

Dominant-negative Pes1 mutants inhibit ribosomal RNA processing and cell proliferation via incorporation into the PeBoW-complex

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ABSTRACT

The nucleolar PeBoW-complex, consisting of Pes1, Bop1 and WDR12, is essential for cell proliferation and processing of ribosomal RNA in mammalian cells. Here we have analysed the physical and functional interactions of Pes1 deletion mutants with the PeBoW-complex. Pes1 mutants M1 and M5, with N- and C-terminal truncations, respectively, displayed a dominant-negative phenotype. Both mutants showed nucleolar localization, blocked processing of the 36S/32S precursors to mature 28S rRNA, inhibited cell proliferation, and induced high p53 levels in proliferating, but not in resting cells. Mutant M1 and M5 proteins associated with large pre-ribosomal complexes and co-immunoprecipitated Bop1 and WDR12 proteins indicating their proper incorporation into the PeBoW-complex. We conclude that the dominant-negative effect of the M1 and M5 mutants is mediated by the impaired function of the PeBoW-complex.

INTRODUCTION

Eukaryotic ribosome biogenesis is a highly regulated, evolutionary conserved process in the nucleolus. A large precursor ribosomal RNA (pre-rRNA) is transcribed by Pol I and rapidly packaged into the 90S ribonucleoprotein particle (90S pre-RNPs) containing ribosomal proteins, non-ribosomal proteins and snoRNA-containing ribonucleoprotein particles (snoRNPs). The 90S pre-RNPs are processed into intermediates, which finally give rise to mature 40S and 60S ribosomal subunits (1).

Ribosome biogenesis is the major metabolic challenge of rapidly proliferating cells, particular in tumour cells, as it consumes up to 80% of the total energy. However, little is known about the molecular mechanism that ensure the equilibrium between cell division and ribosome biogenesis required for balanced cell proliferation (1). Recently it has become evident that ribosome synthesis is cell cycle controlled and sensitive to growth factor and nutrient signalling, and likewise inhibited upon stress signals. Interestingly, several pivotal regulators of cell cycle progression and senescence, such as p19ARF reside within the nucleolus and are also involved in the control of ribosome biogenesis. Moreover, nucleolar proteins like nucleophosmin not only function in the maturation of ribosomes, but are also implicated in the control of the tumour suppressors p53 and p19ARF (2,3). Noteworthy, dysfunction of nucleophosmin is frequently associated with acute myeloid leukaemia and heterozygous mice develop myelodysplastic syndromes (4,5). In conclusion, the recent years have unravelled remarkable links between the nucleolus and cell cycle regulation, thus underlining the importance of the nucleolus far beyond the production of ribosomes.

The nucleolus owns a particular ability in sensitizing cellular stress after ultraviolet (UV) radiation of cells. Using micropore irradiation, Rubbi and Milner (6) demonstrated that large amounts of nuclear DNA damage failed to stabilize p53 unless the nucleolus was affected. In addition, forcing nucleolar disruption by anti-upstream binding factor (UBF) antibody microinjection (in the absence of DNA damage), different chemotherapeutic drugs, or cre-mediated deletion of the Pol I specific transcription factor TIF-IA also caused p53 stabilization and a p53-dependent cell cycle arrest. This suggests that the nucleolus is a stress sensor responsible for the maintenance of low levels of p53, which are automatically elevated as soon as nucleolar function is impaired in response to stress (6,7).

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How does nucleolar stress result in the activation of cell cycle check points? Several mechanisms have been proposed that link ribosome biogenesis to the cell cycle machinery in mammalian cells. A central player in all models is Mdm2, a p53-specific ubiquitin ligase. Disturbance of ribosome biogenesis may decrease the demand for ribosomal proteins and thus lead to an excess of free ribosomal proteins as L5, L11 and L23, which directly bind and inactivate Mdm2 resulting in the accumulation of p53 (8–12). Alternatively, export of ribosomal subunits to the cytoplasm may be a critical step for p53 degradation, which does not take place, if rRNA processing is inhibited (13). However, other models cannot be excluded.

We have recently described the nucleolar complex PeBoW, consisting of Pes1 (Pescadillo), Bop1 (block of proliferation) and WDR12 (WD-repeat protein) in mammalian cells. Knockdown of WDR12 by siRNA technology or expression of a dominant-negative WDR12 mutant blocked processing of the 32S pre-rRNA, evoked stabilization of p53 and induced a strong cell cycle arrest (14). Likewise, expression of dominant-negative mutants of other members of the complex, Pes1 and Bop1, inhibit rRNA processing and cell cycle progression (15,16).

The structure and function of the PeBoW-complex appears to be highly conserved throughout evolution. A homolog complex consisting of Nop7 (Yph1p), Erb1 and Ytm1p has been identified in yeast. As in mammals, mutants of Nop7 and Ytm1 inhibit rRNA processing and cell cycle progression (17). Mutations in Ytm1 disrupt interactions between Ytm1 and Erb1, destabilize the heterotrimer, and significantly reduce association of all three proteins with 66S pre-ribosomes (18).

Even though a function of the PeBoW-complex in processing of the 32S rRNA precursor is established, the structure of the complex and the role of its components Pes1, Bop1 and WDR12 in other cellular processes is largely unresolved e.g. mouse embryos lacking Pes1 arrest at morula stages of development, their nucleoli fail to differentiate and accumulation of ribosomes is inhibited, suggesting an essential role of Pes1 for ribosome biogenesis and nucleologenesis (19). Overexpression of Pes1 can replace the SV40 T antigen in inducing colony formation in soft agar growth but not in inducing cell immortalization (20). Transient depletion of Pes1 resulted in an increase of abnormal mitoses with appearance of binucleate or hyperploid cells, of cells with multipolar spindles and of aberrant metaphase plates (21). Thus, Pes1 appears to be involved in multiple cellular processes, yet it is unclear, whether all these processes require the PeBoW-complex.

Recently, the interaction of Pes1 transposon insertion mutants with Bop1 protein has been studied. Several of the mutants displayed a dominant-negative phenotype for rRNA processing. Interestingly, the dominant-negative phenotype required the interaction of the mutant Pes1 with Bop1, while Pes1 mutants, which did not interact with Bop1, failed to induce a dominant-negative phenotype (15), suggesting that Pes1 mutants might act only in the context of the PeBoW-complex.

Here we report the generation of a set of Pes1 deletion mutants and their interaction with the PeBoW-complex. Two mutants with a N- and C-terminal deletion displayed a

dominant-negative effect on rRNA processing and cell growth. Both mutants were incorporated into the PeBoW-complex and induced accumulation of p53. Our data suggests that essential cellular functions of Pes1 are mediated by its incorporation into the PeBoW-complex.

