The E3 ubiquitin ligase UBR5 interacts with the H/ACA ribonucleoprotein complex and regulates ribosomal RNA biogenesis in embryonic stem cells

Isabel Saez1, Jennifer V. Gerbracht2, Seda Koyuncu1, Hyun Ju Lee1, Moritz Horn3, Virginia Kroef3, Martin S. Denzel3, Christoph Dieterich4, Niels H. Gehring2 and David Vilchez1

1 Institute for Genetics and Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Germany
2 Institute for Genetics, Department of Biology, University of Cologne, Germany
3 Max Planck Institute for Biology of Ageing, Cologne, Germany
4 Section of Bioinformatics and Systems Cardiology, Department of Internal Medicine III and Klaus, Tschira Institute for Computational Cardiology, University Hospital, Heidelberg, Germany

Correspondence
D. Vilchez, Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Joseph Stelzmann Strasse 26, 50931 Cologne, Germany
Tel: +49 22147884172
E-mail: dvilchez@uni-koeln.de

UBR5 is an E3 ubiquitin ligase involved in distinct processes such as transcriptional regulation and development. UBR5 is highly upregulated in embryonic stem cells (ESCs), whereas its expression decreases with differentiation, suggesting a role for UBR5 in ESC function. However, little is known about how UBR5 regulates ESC identity. Here, we define the protein interactome of UBR5 in ESCs and find interactions with distinct components of the H/ACA ribonucleoprotein complex, which is required for proper maturation of ribosomal RNA (rRNA). Notably, loss of UBR5 induces an abnormal accumulation of rRNA processing intermediates, resulting in diminished ribosomal levels. Consequently, lack of UBR5 triggers an increase in p53 levels and a concomitant decrease in cellular proliferation rates. Thus, our results indicate a link between UBR5 and rRNA maturation.

Keywords: embryonic stem cells; protein–protein interactions; ribosomal RNA; ribosome; ubiquitin ligases

UBR5 is an E3 ubiquitin ligase which belongs to the HECT (homologous to the E6-AP carboxy terminus) ligase family [1]. This 300 kDa enzyme is mostly localized in the nucleus [2] and contains several functional domains. Besides its HECT-ubiquitin ligase domain, UBR5 also has a UBR box finger-like domain, which targets proteins containing the destabilizing N-degron signal toward degradation [3]. Moreover, it contains a PABC [polyadenylate-binding protein (PABP) C terminus] domain, also known as MLLE domain, which confers UBR5 the ability to bind mRNA and modulate eukaryotic translation initiation [4]. Notably, UBR5 exhibits E3-independent activity as a transcriptional cofactor for the progesterone receptor and acts as a binding partner for distinct proteins such as GW182, CHK2, and DUBA [5–7]. UBR5 was first discovered as a tumor suppressor in a progestin-modifying screening using breast cancer cells [1]. In these lines, several studies evidenced a role of UBR5 in cell cycle progression and tumorigenesis [8,9]. UBR5 is also an important mediator of the DNA damage response, since it is a substrate of DNA damage response kinases such as ATM [10,11]. Furthermore, UBR5 has a role in transcriptional regulation, as it

Abbreviations
GO, gene ontology; hESCs, human embryonic stem cells; mESCs, mouse embryonic stem cells; PABP, polyadenylate-binding protein; RIP, RNA immunoprecipitation; RNP, ribonucleoprotein; rRNA, ribosomal RNA; snoRNAs, small nucleolar RNAs; snRNA, small nuclear RNA.
binds and promotes the transactivation of the progesterone receptor [2], ubiquitinates the CDK9 subunit of the positive transcription elongation factor b [12], and regulates the miRNA pathway [13]. In addition, Ubr5−/− mice embryos are not viable, indicating that this protein is essential for development [14].

Recently, we have shown that UBR5 is highly expressed in human embryonic stem cells (hESCs) and its expression decreases when hESC differentiate, suggesting a role of UBR5 in ESC identity [15]. In these lines, a study reported that Ubr5 knockdown results in significant loss of pluripotency markers in mouse ESCs (mESCs) [16]. Here we examine the role of UBR5 in ESCs by defining its interactome using immunoprecipitation assays followed by quantitative proteomics. We find that UBR5 interacts with the H/ACA ribonucleoprotein (rRNA) complex, which participates in proper maturation and processing of ribosomal RNA (rRNA). We further characterize the role of UBR5 in this system by analyzing pre-rRNA processing and find that the levels of several pre-rRNA intermediates are altered in Ubr5−/− ESCs. Finally, we show a decreased translational profile in UBR5-defective cells accompanied by increased p53 levels and diminished proliferation rates.

Methods

hESC and mESC lines and culture

The H9 (WA09) hESC line was obtained from the WiCell Research Institute and was maintained on Geltrex (ThermoFisher Scientific, Waltham, MA, USA) using mTeSR1 (Stem Cell Technologies, Cologne, Germany). Undifferentiated hESC colonies were passaged using a solution of dispase (2 mg mL−1), and scraping the colonies with a glass pipette. The mESC AN-13 mESC line was obtained from the Haplobank at the Institute of Molecular Biotechnology. The mESC was cultured in noncoated plates and grown in DMEM supplemented with 7.25% FBS, 1% Pen/Strep, 1% nonessential amino acids, 1% Glutamax, 1% Sodium pyruvate, 50 µM ß-mercaptoethanol and human LIF (12 ng/mL). The cell lines used in this study were tested for mycoplasma contamination at least once every three weeks. No mycoplasma contamination was detected. Research involving hESCs was performed with approval of the German Federal competent authority (Robert Koch Institute).

