase cofactor Trm112 (Figaro et al. 2012; Sardana et al. 2013). We observed that WBSCR22 directly interacts with several human TRM112 isoforms (data not shown). We could, however, not detect any catalytic activity of recombinant WBSCR22 on in vitro transcripts even in the presence TRM112, suggesting that the structural context of the pre-ribosome is required for the catalytic activity.

Taken together, our findings indicate that WBSCR22 has two independent functions in the biogenesis of the small ribosomal subunit in human cells; it mediates m^7G methylation of G1639 in the 18S rRNA and it is required for efficient processing of nuclear 18S rRNA precursors. Depletion of WBSCR22 results in nuclear accumulation of 3′-extended 18SE pre-rRNAs, suggesting an important function of WBSCR22 in facilitating the 3′–5′-trimming of 18SE pre-rRNAs, enabling nuclear export of pre-40S particles. WBSCR22 is associated not only with Williams–Beuren syndrome but also cancer progression and the inflammatory response (Nakazawa et al. 2011; Jangani et al. 2014). While the hemizygous deletion of WBSCR22 in Williams–Beuren syndrome is unlikely to correlate with ribosome biogenesis defects, it will be interesting to see whether the key role of WBSCR22 in ribosome production underlies its links to other cellular pathways.

MATERIALS AND METHODS

Human cell culture and siRNA treatment

HEK293 and HeLa cells were cultured at 37°C and 5% CO_2 in 1× Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS and 2 mM Glutamine. siRNAs (non-target: 5′-UCGUAAGUAAGCGCAACCC-3′; WBSCR22 5′-CUAACAGUGCCAAAGCAAA-3′) were transfected with Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions. Cells were harvested 72 h after siRNA transfection. For generation of tetracycline-inducible stable cell lines the *wbscr22* isoform 1 (NM_001202560.2) was cloned into the pcDNA5 vector with N-terminal Flag tag and transfected into HEK293 Flp In T-Rex cells (Life Technologies) according to the manufacturer's instructions. For expression of siRNA-insensitive WBSCR22 four silent mutations were introduced into the siRNA target site. To generate catalytically inactive WBSCR22 mutants, point mutations (G63E and D82K) were introduced in two highly conserved residues, analogous to *S. cerevisae* Bud23 mutants described previously (White et al. 2008). For rescue experiments the expression of the transgene was induced by addition of 1 μg/μL tetracycline 20 h before harvesting.

Pulse-chase labeling and Northern blotting

Pulse-chase labeling experiments were performed as previously described (Sloan et al. 2014). In brief, 48 h after transfection of siRNA, cells were grown in phosphate-free DMEM for 1 h before addition of 15 μCi/mL ^{32}P-orthophosphate for a further hour. Cells were then grown in normal DMEM for further 3 h before harvesting. Labeled RNA was isolated using Tri-reagent, separated by agarose–gyloxal gel electrophoresis, transferred to nitrocellulose membrane and visualized using a phosphorimager.

For Northern blot analysis, RNA was separated on a 1.2% agarose–glyoxal gel, transferred to a nitrocellulose membrane and incubated with a ^{32}P-labeled probe hybridizing to the 5′ end of ITS1 (5′-CCTCGCCCTCCGGGCTCCGTTAATGATC-3′). RNA was detected using a phophorimager and quantified using the ImageQuant software.

Fractionation of cells and RNA isolation

Cells were washed with 1× PBS and harvested before fractionation in 10 mM Tris, pH 8.4, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM ETDA, 0.5 mM DTT, 0.5% (v/v) NP-40, and 400 units/mL RNAsin and

centrifuged at 10,0000*g* for 10 min at 4°C. The supernatant (cytoplasmic extract) was collected and RNA was extracted with phenol–chloroform–isoamylalcohol (25:24:1) and precipitated. The nuclei containing pellet was washed and nuclear RNA was isolated using Tri-reagent.

RNase H cleavage of total RNA

Total RNA was annealed to DNA oligonucleotides (oligo1: 5′-TTT ACTTCCTCTAGATAGTCAAGTTCGACC-3′; oligo2: 5′-TGTTA CGACTTTTACTTCCTCTAGATAGTC-3′). The RNA was then incubated with RNase H (NEB), 1× RNase H buffer, 20 units RNAsin, and 1 mM DTT at 37°C for 30 min. Reactions were stopped by addition of 0.2 mM EDTA. RNA was extracted using phenol–chloroform–isoamylalcohol (25:24:1), precipitated, separated on a 12% denaturing polyacrylamide gel and analyzed by Northern blotting.

3′-RACE

Total RNA from cells treated with nontarget or WBSCR22 siRNAs was subjected to poly(A) tailing using poly(A) polymerase from *E. coli* (Ambion) according to the manufacturer's instructions. cDNA was synthesized using an oligo(dT) reverse transcription primer including an additional primer binding site and VN (V = C, G, A; N = C, G, A, T) at the 3′ end for anchoring the primer at the 5′end of the poly(A) tail (5′-CGAATTCTAGAGCTCGAGGC AGGTCCTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3′). The 3′ end of 18SE pre-ribosomal RNA was specifically amplified by PCR using a forward primer reaching 13 nt into the ITS1 region (Preti et al. 2013) and a reverse primer corresponding to the specific sequence introduced during reverse transcription. The amplicons were sequenced on a HiSeq2000 (GATC Biotech). Bioinformatic analysis included demultiplexing of samples and removal of the poly(A) tail with "Flexbar." Remaining sequences (totals of 1,538,025 and 2,609,761 reads for nontarget and WBSCR22 siRNA-treated cells, respectively) were mapped to human 47S rRNA using "Bowtie 2." Counting of the reads was done using python scripts.

Aniline cleavage assays

Total RNA was dissolved in reduction buffer containing 200 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl. The RNA was reduced by addition of $NaBH_4$ to a final concentration of 0.5 M for 30 min on ice and subsequently precipitated. The pellet was dissolved in 20 µL of 1 M aniline (pH 4.5), incubated at 60°C for 15 min and reprecipitated. RNA was then analyzed by agarose–glyoxal gel electrophoresis, followed by Northern blotting using a probe hybridizing to mature 18S rRNA (5′-CCAGTCAGTGTAGCGCGCG-3′).

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