MATERIALS AND METHODS

Cloning/plasmids

Pes1 was cloned from human cDNA using the following primers: HsPescadillo fwd: 5'-GCCACCATGGGAGGCCTT-GAGAAGAAG-3'; HsPescadillo bwd: 5'-CTCCGGCCTTG-CCTTCTTGGCCTTC-3'. Cloning into the modified pUC18 vector resulted in the addition of a C-terminal HA-tag to the Pes1 open reading frame. The mutant Pes1-HA alleles were created by standard techniques of molecular biology using restriction enzymes and site directed mutagenesis. Pes1-HA wild-type and mutants were cloned into the vector pRTS-1 using the SfiI restriction site (22).

Tissue culture

TGR-1 rat fibroblasts (provided by J. Sedivy, Brown University, Providence, RI) and U2OS osteosarcoma cells were cultured in DMEM with 10% FBS at 8% CO₂. For generation of polyclonal cell lines, 6 × 10⁵ cells were transfected with 7.5 µg pRTS-1 plasmids using Polyfect (QIAGEN) and selected in the presence of 200 µg/ml hygromycin B for 10 to 14 days. Conditional gene expression was induced with 1 µg/ml doxycycline. The percentage of induced cells was monitored by FACS analysis for EGFP expression.

BrdU light assay

BrdU light assays were performed essentially as described previously (23). Briefly, stable polyclonal TGR-1 cells were seeded in the presence of 1 µg/ml doxycycline at a density of 10⁵ cells per 100 mm well. After 30 h seeding, the cells were incubated with 100 µM BrdU and doxycycline for 72 h. Culture medium was then removed and replaced by medium containing doxycycline and Hoechst 33258 at 2 µg/ml for 3 h. Finally, cells were placed on sheet of glass 11 cm above a 30 W fluorescent daylight bulb and irradiated from beneath for 15 min. Cells were washed in phosphate-buffered saline (PBS) two times and regular culture medium without doxycycline was added.

RNA analysis and ³²P *in vivo* labelling

Total RNA was isolated from TGR-1 cells using Trifast (Peq-Lab). A total of 2 µg of RNA were separated on a 1% agarose-formaldehyde gel and blotted on Hybond N⁺ membranes (GE Healthcare). The following ³²P-labelled oligonucleotides were used to visualize rRNA precursors: ITS-1, 5'-CCGGAGAGATCACGTACCACCCCGGTGCACACGAGATCACGGAGCCG-3'; ITS-2, 5'-GGAGCGGTCCGGCCCGGTAGAGGGAGCGGGGGAGGAGAGGGACGCGAG-3'; 18S, 5'-CACCCGTGGTCACCATGGTAGGCACGGCGACTACCATCGAAAGTTGATAG-3'.

For metabolic labelling of rRNA, TGR-1 cells were induced with doxycycline for 24 h, followed by pre-incubation in phosphate-free DMEM (Gibco) with dialyzed

FBS (Gibco) for 30 min. The medium was then replaced by phosphate-free DMEM/10% dialyzed FBS containing 15 $\mu\text{Ci/ml}$ ^{32}P -orthophosphate (Amersham). After 60 min the radioactive medium was removed and cells were incubated in regular DMEM/10% FBS for indicated times. A total of 2 μg of RNA were separated on 1% agarose-formaldehyde gels. After electrophoresis, gels were placed on whatman-paper and dried at 80°C under vacuum suction. Dried agarose gels were exposed to regular X-ray films (Kodak) and rRNA was visualized by autoradiography. A PhosphoImager (Fuji) was used for the quantification of signal intensities.

Sucrose gradients

Sucrose solutions were prepared in 50 mM Tris-HCl (pH 8.0), 20 mM NaCl. Sucrose gradients from 50–5% were prepared on top of a 300 μl CsCl (1.4 g/ml) cushion. A total of 1×10^7 cells, without pre-treatment with cycloheximide, were lysed in 1 ml lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.1% NP-40, protease inhibitors] at 4°C for 5 min. A total of 20 μg of glass beads were added each and total cell lysates were sonicated 10 times for 1 s. A total of 100 μl of total cell lysate were loaded on top of the sucrose gradients. Subsequently, sucrose gradients were centrifuged at 30 000 r.p.m. for 2.5 h using a Ti60W rotor. Fractions of 200 μl each were collected. A total of 20 μl of each fraction were used for SDS-PAGE electrophoresis.

siRNA transfection

The day before transfection, 5×10^4 to 10^5 U2OS cells were seeded in 6-well plates. A total of 5 μl of 20 μM control or Pes1-specific siRNA were diluted in 150 μl Optimem (Invitrogen). A total of 150 μl Optimem containing 5 μl Oligofectamine (Invitrogen) was added and incubated for 15 min. Finally, 600 μl Optimem was added and applied to cells after aspiration of the culture medium. Cells were incubated for 6 h. The following sequences (sense) were used: Pes1, CCA-GAGGACCUAAGUGUGAdTdT; Control (nonspecific siRNA), UUCUCCGAACGUGUCACGUDtT.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2 \times SDS-loading buffer (100 mM Tris-HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromophenol blue, 20% glycerol). Cell lysates were separated by SDS-PAGE and blotted on nitrocellulose membranes (GE Healthcare). Equal loading of samples was controlled by Ponceau S staining. Immunodetection was performed with anti-HA (3F10; Roche), anti-WDR12 (1B8), anti-Bop1 (6H11), anti-Pes1 (8E9) [Holzel *et al.* (14)], and anti-p53 (Pab-240; Dianova) antibodies.

For immunofluorescence, cells were grown on cover slides, fixed with ice cold methanol or methanol-aceton 1:1 and air dried. Unspecific binding was blocked with PBS/10% FBS. p53 and HA-tagged Pes1 were detected with a 1:100 dilution of anti-p53 antibody (Pab122; Dianova) and a 1:1000 dilution of 3F10 anti-HA antibody, respectively. Primary antibodies were incubated overnight at 4°C in a humidified chamber. Cy3 labelled secondary antibodies (Dianova) were incubated for 1 h at RT. Nuclei were counterstained with DAPI (Sigma-Aldrich). Digital images were acquired using the Openlab

acquisition software (Improvision) and a microscope (model Axiovert 200 M; Carl Zeiss MicroImaging, Inc.) with 63 (1.15) and 100 (1.30) plan oil objectives connected to a five charge-coupled device camera (model ORCA-479; Hamamatsu).

Immunoprecipitation

For immunoprecipitations, U2OS cells were seeded at sub-confluent density and treated with 1 $\mu\text{g/ml}$ doxycycline for 20 h. Cells were harvested by trypsination and washing three times with PBS. For lysis, cells were resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, protease inhibitors, phosphatase inhibitors] and incubated on ice for 20 min. After centrifugation, protein G beads (incubated with antibodies for at least 1 h, washed twice after incubation) were incubated with the lysate over night at 4°C. The beads were washed three times with lysis buffer (lacking phosphatase- and protease inhibitor) and resuspended in SDS-loading buffer/lysis buffer 1:1 with the volume equal to the volume of cell lysate used for the IP.