Lentiviral infection of hESCs

Lentivirus (LV)-nontargeting shRNA control, LV-UBR5 shRNA #1 (TRCN000003411), and LV-UBR5 shRNA #2 (TRCN0000226458) in pLKO.1-puro vector were obtained from Mission shRNA (Sigma, Hamburg, Germany). Transient infection experiments were performed as follows. H9 colonies growing on Geltrex were individualized using Accutase. Hundred thousand cells were plated on Geltrex plates and incubated with mTeSR1 medium containing 10 µM ROCK inhibitor for 1 day. Then, cells were infected with 5 µL of concentrated lentivirus. Cells were fed with fresh media the day after to remove the virus. After 1 day, cells were selected for lentiviral integration using 2 µg·mL−1 puromycin (ThermoFisher Scientific).

CRISPR/Cas9- mediated KO of mESCs

UBR5 gene knockout was done in mouse embryonic stem cells carrying a haploid chromosome set [[17]]. These cells become diploid over time and mutations thus remain homozygous. For genome engineering CRISPR/Cas9 was used as previously described [18]. Sequences for small guide RNAs were designed online (http://crispor.org) and purchased from Sigma (FGuide 1: CACCGTGAAATGTTACCATACCGGT, RGuide 1: AAACACCGTAGGTAATATC TTAC, FGuide 2, CACCGATGCTTTAAACCTCCAC TT, RGuide 2: AAACAAGTAGGAGTTTTAAGCAATC). The primers were cloned into the Cas9-GFP expressing plasmid PX458 (Addgene #48138, gift from Feng Zhang). A combination of guides and the Cas9-expressing plasmid were transfected using lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer’s instructions. Twenty-four hours post transfection a mixed population of haploid and diploid ES cells were stained with 10 µg/mL Hoechst 33342 (Thermo Fisher Scientific) for 30 min and sorted according to their DNA content and GFP expression on a FACSAria Fusion sorter (BD, Franklin Lakes, NJ, USA). Haploid GFP-positive cells were single cell sorted into 96-well plates. Emerging clones were transferred to 24-well plates 7 days later and genotyped following DNA extraction (DNA extraction solution, Epicentre Biotechnologies, Madison, WI, USA) using the following primers; F: GAGACCGCCTGTGGTTT, R: CCCAATTGATCTCTGAGCCA. Sanger sequencing of PCR products was performed at Eurofins Genomics GmbH, Ebersberg, Germany. UBR5 knockout clones were selected for diploid cells (FACS after Hoechst staining) prior to further experiments.

Transfection of HEK293T cells

HEK293T cells (ATCC) were plated on 0.1% gelatin-coated plates and grown in DMEM supplemented with 10% FBS and 1% MEM nonessential amino acids (ThermoFisher Scientific). Cells were transfected once they reached 80-90% confluency. About 1 µg GFP-UBR5 wild-type or GFP-UBR5 ΔHECT overexpression plasmid and 1 µg of GAR1-FLAG, NHP2-FLAG or DKC1-FLAG were used for transfection, using Fugene HD
(Promega, Mannheim, Germany) following manufacturer’s instructions. After 24 h incubation in normal medium, the cells were harvested for further experiments. GFP-UBR5 wild-type and GFP-UBR5 ΔHECT overexpression plasmids (Addgene plasmids #52050 and #52051, respectively) were a gift from D. Saunders and were first published in ref [19].

DKC1, GAR1, and NHP1 were PCR-amplified from HeLa cDNA and cloned between Xho I/Not I into a modified pCI-neo (Clontech, Saint-Germain-en-Laye, France) plasmid, which contains an N-terminal FLAG-tag sequence (pCI-FLAG).

Immunoprecipitation of UBR5 for interactome analysis

The immunoprecipitation was performed under the conditions described in [20]. Briefly, hESCs were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton x-100, 1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) supplemented with protease inhibitor cocktail (Roche) and centrifuged at 13 000 g for 15 minutes at 4 °C. About 350 µg of protein was incubated with UBR5 Antibody (Cell Signaling, Frankfurt am Main, Germany, #8755, 1 : 50) or FLAG antibody as a control (Sigma, F7425, 4 µg). Subsequently, samples were incubated with 100 µL of µMACS Micro Beads for 1 h at 4 °C and loaded to precleared µMACS column (#130-042-701). Beads were washed three times with 50 mM Tris (pH 7.5) buffer containing 150 mM NaCl, 5% glycerol, and 0.05% Triton and then washed five times with 50 mM Tris (pH 7.5) and 150 mM NaCl. Then, columns were subjected to in-column tryptic digestion containing 7.5 mM ammonium bicarbonate, 2 mM urea, 1 mM DTT, and 5 ng·mL⁻¹ trypsin. Digested peptides were eluted using 50 µL of elution buffer 1 containing 2 mM urea, 7.5 mM Ambic, and 5 mM IAA two times. Digests were incubated over night at room temperature and samples were stage-tipped the next day for label-free quantitative proteomics. All samples were determined with a standard BCA protein assay (ThermoFisher Scientific). Approximately 30 µg of total protein was separated by SDS/PAGE, transferred to PVDF membranes (Millipore, Darmstadt, Germany) and subjected to immunoblotting. Western blot analysis was performed with anti-UBR5 (Cell Signaling, #8755, 1 : 1000), anti-FLAG (Sigma, F7425, 1 : 5000), anti-DKC1 (Abcam, Cambridge, UK, ab156877, 1 : 1000), anti-RPL7 (Genetex, Irvine, CA, USA, #114727, 1 : 500), anti-RPS27 (Proteintech, Manchester, UK, 15355-1-AP, 1 : 500), anti p53 (Proteintech, 10442-1-AP, 1 : 1000), and anti-β Actin (Abcam, ab8226, 1 : 10 000).

Immunocytochemistry

Cells were fixed with paraformaldehyde (4% in PBS) for 20 min, followed by permeabilization (0.2% Triton X-100 in PBS for 10 min) and blocking (3% BSA in 0.2% Triton X-100 in PBS for 10 min). Primary antibody was incubated for 1.5 h at room temperature [anti-FLAG (Sigma F7425, 1 : 100)] and incubated with secondary antibody [Alexa Fluor 488 Goat anti-Mouse (ThermoFisher Scientific, #A-11029, 1 : 500), Alexa Fluor 568 F(ab')2 Fragment of Goat Anti-Rabbit IgG (H+L) (ThermoFisher Scientific, #A-21069, 1 : 500) ,and Hoechst 33342 (Life Technologies, Darmstadt, Germany, #1656104) for 1 h at room temperature. PBS and distilled water wash were followed before the cover slips were mounted on Mowiol (Sigma, #324590).

Ribosome fractionation

About 5% (w/v) to 50% (w/v) sucrose gradients were prepared from 5% and 50% sucrose solutions [20 mM Tris-
UBR5 regulates rRNA biogenesis

I. Saez et al.

A

Box H/ACA RNP

B

Input

UBR5

β-actin

anti-UBR5 IP

UBR5

Empty vector

GAR1- FLAG

NHP2- FLAG

DKC1- FLAG

+ – + + +

+ – – – –

– – – + +

– – – + +

UBR5

UBR5-GFP

Empty Vector

UBR5-GFP

GAR1- FLAG

UBR5-GFP

NHP2- FLAG

UBR5-GFP

DKC1- FLAG

→

→

→

C

50

25

20

37

5S rRNA

SNORA5

SNORA30

SNORA34

SNORD3/A/U17

SNORD3/U3

SNORD46

SNORD68

SNORD104

0.0

0.5

1.0

1.5

2.0

2.5

* *

H/ACA box

C/D box

mRNA relative enrichment (UBR5/FLAG)

D

UBR5 antibody

FLAG antibody

mRNA relative enrichment (UBR5/FLAG)

E

Input

UBR5

β-actin

RNase A + anti-UBR5 IP

UBR5

UBR5

Empty vector

GAR1- FLAG

NHP2- FLAG

DKC1- FLAG

+ – + + +

– – – + +

– – – – –

– – – – –

– – – – –

– – – – –

– – – – –

– – – – –

– – – – –

FEBS Letters 594 (2020) 176–188 © 2019 The Authors. FEBS Letters published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.
with protease inhibitor (Roche, Berlin, Germany), 400 μg/ml vanadyl ribonucleoside complexes, 1 mM DTT, and 20 mM EDTA. The cell lysate was mixed with antibody-coated beads and incubated 2 h at room temperature. The beads were washed five times with ice cold NT2 buffer and finally washed with NT2 buffer with 1% Triton X-100. RNA extraction was done from the immunoprecipitated pellet using RNAbee (Tel-Test Inc., Friendswood, TX, USA). cDNA was generated using qScript CDNA SuperMix (Quantabio, Beverly, MA, USA). SybrGreen real-time qPCR experiments were performed with a 1 : 20 dilution of cDNA using a CFC384 Real-Time System (Bio-Rad) following the manufacturer’s instructions. Data were analyzed with the comparative ΔΔCt method (RNA relative fold enrichment in anti-UBR5 RIP over RIP performed with control FLAG antibody) after normalization to the corresponding input values. See Table S1 for details about the primers used for this assay.

Bromodeoxyuridine proliferation assay

The mESCs were incubated with media containing 10 μM 1-bromodeoxyuridine (BrdU) for the indicated times. Cells were fixed with formaldehyde 4% in PBS. Then, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 3% BSA-PBS for 1 h at room temperature. 2N HCl was added for 15 min at room temperature. After this, cells were incubated in 0.1 M sodium tetra-borate for 15 min at room temperature. We performed overnight incubation with rabbit anti-BrdU (ABD Serotech, OBT0030, 1 : 1000) at 4 °C followed by incubation with an anti-rat-AlexaFluor 546 Cross-Adsorbed (ThermoFisher Scientific, #A11081, 1 : 500) for 1 h. Hoechst 33342 was used to visualize nuclei.

RNA extraction and northern blotting

Total RNA was extracted using RNAbee (Tel-Test Inc.). About 2.5 μg of total RNA was resolved on a 1% agarose/
0.4 M formaldehyde gel using the tricine/triethanolamine buffer system (ref PMID 23800830). The RNA was blotted on an uncharged nylon membrane (Roth) by capillary transfer overnight in 10x SSC buffer. Following UV-crosslinking, the blots were prehybridised for 1 h in Church buffer. Hybridization with $5'\text{-}32\text{P}$-labeled oligonucleotides in Church buffer was carried out overnight at 40 °C. The oligonucleotide sequences were as follows: ITS1 mouse: (5'-GCCGCTCCTCCACAGTCTCCGTATTTATGATC-3'), ITS2 human: (5'-CTGCCAGGAAACCCCCACGGCAGGCA-3'), ITS2 mouse: (5'-CTGCCAGAAACTCCGCAGGCAGCCGAGCAC-3'). The ITS1 (human) probe was generated by in vitro transcription with [$\alpha$-32P]-GTP using the BamHI linearized pGEM4Z-ITS1 plasmid as a template. The signals were detected using a Typhoon FLA 7000 (GE Healthcare) and quantification was performed with Image Quant TL (GE Healthcare, Solingen, Germany).

**Results**

**UBR5 interacts with the H/ACA ribonucleoprotein complex**

UBR5 is upregulated in both hESCs and mESCs compared with their differentiated counterparts [15,16,20], suggesting a role for this protein in ESC function. To gain insights into the function of UBR5, we performed co-immunoprecipitation experiments of endogenous UBR5 in hESCs followed by single-shot proteomic analysis. We identified 101 interactors of UBR5 (Data S1). Gene Ontology (GO) analysis of UBR5 potential interactors indicated enrichment for proteins involved in biological processes such as spliceosome assembly, small nuclear RNA (snRNA) modification, and rRNA pseudouridine synthesis (Data S1 and Fig. S1). Interestingly, we found that UBR5 interacts with the proteins (i.e., DKC1, GAR1, NOP10, and NHP2) of the nucleolar H/ACA RNP complex (Data S1).