RESULTS

Cloning and conditional expression of human Pes1 mutants

The Pes1 protein is highly conserved in eukaryotes and several domains and motifs have been predicted based on sequence analysis (Figure 1A). Pes1 contains an evolutionary highly conserved N-terminal pescadillo-like protein domain (NPLP-domain, Pfam-database PF06732) at the N-terminus, a BRCT domain located in the middle of the protein, three classical nuclear localization sequences (NLS) distributed over the protein, six bipartite NLS at the C-terminus and two stretches of acidic amino acids near the C-terminus (24). Pes1 is modified by SUMOylation, but SUMOylation at the consensus site, ψKXE , located at the C-terminus has yet not been experimentally confirmed (25). Human and mouse Pes1 proteins are 89% identical (Figure 1A).

Systematic deletions are an appropriate tool for the investigation of domains involved in subcellular localization and interactions as well as the generation of dominant-negative mutants. A panel of *pes1* deletion mutants covering the entire open reading frame and a point mutation of the lysine in the consensus SUMOylation site were constructed and tagged with the hemagglutinin (HA) epitope at the C-terminus (Figure 1A). For the conditional expression in mammalian cells, wild-type (wt) and mutant *pes1* alleles were cloned into the pRTS-1 vector (Figure 1B) (14,22).

The expression of the gene of interest is tightly controlled by a doxycycline-regulable trans-activator and trans-repressor. Thus, this tight control prevents any selection against vectors carrying dominant-negative *pes1* alleles during normal tissue culture due to leakiness of the promoter. Moreover, the pRTS-1 vector allows for high expression levels of the gene of interest. In stably transfected polyclonal cell cultures, conditional gene activation is achieved in >95% of cells (Figure 1C). As the bidirectional promoter of the pRTS-1 vector accomplishes simultaneous expression of EGFP in addition to the gene of interest, conditional gene

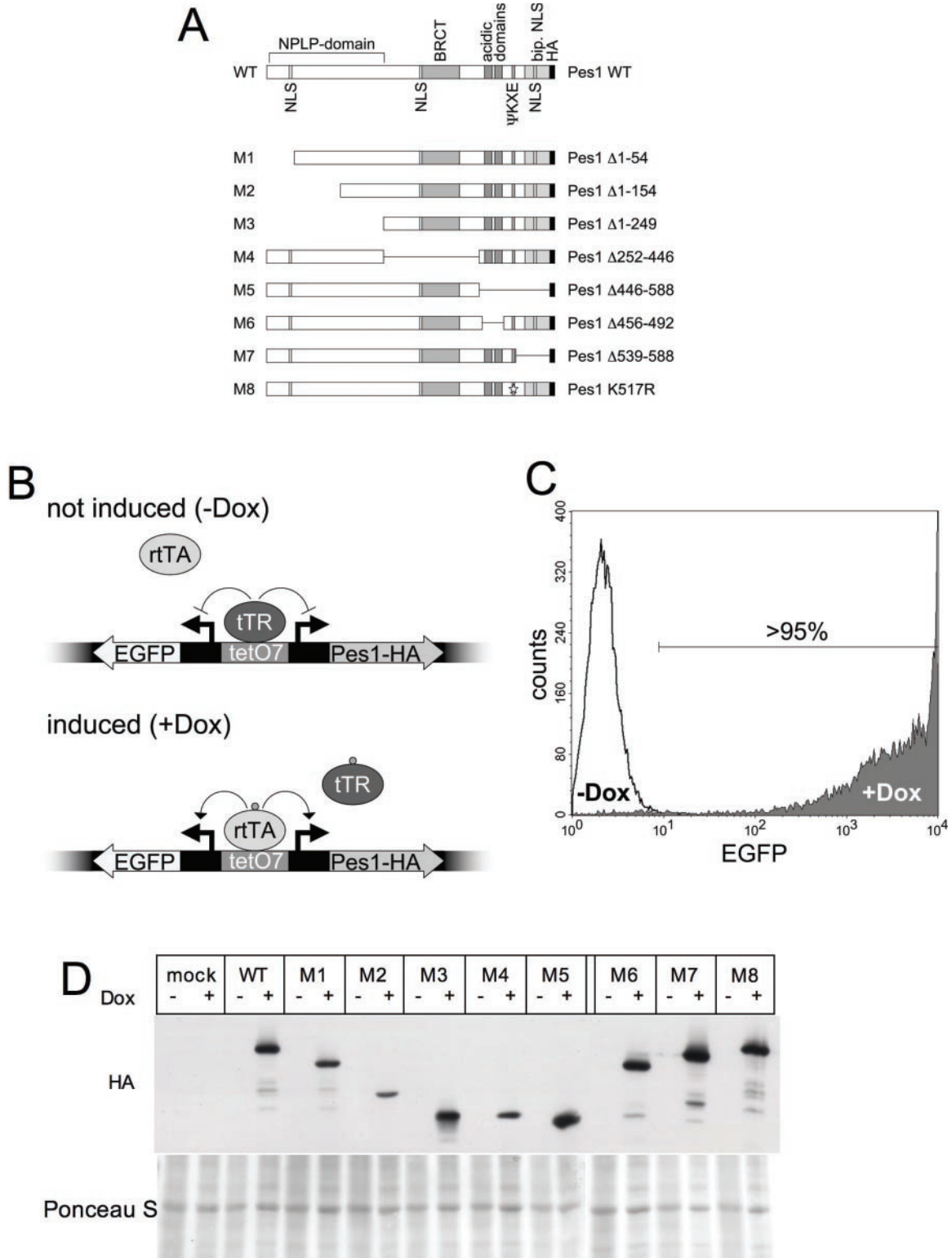


Figure 1. Pes1 mutagenesis and conditional Pes1 expression. (A) A panel of Pes1 deletion mutants (M1–M8) were constructed. Recombinant Pes1 wt and mutant proteins carry a C-terminal HA-tag. NLS: classical nuclear localization sequence; the N-terminal NLS consists of three overlapping NLS; bip. NLS: six overlapping bipartite NLS; BRCT: BRCT domain; acidic domains: two glutamic acid rich regions; ΨKXE: consensus SUMOylation site; HA: hemagglutinin-tag. Mutant M8 has replaced Lys517 by Arg. (B) Pes1 and its mutants were expressed in rat fibroblasts (TGR-1) stably transfected with the inducible EBV-based vector pRTS-1. pRTS features a bidirectional promoter expressing simultaneously the gene of interest and EGFP. After induction by doxycycline, the vector switches from active silencing to transactivation. (C) EGFP expression was induced in >95% of cells 24 h after addition of doxycycline, as determined by FACS analysis. (D) Western blot analysis of wt or mutant Pes1 proteins in TGR-1 fibroblasts by anti-HA antibodies (3F10). The mock cell line expresses luciferase instead of Pes1-HA. The indicated cell lines were treated with 1 μg/ml doxycycline for 30 h (+) or left untreated (–). Bottom panel shows Ponceau S staining as a loading control.

activation can be easily monitored by FACS analysis or fluorescence microscopy (Figure 1B and C). Pes1 wt and mutants M1–M8 were conditionally expressed in the rat fibroblast cell line TGR-1. Expression levels were determined 30 h after addition of doxycycline by western blot analysis. Most of the mutant forms were detected at the expected size, however deletion of the acidic regions in M5 and M6 significantly enhanced migration (Figure 1D).