The H/ACA RNP box is formed by four different proteins bound to small nucleolar RNAs (snoRNAs) (Fig. 1A), and modulates the modification of rRNAs as well as other RNAs by catalyzing their pseudouridylation (i.e., conversion of uridine to pseudouridine) [23,24]. The noncoding H/ACA snoRNAs act as adaptors that link the catalytic protein DKC1 to its targets [25,26]. The three other proteins of the H/ACA RNP box – GAR1, NOP10, and NHP2 – act as scaffolding proteins and they are essential for the proper functioning of the complex [27,28]. We identified DKC1, GAR1, NOP10, and NHP2 as putative binding partners of UBR5 in our analysis, suggesting

![Fig. 2.](image-url)

**UBR5 regulates rRNA biogenesis**

I. Saez et al.
a role of UBR5 in the regulation of the H/ACA RNP complex (Data S1). To further assess this interaction, we overexpressed UBR5 together with GAR1, NHP2, or DKC1 in HEK293 human cells and performed co-immunoprecipitation assays followed by western blot. These experiments confirmed the interaction of UBR5 with GAR1, NHP2, and DKC1 (Fig. 1B). Prompted by these results, we analyzed the intracellular localization of UBR5 and GAR1, NHP2, or DKC1 in HEK293. Notably, these components of the H/ACA RNP complex co-localized with UBR5 in the nucleus, further supporting our interactome analysis (Fig. 1C).

**Fig. 3.** UBR5 is required for the correct maturation of ribosomal RNA. (A) Schematic representation of the 47S/45S pre-rRNA processing pathway and the binding sites of the internal probes ITS1 and ITS2. The 47S pre-rRNA is subjected to several internal and external cleavages to finally convert into the mature 18S, 5.8S and 28S rRNA. The intermediate species observed on the northern blots in (B)–(E) are listed. (B) Representative northern blots of total RNA obtained from hESCs upon UBR5 knockdown (KD) and mESCs upon Ubr5 knockout (KO). The hybridization was performed with probes targeting the ITS1 region of ribosomal precursor RNAs. NT: nontargeting shRNA control. (C) Quantification of the northern blots shown in (B). The amounts of the 30S/34S or 21S/20S precursor rRNAs were normalized to the corresponding amount of 47/45S pre-rRNA. The mean values ± SD were calculated from n = 9 (hESCs) and n = 5 (mESCs) independent experiments. Statistical comparisons were made by Student’s t-test for paired samples [P-value: **(P < 0.01), ***P < 0.001], ns: not significant. (D) As performed in (B), but with a probe targeting the ITS2 region of ribosomal precursor RNAs. (E) Quantification of the blots shown in (D). The amounts of the 32S or 12S precursor rRNAs were normalized to the corresponding amount of 47/45S pre-rRNA. The mean values ± SD were calculated from n = 6 (hESCs) and n = 4 (mESCs). Statistical comparisons were made by Student’s t-test for paired samples. [P-value: **(P < 0.01), ns: not significant].

I. Saez et al. UBR5 regulates rRNA biogenesis
UBR5 regulates rRNA biogenesis

I. Saez et al.
With the strong interaction between UBR5 and proteins of the H/ACA RNP complex, we examined whether UBR5 protein also pulls down snoRNAs by RNA immunoprecipitation (RIP) assays. We assessed four snoRNAs of the H/ACA RNP complex and found interaction of UBR5 protein with SNORA73A/U17 and SNORA5A (Fig. 1D). In contrast, we did not observe significant interaction of UBR5 with snoRNAs of the C/D box RNP, a distinct nucleolar complex that catalyzes 2′ O-methylation of rRNA (Fig. 1D). Since UBR5 protein pulled down snoRNAs of the H/ACA RNP, we asked whether the interaction of UBR5 with proteins of this complex is mediated by RNA. To assess this hypothesis, we performed co-immunoprecipitation assays treating the samples with RNase A prior to immunoprecipitation with UBR5 antibody. Notably, the interactions with GAR1, NHP2, and DKC1 proteins remained upon RNase A treatment (Fig. 1E), indicating that this binding is not due to secondary, RNA-mediated interactions.

Given the role of UBR5 in polyubiquitination and proteasomal degradation of distinct substrates [15], we asked whether its interaction with H/ACA components triggers their degradation through the proteasome. For this purpose, we overexpressed GAR1, NHP2, or DKC1 with either wild-type UBR5 (UBR5WT) or a catalytically dead mutant of UBR5 (UBR5ΔHECT) which lacks the ubiquitin ligase activity. We assessed the levels of DKC1, NHP2, and GAR1 by western blot and found no differences upon overexpression of either UBR5WT or UBR5ΔHECT, indicating that UBR5 does not target them for proteasomal degradation (Fig. 2A). In addition, we generated two independent hESC lines with reduced levels of UBR5 (hESCUBR5 KD) and found no changes in the endogenous levels of DKC1 (Fig. 2B). Likewise, endogenous DKC1 levels were not affected in a mESC line in which UBR5 was knocked down using CRISPR/Cas9 (mESCUBR5 KO) (Fig. 2C). Thus, our results indicate that UBR5 interacts with distinct proteins of the H/ACA ribonucleoprotein complex but it does not induce their proteasomal degradation.