Subcellular localization of Pes1 mutants

Pes1 protein has been reported to localize predominantly to the nucleolus. To determine the subcellular localization of the Pes1 mutants, we performed indirect immunofluorescence using HA-tag specific antibodies. As expected, the recombinant Pes1 wt protein showed predominantly nucleolar

localization and also some nucleoplasmic staining in cells with high expression of Pes1 (Figure 2, wt). The deletion of 54 N-terminal amino acids in mutant M1 did not affect the nucleolar localization whereas the extension of this deletion to amino acid 154 (M2) or 249 (M3) results in a diffuse nuclear staining. Deletion of the central part of Pes1 containing the BRCT domain and adjacent uncharacterized regions in mutant M4 leads to a diffuse nucleoplasmic staining but with nucleolar staining in very few cells (Figure 2, data not shown). The C-terminal deletion mutants M5 to M7, together with mutant M8 harbouring a mutated consensus SUMOylation site, located to the nucleolus as Pes1. Therefore, our mutational analysis suggests that the region between 55 and 154 amino acid plays an important role for the nucleolar localization of the Pes1 protein. A contribution of the region extending from amino acid 155 to 249 remains elusive for

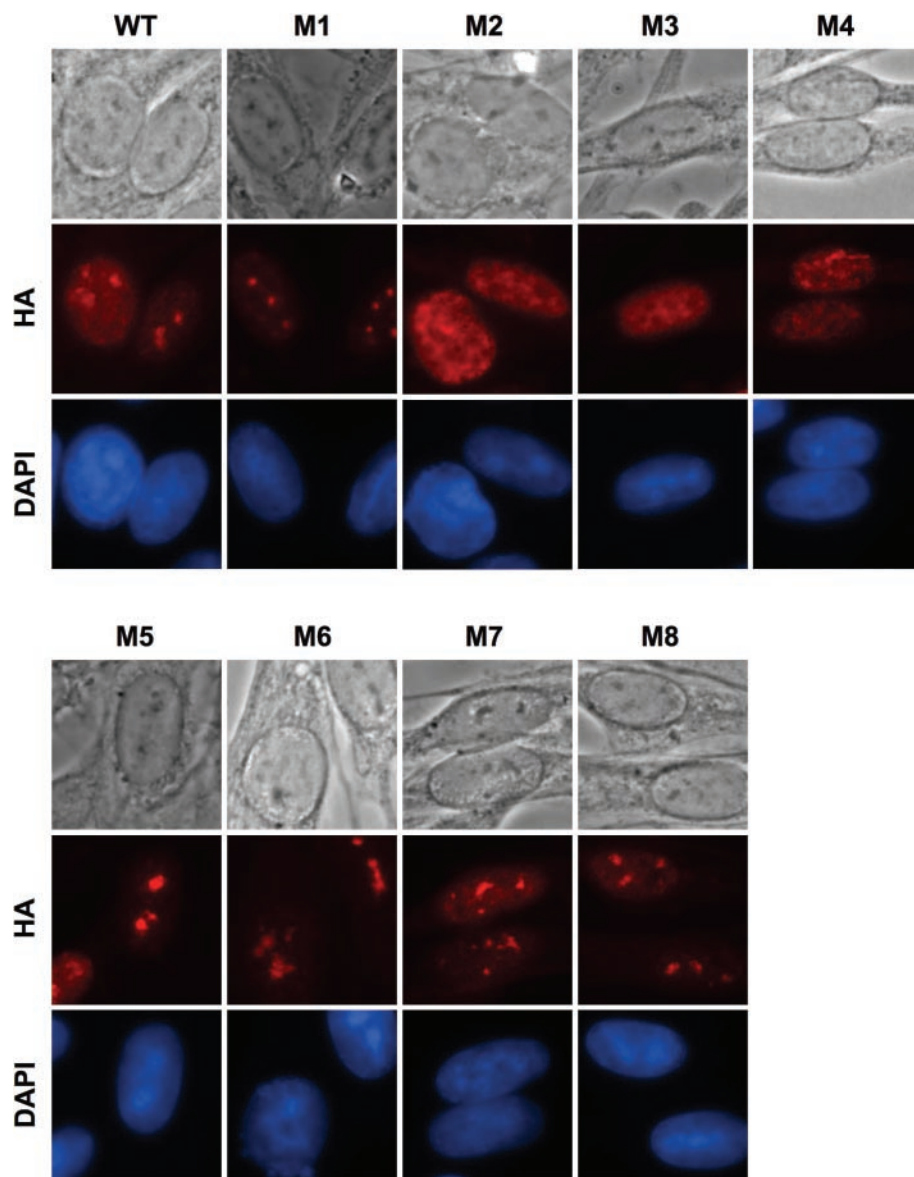


Figure 2. Cellular localization of wt and mutant Pes1 proteins by indirect immunofluorescence. TGR-1 cells transfected with the indicated constructs were fixed with methanol/acetone after induction of the Pes1-HA alleles with doxycycline for 30 h. The HA-tagged proteins were stained by anti-HA antibodies (3F10), the nuclei were counterstained with DAPI.

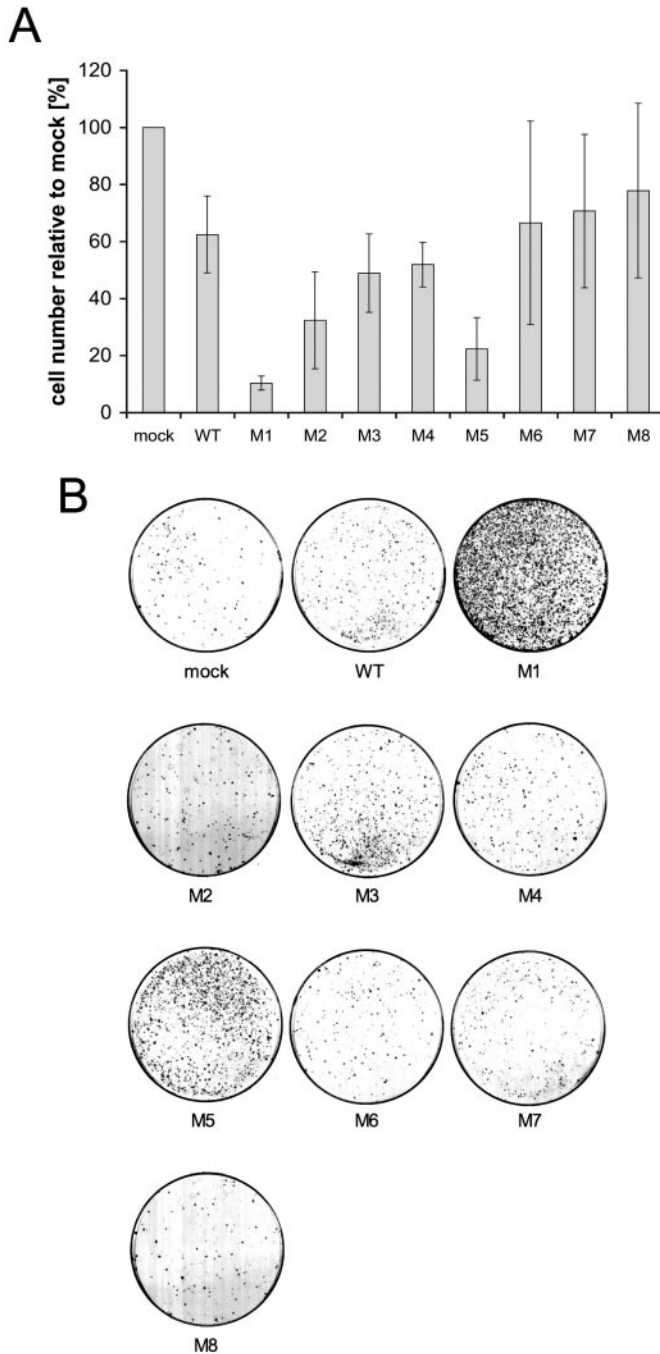


Figure 3. Pes1 mutants M1 and M5 inhibit cell proliferation and elicit a reversible cell cycle arrest. (A) Equal numbers of TGR-1 cells stably transfected with the indicated constructs were seeded in multiples in the presence of 1 μ g/ml doxycycline. Cells were trypsinized and counted by trypan blue exclusion after 6d. The histogram depicts the cell counts relative to the mock cell line after 6d. Error bars indicate SD. (B) Reversible cell cycle arrest by overexpression of mutants M1 and M5. Stably transfected TGR-1 cell lines were seeded at low density and treated with 1 μ g/ml doxycycline for 30 h to induce expression of the specified constructs. Subsequently, the cells were incubated with 100 μ M BrdU for 72 h to label proliferating cells. Visible light irradiation in the presence of Hoechst 33 258 selectively kills cells that have incorporated BrdU in their DNA. Arrested cells survive BrdU light treatment and give rise to colonies after withdrawal of doxycycline. Images show representative BrdU light assays conducted with the indicated cell lines.

nucleolar localization in this study. Noteworthy, none of the mutants showed cytoplasmic staining suggesting that neither of the deletions affected the nuclear transport of Pes1. Since all mutants retained at least two putative NLS, the functionality of a single NLS could not be addressed by this set of mutants.

N-terminal (M1) and C-terminal (M5) truncation mutants potently inhibit proliferation and trigger a reversible cell cycle arrest

Next, we tested the effect of Pes1 deletion mutants on cell proliferation. Equal numbers of cells were seeded and expression of wt Pes1, mutants M1 to M8 and luciferase (mock) was induced by addition of doxycycline. Cell numbers were determined after 6d (Figure 3A). Overexpression of Pes1 wt reduced the cell count to 62% compared to mock cells. A likewise decrease was observed for the mutants M6, M7 and M8, and to a higher extent for the mutants M2, M3 and M4. Expression of the mutants M1 and M5 resulted in the strongest reduction, 10 and 22% of mock cell count, respectively. FACS analysis revealed a significant increase of cells in the G_0/G_1 phase of the cell cycle 48 h after expression of mutants M1 and M5 (Table 1, Supplementary Figure S1). Hence, the mutants M1 and M5 suppressed cell proliferation in rat fibroblasts most efficiently.

Further, we studied the ability of mutants M1 and M5 to mediate a reversible cell cycle arrest in a BrdU/light assay. In brief, dividing cells that incorporate BrdU into their DNA become highly photosensitive if they are additionally labelled with the Hoechst dye 33 258 and undergo apoptosis upon irradiation with visible light. Cell cycle arrested cells are protected from increased photosensitivity and survive BrdU/light treatment. Thus, cells that were arrested by conditional expression of an anti-proliferative Pes1 mutant, would subsequently give rise to colonies after withdrawal of doxycycline. Mock cells expressing luciferase showed only little colony outgrowth and thus demonstrating the low background of this assay. Expression of Pes1 wt did not increase the number of colonies in comparison to the mock situation. The Pes1 mutant M1 provoked a pronounced rescue effect and subsequent colony outgrowth, consistent with a strong reversible cell cycle inhibition. The effect of mutant M5 was less intense but still significant. Mutant M3 showed a colony number slightly over the background level, while all other Pes1 mutants remained at the background level. The reduced proliferation rates of Pes1 wt (62% in the proliferation assay) and mutants M2–M4 and M6–M8 is not accompanied by an increased apoptosis rate (data not shown) or altered cell cycle distribution (Table 1), thus the reason for the reduced cell count remains currently unclear. Taken together, the mutants M1 and M5 mediated a potent inhibitory effect on cell proliferation and triggered a reversible G_0/G_1 -specific cell cycle arrest (Table 1).

The Pes1 mutants M1 and M5 inhibit pre-rRNA processing

Pes1 is involved in ribosome biogenesis and therefore we tested the ability of mutants M1 and M5 to inhibit maturation of ribosomal RNA. A short overview of the major mammalian rRNA processing pathway is depicted in Figure 4A.

Table 1. Cell cycle distribution of Pes1 mutants

Cell line	G ₀ /G ₁ %	S %	G ₂ %
Mock	46.6	23.1	30.4
WT	47.7	24.6	27.7
M1	62.3	17.8	19.9
M2	43.7	26.5	29.9
M3	53.5	18.2	28.3
M4	47.6	21.7	30.7
M5	59.1	18.9	21.9
M6	48.6	24.8	26.6
M7	49.5	22.6	27.9
M8	46.7	24.8	28.5

Stably transfected TGR-1 cells were induced at subconfluent density for 48 h with doxycycline and subjected to FACS analysis. The percentage of cells in different cell cycle phases are indicated.

Pes1 wt and mutant forms were expressed in TGR-1 cells for 30 h. Total RNA was isolated, and analysed by northern analysis with hybridization probes specific for the internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) of the ribosomal pre-rRNA (Figure 4A). Expression of M1 and M5-induced a significant accumulation of the 36S precursor as visualized by hybridization with the ITS-1 specific probes (Supplementary Figure S2). Inhibition of pre-rRNA processing becomes even more pronounced, if rRNAs were hybridized with the ITS-2 specific probe. Mutants M1 and M5 lead to a strong increase of the amount of 32S rRNA precursor (Figure 4B, upper arrows). While both mutants, M1 and M5, induced accumulation of the 36S/32S rRNA, their effect on processing of the 12S rRNA intermediate differed. The level of 12S pre-rRNA was reduced for the mutant M1, but appeared unaffected for the mutant M5 (Figure 4B, lower arrows). This may indicate separable and common functions of the mutants M1 and M5 in rRNA processing.