**UBR5 is required for the correct maturation of ribosomal RNA**

Given the interaction of UBR5 with the H/ACA RNP complex, we asked whether UBR5 has a role in the regulation of rRNA in ESCs. For this purpose, we assessed the maturation of rRNAs by northern blot analysis of their precursors in hESCUBR5 KD and mESCUBR5 KO. In eukaryotes, the ribosome contains four distinct rRNAs: the 18S rRNA is present in the 40S small ribosomal subunit, while the large 60S subunit contains the 5.8S, 28S, and 5S rRNAs. The 18S, 5.8S, and 28S rRNA are transcribed by RNA polymerase I as a single rRNA precursor transcript, the 47S/45S pre-ribosomal RNA (pre-rRNA) [29,30]. Ribosomal proteins and assembly factors bind co-transcriptionally to the pre-rRNA, which then undergoes a series of modifications and processing steps including endonucleolytic cleavage and exonucleolytic trimming [31]. The maturation process can be assessed by radioactive labeled probes (ITS1 and ITS2) which bind to internal sequences of the pre-rRNA. These sequences flank the mature rRNA and are eliminated during the sequential cleavages of the pre-rRNA [32] (Fig. 3A).

rRNA precursors of the 18S rRNA were detected with an ITS1 probe (Fig. 3B,C). In hESCUBR5 KD, the amount of 34/30S rRNA relative to the 47/45S pre-rRNA was significantly reduced compared with control hESCs. The 30S rRNA is further processed to 21S rRNA by additional cleavage steps at positions 01 and 1. Interestingly, the levels of 21S rRNA remained unchanged in hESCUBR5 KD when compared with control hESCs. Since the 47S pre-rRNA can also be processed involving an alternative pathway via the 41S rRNA, the reduced cleavage into 30S rRNA could
potentially be compensated. In mESC^{Ubr5 KO}, the reduction of 34/30S rRNA compared to 47/45S pre-rRNA was even more dramatic. Moreover, the levels of 20S rRNA were also affected in these cells. However, the amounts of 41S rRNA, the intermediate of the alternative pathway of cleavage at position 2, were unchanged. Using a probe targeting ITS2, we examined the 32S and 12S precursor rRNAs in hESC and mESCs (Fig. 3D,E). Although in hESC^{UBR5 KD}, the levels of 12S rRNA relative to 47/45S pre-rRNA were significantly increased, we did not observe these effects in mESC^{Ubr5 KO}. In both hESCs and mESCs, the levels of 32S rRNA were not significantly changed upon loss of UBR5 (Fig. 3D,E). Taken together, our results indicate that dysfunction of UBR5 could affect rRNA maturation, particularly conversion into 34/30S rRNA.

UBR5 deficiency impairs translational profile and triggers a stress response in mESCs

Since alterations in rRNA might originate defects in the proper assembly of the ribosomes, we assessed ribosome pools in mESC^{Ubr5 KO} (Fig. 4A). When compared with wild-type ESCs, these cells exhibited a decrease in 40S and 60S, as well as in the monosome and polysome fraction, suggesting lower translational rates in the absence of UBR5. The levels of 40S (i.e., RPS27) and 60S (i.e., RPL7A) proteins in mESC^{UBR5 KO} cells were also altered in different ribosome fractions, although the decrease in RPL7A was more evident than RPS27 (Fig. 4B). Alterations in rRNA synthesis can trigger a cellular stress response [33,34], which, in turn, increases p53 levels. Notably, we found increased p53 levels in mESC^{Ubr5 KO}, suggesting the activation of a stress response in these cells (Fig. 4C). p53-mediated response toward stress promotes cell cycle arrest to maintain cellular homeostasis [35]. Importantly, we observed that the proliferation rates in mESC^{Ubr5 KO} cells were significantly diminished (Fig. 4D). We then asked whether the lack of UBR5 sensitized mESCs toward other stressors which inhibit translation. For this purpose, we exposed mESC^{Ubr5 KO} cells to either heat stress (Fig. 4E) or cycloheximide (Fig. 4F) and determine the degree of apoptosis by measuring cleaved caspase-3 levels. However, we did not find notable differences in the apoptosis rates in the absence of UBR5. Altogether, our results suggest that UBR5 participates in the maturation of rRNA, a process necessary for proper ribosomal assembly. In the absence of UBR5, ESCs exhibit defects in their ribosomal content and increased p53-mediated stress response, resulting in lower proliferation rates.

Discussion

Growing evidence indicates that UBR5 is involved in the regulation of distinct RNA-related pathways [4,12,13]. In mESCs, UBR5 is required for the proper function of the miRNA machinery by directing the scaffolding protein GW182 to the miRNA complex through a ubiquitin ligase-independent mechanism [13]. This process results in the recruitment of other downstream effectors like DDX6 and Tob1/2, which promote the deadenylation and consequent destabilization of target mRNAs [36–38]. Moreover, UBR5 phosphorylation by p90 ribosomal S6 kinase (p90RSK) is necessary for the effect of UBR5 on miRNA repression activity, adding an additional regulatory level [39]. The effects of UBR5 on the miRNA pathway could be evolutionary conserved among species as similar effects were also reported in Drosophila melanogaster [40]. Additionally, upon UV-induced DNA damage, UBR5 represses the transcription at the damaged sites by inhibiting the elongation of polymerase II [41]. UBR5 also interacts with the phosphorylated form of PIH1D1, which is a subunit of the co-chaperone complex R2TP [42]. R2TP is implicated in the assembly of large complexes such as the H/ACA RNP complex [43]. However, a direct link between UBR5 and the H/ACA RNP complex has not been previously reported. Notably, we find an interaction between UBR5 and the four proteins of the nucleolar H/ACA complex (i.e., DKC1, GAR1, NOP10, and NHP2). Besides these proteins, the H/ACA RNP complex contains small nucleolar RNAs (snoRNAs) that complement rRNA sequences and guide their pseudouridylation, a modification required for proper rRNA processing. As a further indication of the link of UBR5 with H/ACA RNP, RIP experiments showed that UBR5 protein also pulls down snoRNAs of this complex while it does not interact with snoRNAs of the nucleolar C/D Box RNP. Importantly, our data suggest that the interaction of UBR5 with proteins of the H/ACA RNP complex is RNA-independent. Thus, these direct protein–protein interactions raise the interesting hypothesis that increased levels of UBR5 could titrate out proteins from the H/ACA RNP complex, leading to a lower stability of this complex. However, our results do not support this hypothesis because we observe that loss of UBR5 impairs the maturation of rRNA and increases the levels of intermediates, a process that could ensue from impaired functioning of the H/ACA RNP complex. In addition, we show that the levels of the 40S and 60S subunits of the ribosome, as well as the monosome and polysomes fractions, are reduced upon loss of UBR5, correlating with impaired
rRNA biogenesis. Taken together, these findings support that UBR5 is important for the function of the H/ACA RNP complex, but it does not act as a suppressor of its stability/activity. In support of this hypothesis, ESCs express intrinsic higher levels of UBR5 protein when compared with their differentiated counterparts, while they exhibit enhanced global translation rates [44].