In addition we studied the impact of mutants M1 and M5 on rRNA processing in TGR-1 cells by metabolic ³²P-labelling (Figure 4C). The production of mature 28S rRNA was severely reduced resulting from an inefficient processing of the 32S rRNA precursor, as concluded from the relative high abundance of metabolically labelled 32S rRNA (Figure 4D). In contrast, synthesis of mature 18S rRNA was almost unaffected. These results are in line with our northern blot analysis. In conclusion, the deletion of the N-terminus or the C-terminus of Pes1 either compromises ribosome biogenesis by blocking rRNA processing at the level of the 32S rRNA precursor.

Endogenous Pes1 is required for rRNA processing and cell proliferation

To confirm the role of Pes1 in ribosome biogenesis and cell proliferation, we performed small interfering RNA (siRNA) knockdown experiments of endogenous Pes1. We used human osteosarcoma U2OS cells, because we had generated a monoclonal antibody directed against human Pes1 (14). Cells were transfected at day 0, 1 and 2 with either control or Pes1-specific siRNA. Expression of endogenous Pes1 was dramatically reduced, as monitored by western blot analysis 2d after the last transfection (Figure 5A). Moreover, proliferation of Pes1-depleted cells was severely impaired (data not shown). Further, we investigated the impact of Pes1

knockdown on ribosome biogenesis after *in vivo* labelling of rRNA. The production of the mature 28S rRNA was strongly compromised (Figure 5B). Notably, synthesis of the large 45/47S precursor was not affected, indicating that Pes1 is not involved in rRNA transcription. In conclusion, expression of dominant-negative Pes1 and depletion of endogenous Pes1 block rRNA processing of the 32S rRNA precursor.

Mutants M1 and M5 induce p53 accumulation in proliferating, but not in starving cells

As mentioned before, p53 is believed to induce cell cycle arrest following nucleolar stress. This raised the question if the observed impact of mutants M1 and M5 on pre-rRNA processing triggers the accumulation of p53. To address this question, we analysed p53 levels in TGR-1 cells 30 h after expression of wt and mutant Pes1 proteins by western blotting (Figure 6A). Pes1 wt and mock cells did not accumulate p53 (Figure 6A, lanes 1–4). Expression of mutant M1 provoked a strong increase of p53 protein (lanes 5 and 6). A less pronounced accumulation was seen for the mutants M3 and M5 (lanes 9, 10 and 13, 14), whereas all other mutants caused no change in p53 levels. If disturbance in ribosome biogenesis is the reason for p53 accumulation, then the accumulation of p53 should be diminished in cells in the absence of active ribosome biogenesis, such as serum-starved cells. To test this assumption, subconfluent TGR-1 cells were cultured with 0.1% FBS for 72 h prior to the addition of doxycycline. The cells were lysed 30 h later. Serum starvation did not affect the induction rate of recombinant proteins (Figure 6B, lower panel, see also Figure 1D), however, neither of the Pes1 mutants was able to trigger the accumulation of p53 (Figure 6B, upper panel).

To further investigate the p53 accumulation induced by the mutant Pes1 proteins on the single cell level, we analysed p53 accumulation by immunofluorescence (Figure 6C). In proliferating cells expressing Pes1 wt, 8.9% of nuclei stained positive for p53. Expression of Pes1 mutants M1 and M5 increased the number of positive cells to 82.0 and 53.4%, respectively (Figure 6D). Serum starvation reduced the number of p53-positive cells for Pes1 wt to 0.71%, and for the mutants M1 and M5 to 8.7 and 3.3%, respectively. Thus neither of the mutants were able to induce p53 significantly in serum-starved cells supporting the notion, that ribosome biogenesis is a prerequisite for the dominant-negative action of Pes1 mutants M1 and M5.

As previously demonstrated, N-terminally deleted WDR12 elicited a p53-dependent cell cycle arrest that was attenuated by the coexpression of the human papillomavirus protein E6 (14), a ubiquitin ligase targeting p53 for degradation. In line with these studies, mutants M1 and M5-induced cell cycle arrest was alleviated in TGR-1 cells stably transfected with HA-tagged E6 (Supplementary Figure S3).

Mutants M1 and M5 associate with the large pre-ribosomal subunit

The association of Pes1 mutants with pre-ribosomal complexes was studied by sucrose gradient fractionation using TGR-1 total cell lysates (Figure 7). All recombinant proteins accumulated in low molecular fractions, most likely due to an excess of overexpressed free protein in total cell lysates. However, Pes1,

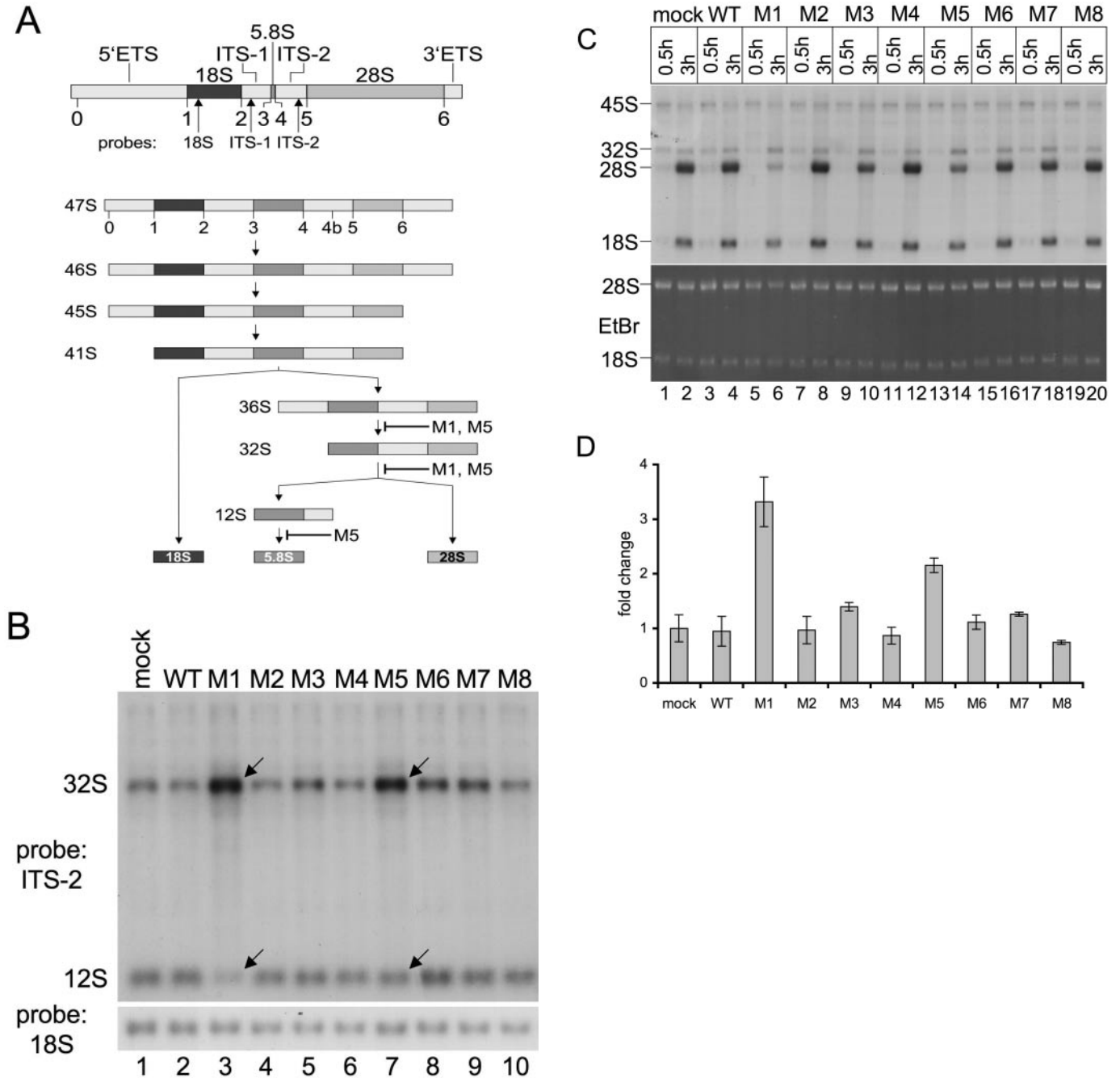


Figure 4. Dominant-negative Pes1 mutants inhibit pre-rRNA processing. (A) Schematic of eukaryotic pre-rRNA processing. The top diagram shows the primary 47S transcript at the correct scale and the position of the probes used in the northern blots. The lower diagram shows the processing of the pre-rRNA schematically. The effects of M1 and M5 overexpression are indicated. (B) Northern blot for pre-rRNA probed with ITS-2. Total RNA was extracted from subconfluent TGR-1 cells transfected with the indicated constructs after 30 h of induction. Hybridization with a probe against the 18S rRNA confirms equal loading of the blot. (C) Expression of dominant-negative Pes1 mutants impairs formation of mature 28S rRNA. Asynchronously growing TGR-1 cells were treated with doxycycline for 24 h. Cells were pulse labelled with ³²P-orthophosphate for 1 h and chased in regular medium for 0.5 and 3 h. (D) Ratio of 32S/28S rRNA at 3 h after metabolic labelling with ³²P-orthophosphate as described in (C).

and mutants M1 and M5, but not the mutant M3, exhibited specific enrichment in high molecular weight fractions that co-fractionated with ribosomal particles. Given the fact that M1 and M5 impair rRNA processing at the level of the 32S pre-rRNA and co-sediment in fractions similar to the 60S peak (see 28S RNA), our data suggests that mutants M1 and M5 accumulate with the 66S pre-ribosomal large subunit.

Incorporation of mutant Pes1 proteins into the PeBoW-complex

Previously we showed that Pes1 forms a stable complex with two other nucleolar proteins, Bop1 and WDR12, respectively (PeBoW-complex). Interestingly, several of the deletion mutants generated in this study localized to the nucleolus

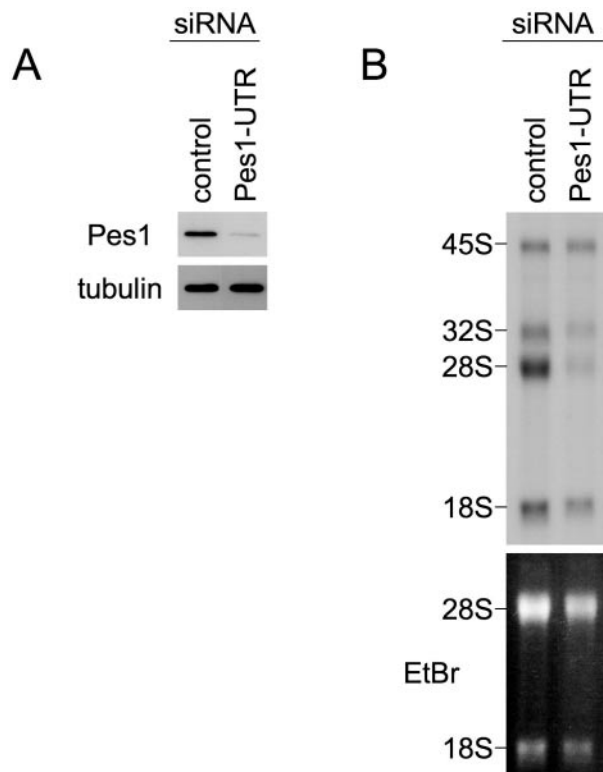


Figure 5. Endogenous Pes1 is required for rRNA processing. (A) U2OS cells were transfected at day 0, 1 and 2 with either control or Pes1-specific siRNA. Endogenous Pes1 levels were analysed by western blot analysis 2d after the last transfection. Tubulin is shown as a loading control. (B) U2OS cells were transfected only twice at day 0 and 1 and metabolically labelled with ^{32}P -orthophosphate for 60 min at day 3. Subsequently, cells were incubated for 3 h in regular culture medium. Labelled rRNAs are indicated.

with or without a dominant-negative phenotype. Thus we asked, if these mutants are nucleolar upon incorporation into the PeBoW-complex or if nucleolar localization is a feature of Pes1 independent of PeBoW-association. Further, it was unclear if the Pes1 mutants interact with Bop1 or WDR12 in conjunction or independently of each other. Moreover, analysing the incorporation of the dominant-negative mutant proteins M1 and M5 into the PeBoW-complex would help to unravel the mechanism of their inhibitory function. To address these issues, we performed a series of vice-versa immunoprecipitation experiments. As our antibodies against the PeBoW components WDR12 and Bop1 are human specific, we performed the experiments in the human osteosarcoma cell line U2OS. This cell line also allowed proper conditional expression of Pes1 mutants by the pRTS-1 vector.

U2OS cells were plated at a subconfluent density and the Pes1 constructs were induced for 20 h with doxycycline. The immunoprecipitation experiments were carried out with antibodies against the HA-tag, WDR12 and Bop1, as well as an isotype control. Western blot analysis was then performed with a HA-tag specific monoclonal antibody (3F10) to detect the recombinant Pes1 protein and monoclonal antibodies against WDR12 and Bop1 to detect the association of the recombinant Pes1 protein with endogenous PeBoW members. In the mock cell line, the lack of the HA-tag, WDR12 and Bop1 specific signals in the IP against the HA-tag

revealed no unspecific reactivity of the antibodies (Figure 8A). The endogenous PeBoW-complex was precipitated in IPs against WDR12 and Bop1, as expected (Figure 8A, lanes 3 and 4). HA-tagged Pes1 wt protein co-immunoprecipitated high amounts of WDR12 and Bop1 indicating a proper incorporation into the PeBoW-complex (Figure 8B, lane 2). The IPs against WDR12 and Bop1 could co-precipitate only a small fraction of HA-tagged Pes1 protein (Figure 8B, lanes 3 and 4). This might be due to the fact that 20 h after addition of doxycycline not all PeBoW-complexes have incorporated the HA-tagged Pes1 protein. Hence, the co-immunoprecipitation of WDR12 and Bop1 with the anti-HA antibody is the most reliable readout for the incorporation of HA-tagged Pes1 proteins in the PeBoW-complex.