Besides the nucleolar H/ACA RNP complex, small Cajal body-specific RNAs (scaRNAs) confined to Cajal bodies in the nucleus also contain similar box H/ACA domains as well as box C/D domains [45]. As such, scaRNAs are involved in the pseudouridylation or 2’ O-methylation required for maturation of not only small nuclear RNAs (snRNAs) but also nucleolar snoRNAs [45]. Since Cajal bodies contribute to the biogenesis of nucleolar RNPs [45], it will be fascinating to examine whether UBR5 interacts with nuclear RNPs in Cajal bodies and the impact of this interaction in nucleolar rRNA metabolism. Besides its effects in nucleolar RNP biogenesis, Cajal bodies are also involved in the proper maturation of snRNAs required for the assembly of distinct nuclear RNP complexes such as the spliceosome, which regulates RNA splicing [46]. Notably, the interactome of UBR5 is highly enriched in proteins involved in the assembly and activity of the spliceosome RNP complex (Fig. S1 and Data S1). Since these data support an interaction between UBR5 and spliceosome, it will be interesting to assess a role of UBR5 in the assembly and/or activity of the spliceosome RNP complex.

The link of UBR5 with rRNA biogenesis indicates a role in translational rates. Importantly, UBR5 contains a PABC domain at the C terminus, also known as MLLE domain, which mediates protein–protein interaction through the binding of the (PABP-interacting motifs) PAM2 peptide motif [47,48]. This PABC domain is equivalent to the one found in the poly(A) binding protein (PABPC1), a protein that recognizes 3’ mRNA poly(A) tails and plays an essential role in eukaryotic translation initiation [49,50]. UBR5 and PABPC1 share common interacting partners, such as Paip1 [47] and Paip2 [51]. Paip2 is a repressor of translation initiation which can interact with both PABPC1 and UBR5. When bound to UBR5, Paip2 is ubiquitinated and degraded by the proteasome [51]. Conversely, the levels of the repressor increase in the absence of UBR5 and translation are consequently inhibited. Thus, UBR5 could modulate translational rates via both Paip2-mediated mechanisms and the maturation of rRNA.

As a consequence of perturbations in ribosomal biogenesis, cells enter into the so called ‘nucleolar stress’ [52]. Under normal conditions, the E3 ligase Mdm2 interacts with p53 and results in the ubiquitination and degradation of p53. During nucleolar stress, several assembly factors, such as Arf or Nucleophosmin1, and ribosomal proteins bind to Mdm2 preventing its interaction with p53, a process that results in stabilization of p53 levels [53,54]. In addition, the 5S rRNA also interacts and blocks Mdm2 [55]. As a consequence of p53 accumulation, cell enters in cell cycle arrest to ensure cellular homeostasis until the stress is over. Remarkably, we find that the levels of p53 levels are increased in mESC-UBR5 KO cells. This could indicate that UBR5-lacking cells undergo nucleolar stress, as suggested by the altered rRNA precursors and polysome levels. Moreover, loss of UBR5 induces a decline in the proliferation rates of ESCs, which could be ensue from increased p53 levels. In these lines, UBR5 is deregulated in many cancer types and alters p53 levels [56–58]. However, it is unclear whether UBR5 would act as a tumor suppressor or oncogene, since both amplifications as well as loss-of-function mutations have been linked to tumorigenesis. Thus, the cellular context might be determinant for the role of UBR5 in p53 regulation and cellular proliferation [59–61]. Whereas previous studies reported that the role of UBR5 in the regulation of mRNA machinery is necessary for cell proliferation [13,62], here we provide data indicating that UBR5 also modulates cell proliferation via modulation of rRNA maturation. Thus, UBR5 could impinge upon cellular proliferation through multiple pathways.

Taken together, our results indicate that UBR5 participates in the maturation of rRNA through regulating the nucleolar H/ACA RNP complex. This regulatory pathway is required for proper rRNA processing and ribosomal assembly. Upon loss of UBR5, rRNA intermediates accumulate, decreasing the pool of ribosomes. This perturbation may activate the nucleolar stress response, triggering the accumulation of p53 and consequent proliferation arrest. Taking into consideration the multiple roles of UBR5 in cell cycle and cellular homeostasis, our findings add an additional regulation layer to this process.

Acknowledgements

This work was supported by The Deutsche Forschungsgemeinschaft (DFG) (CECAD and VI742/1-2). We thank Volker Böhm for helping with data analysis.

Author contributions

IS and DV conceived and supervised the study. IS performed most of the experiments, data analysis and
UBR5 regulates rRNA biogenesis

I. Saez et al.

interpretation through discussions with NHG and DV. JVG assessed rRNA maturation. SK performed western blot experiments. HJL performed RIP experiments. MH, VK and MSD generated mESCubr5 KO line and contributed to experiment design. NHG and CD contributed with their expertise on RNA metabolism and performed data analysis. IS, JVG, NHG and DV wrote the manuscript. All the authors discussed the results and commented on the manuscript.

Data Accessibility

Research data pertaining to this article is located at figshare.com: https://dx.doi.org/10.6084/m9.figshare.9153215.

References

1 Callaghan MJ, Russell AJ, Woollatt E, Sutherland GR, Sutherland RL and Watts CK (1998) Identification of a human HECT family protein with homology to the Drosophila tumor suppressor gene hyperplastic discs. Oncogene 17, 3479–3491.

2 Henderson MJ, Russell AJ, Hirid S, Muñoz M, Clancy JL, Lehrbach GM, Calanni ST, Jans DA, Sutherland RL and Watts CK (2002) EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. J Biol Chem 277, 26468–26478.

3 Tasaki T, Mulder LC, Iwamatsu A, Lee MJ, Davydov IV, Varshavsky A, Muesing M and Kwon YT (2005) A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. Mol Cell Biol 25, 7120–7136.

4 Kozlov G, Trempe JF, Khaleghpour K, Kalvejian A, Ekiel I and Gehring K (2001) Structure and function of the C-terminal PABC domain of human poly(A)-binding protein. Proc Natl Acad Sci USA 98, 4409–4413.

5 Henderson MJ, Munoz MA, Saunders DN, Clancy JL, Russell AJ, Williams B, Pappin D, Kharina KK, Jackson SP, Sutherland RL et al. (2006) EDD mediates DNA damage-induced activation of CHK2. J Biol Chem 281, 39990–40000.

6 Munoz-Escobar J, Matta-Camacho E, Kozlov G and Gehring K (2015) The MLLE domain of the Ubiquitin Ligase UBR5 binds to its catalytic domain to regulate substrate binding. J Biol Chem 290, 22841–22850.

7 Rutz S, Kayagaki N, Phung QT, Eideneschken C, Noubade R, Wang X, Lesch J, Lu R, Newton K, Huang OW et al. (2015) Deubiquitinase DUBA is a post-translational brake on interleukin-17 production in T cells. Nature 518, 417–421.

8 Eblen ST, Kumar NV, Shah K, Henderson MJ, Watts CK, Shokat KM and Weber MJ (2003) Identification of novel ERK2 substrates through use of an engineered kinase and ATP analogs. J Biol Chem 278, 14926–14935.

9 Munoz MA, Saunders DN, Henderson MJ, Clancy JL, Russell AJ, Lehrbach G, Musgrove EA, Watts CK and Sutherland RL (2007) The E3 ubiquitin ligase EDD regulates S-phase and G(2)/M DNA damage checkpoints. Cell Cycle 6, 3070–3077.

10 Mu JJ, Wang Y, Luo H, Leng M, Zhang J, Yang T, Besusso D, Jung SY and Qin J (2007) A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. J Biol Chem 282, 17330–17334.

11 Kim MA, Kim HJ, Brown AL, Lee MY, Bae YS, Park JI, Kwak JY, Chung JH and Yun J (2007) Identification of novel substrates for human checkpoint kinase Chk1 and Chk2 through genome-wide screening using a consensus Chk phosphorylation motif. Exp Mol Med 39, 205–212.

12 Cojocaru M, Bouchard A, Cloutier P, Cooper JJ, Varzavand K, Price DH and Coulombe B (2011) Transcription factor IIS cooperates with the E3 ligase UBR5 to ubiquitinate the CDK9 subunit of the positive transcription elongation factor B. J Biol Chem 286, 5012–5022.

13 Su H, Meng S, Lu Y, Trombly MI, Chen J, Lin C, Turk A and Wang X (2011) Mammalian hyperplastic discs homolog EDD regulates miRNA-mediated gene silencing. Mol Cell 43, 97–109.

14 Saunders DN, Hird SL, Withington SL, Dunwoodie SL, Henderson MJ, Biben C, Sutherland RL, Ormandy CJ and Watts CK (2004) Edd, the murine hyperplastic disc gene, is essential for yolk sac vascularization and chorioallantoic fusion. Mol Cell Biol 24, 7225–7234.

15 Koyuncu S, Saez I, Lee HJ, Gutierrez-Garcia R, Pokrzywa W, Fatima A, Hoppe T and Vilchez D (2018) The ubiquitin ligase UBR5 suppresses proteostasis collapse in pluripotent stem cells from Huntington’s disease patients. Nat Commun 9, 2886.

16 Buckley SM, Aranda-Orgilles B, Strikoudis A, Apostolou E, Loizou E, Moran-Crusio K, Farnsworth CL, Koller AA, Dasgupta R, Silva JC et al. (2012) Regulation of pluripotency and cellular reprogramming by the ubiquitin-proteasome system. Cell Stem Cell 11, 783–798.

17 Elling U, Taubenschmid J, Wirsberger G, O’Malley R, Demers SP, Vanhaelen Q, Shukalyuk AI, Schmauss G, Schramek D, Schnuetergen F et al. (2011) Forward and reverse genetics through derivation of haploid mouse embryonic stem cells. Cell Stem Cell 9, 563–574.
The post-transcriptional steps of eukaryotic ribosome biogenesis. *Cell Mol Life Sci* 65, 2334–2359.

32 Granneman S and Baserga SJ (2004) Ribosome biogenesis: of knobs and RNA processing. *Exp Cell Res* 296, 43–50.

33 Donati G, Montanaro L and Derenzini M (2012) Ribosome biogenesis and control of cell proliferation: p53 is not alone. *Cancer Res* 72, 1602–1607.

34 Deisenroth C and Zhang Y (2010) Ribosome biogenesis surveillance: probing the ribosomal protein-Mdm2-p53 pathway. *Oncogene* 29, 4253–4260.

35 Vogelstein B, Lane D and Levine AJ (2000) Surfing the p53 network. *Nature* 408, 307–310.

36 Ezzeddine N, Chang TC, Zhu W, Yamashita A, Chen CY, Zhong Z, Yamashita Y, Zheng D and Shyu AB (2007) Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Mol Cell Biol* 27, 7791–7801.

37 Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA et al. (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell* 35, 868–880.

38 Zekri L, Huntzinger E, Heimstadt S and Izaurralde E (2009) The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. *Mol Cell Biol* 29, 6220–6231.

39 Cho JH, Kim SA, Seo YS, Park SG, Park BC, Kim JH and Kim S (2017) The p90 ribosomal S6 kinase-UBR5 pathway controls Toll-like receptor signaling via miRNA-induced translational inhibition of tumor necrosis factor receptor-associated factor 3. *J Biol Chem* 292, 11804–11814.

40 Zhou R, Hotta I, Denli AM, Hong P, Perrimon N and Hannon GJ (2008) Comparative analysis of argonaute-dependent small RNA pathways in Drosophila. *Mol Cell* 32, 592–599.

41 Sanchez A, De Vivo A, Uprety N, Kim J, Stevens SM Jr and Kee Y (2016) BMI1-UBR5 axis regulates transcriptional repression at damaged chromatin. *Proc Natl Acad Sci USA* 113, 11243–11248.

42 Horejsi Z, Stach L, Flower TG, Joshi D, Flynn H, Skehel JM, O’Reilly NJ, Ogrodowicz RW, Smerdon SJ and Boulton SJ (2014) Phosphorylation-dependent PH1DI interactions define substrate specificity of the R2TP cochaperone complex. *Cell Rep* 7, 19–26.

43 Machado-Pinilla R, Liger D, Leulliot N and Meier UT (2012) Mechanism of the AAA + ATPases pontin and reptin in the biogenesis of H/ACA RNPs. *RNA* 18, 1833–1845.

44 You KT, Park J and Kim VN (2015) Role of the small subunit processome in the maintenance of pluripotent stem cells. *Genes Dev* 29, 2004–2009.
45 Meier UT (2017) RNA modification in Cajal bodies. RNA Biol 14, 693–700.
46 Cioce M and Lamond AI (2005) Cajal bodies: a long history of discovery. Annu Rev Cell Dev Biol 21, 105–131.
47 Deo RC, Sonenberg N and Burley SK (2001) X-ray structure of the human hyperplastic discs protein: an ortholog of the C-terminal domain of poly(A)-binding protein. Proc Natl Acad Sci USA 98, 4414–4419.
48 Kozlov G, De Crescenzo G, Lim NS, Siddiqui N, Fantus D, Kahvejian A, Trempe JF, Elias D, Ekiel I, Sonenberg N et al. (2004) Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. EMBO J 23, 272–281.
49 Kahvejian A, Svitkin YV, Sukarieh R, M’Boutchou MN and Sonenberg N (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev 19, 104–113.
50 Mangus DA, Evans MC and Jacobson A (2003) Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol 4, 223.
51 Yoshida M, Yoshida K, Kozlov G, Lim NS, De Crescenzo G, Pang Z, Berlanga JJ, Kahvejian A, Gehring K, Wing SS et al. (2006) Poly(A) binding protein (PABP) homeostasis is mediated by the stability of its inhibitor, Paip2. EMBO J 25, 1934–1944.
52 James A, Wang Y, Raje H, Rosby R and DiMario P (2014) Nucleolar stress with and without p53. Nucleus 5, 402–426.
53 Zhang Y and Lu H (2009) Signaling to p53: ribosomal proteins find their way. Cancer Cell 16, 369–377.
54 Chen D, Zhang Z, Li M, Wang W, Li Y, Rayburn ER, Hill DL, Wang H and Zhang R (2007) Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene 26, 5029–5037.
55 Nishimura K, Kumazawa T, Kuroda T, Katagiri N, Tsuchiya M, Goto N, Furumai R, Murayama A, Yanagisawa J and Kimura K (2015) Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation. Cell Rep 10, 1310–1323.
56 Shearer RF, Iconomou M, Watts CK and Saunders DN (2015) Functional roles of the E3 Ubiquitin Ligase UBR5 in cancer. Mol Cancer Res 13, 1523–1532.
57 Ling S and Lin WC (2011) EDD inhibits ATM-mediated phosphorylation of p53. J Biol Chem 286, 14972–14982.
58 Smits VA (2012) EDD induces cell cycle arrest by increasing p53 levels. Cell Cycle 11, 715–720.
59 Tomaic V, Pim D, Thomas M, Massimi P, Myers MP and Banks L (2011) Regulation of the human papillomavirus type 18 E6/E6AP ubiquitin ligase complex by the HECT domain-containing protein EDD. J Virol 85, 3120–3127.
60 Ong SS, Goktug AN, Elias A, Wu J, Saunders D and Chen T (2014) Stability of the human pregnane X receptor is regulated by E3 ligase UBR5 and serine/threonine kinase DYRK2. Biochem J 459, 193–203.
61 Matsuura K, Huang NJ, Cocce K, Zhang L and Kornbluth S (2017) Downregulation of the proapoptotic protein MOAP-1 by the UBR5 ubiquitin ligase and its role in ovarian cancer resistance to cisplatin. Oncogene 36, 1698–1706.
62 Plank M, Hu G, Silva AS, Wood SH, Hesketh EE, Janssens G, Macedo A, de Magalhaes JP and Church GM (2013) An analysis and validation pipeline for large-scale RNAi-based screens. Sci Rep 3, 1076.

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Gene Ontology analysis of UBR5 potential interactors indicated enrichment for proteins involved in RNA metabolism.

Table S1. List of primers used for qPCR.

Data S1. Analysis of proteomics data from co-immunoprecipitation experiments with UBR5 and FLAG antibodies in hESCs (FDR (q-value) <0.2 was considered significant).