The dominant-negative mutant M1 co-precipitated WDR12 and Bop1 proteins (Figure 8C, lane 2). Mutant M1 was also detectable in IPs against WDR12 and Bop1. This demonstrates that the Pes1 mutant M1 is efficiently incorporated into the PeBoW-complex (Figure 8C, lane 3 and 4). The mutant proteins M2 and M3 are not incorporated into PeBoW. Both proteins did not co-precipitate WDR12 and Bop1 (Figure 8D and E, lane 2) and could not be precipitated in IPs against WDR12 and Bop1 (Figure 8D and E, lane 3 and 4). This finding is compatible with a previous report, that the region in Pes1 needed for nucleolar localization is also needed for binding of Bop1 (15). Pes1 mutant M4 is also not incorporated into the PeBoW-complex according to the failure to co-precipitate significant amounts of WDR12 and Bop1 (Figure 8F, lane 2). The dominant-negative mutant M5, similar to Pes1 wt and mutant M1 protein, co-precipitates WDR12 and Bop1 (Figure 8G, lane 2) and is co-precipitated by WDR12 and Bop1 (Figure 8G, lane 3 and 4). Mutants M6, M7 and M8 are all incorporated into the PeBoW-complex, as they co-precipitate WDR12 and Bop1 (Figure 8H–J, lane 2) and in reciprocal IPs (Figure 8H–J, lanes 3 and 4). Taken together, our immunoprecipitation studies revealed that all mutants are efficiently incorporated in the PeBoW-complex except the mutants M2, M3 and M4. Notably, the mutants M2, M3 and M4 are the only mutants showing no proper nucleolar localization, suggesting that the nucleolar localization of Pes1 is linked to the PeBoW-association. Interestingly, we observed no independent interaction of Pes1 mutants with either Bop1 or WDR12. Further, our results propose that dominant-negative forms of Pes1 require incorporation into the PeBoW-complex for their negative effect on ribosomal rRNA processing and cell cycle progression.

DISCUSSION

In mammals, Pes1, Bop1 and WDR12 proteins are components of the evolutionary highly conserved PeBoW-complex, which is required for maturation of the 60S ribosomal subunit. Although PeBoW is highly abundant in the nucleolus, the way and the place of its assembly is unclear. A homologous complex in yeast, composed of Nop7, Erb1 and Ytm1, associates with four consecutive 66S pre-ribosomal subunits containing the 27SA2, 27SA3, 27SB and the 25.5S plus 7S pre-rRNAs (18,26). Similar as in mammals, the yeast complex is required for proper processing of the 27S pre-rRNA to the mature

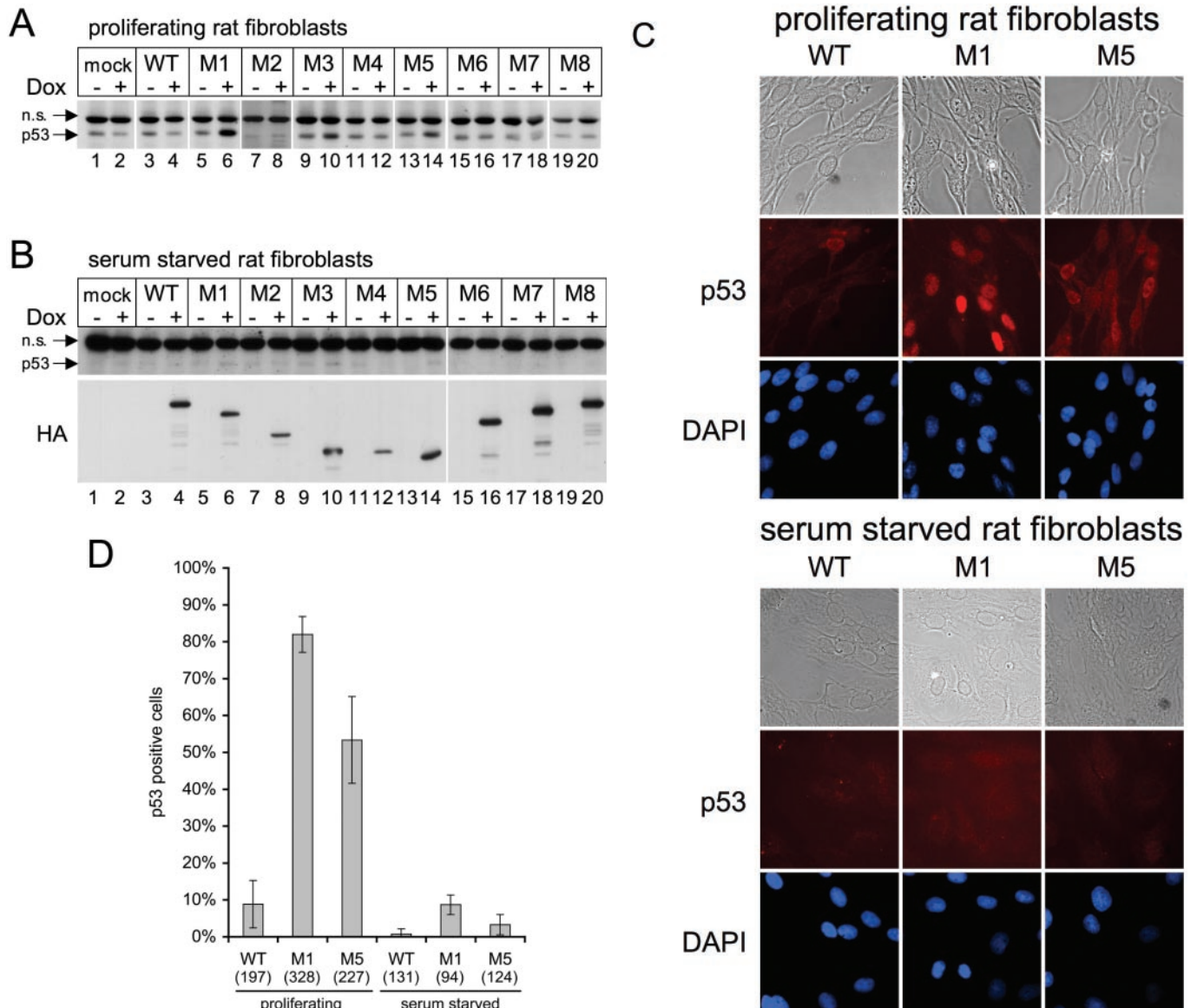


Figure 6. Dominant-negative Pes1 mutants induce p53 accumulation in proliferating cells but not in quiescent cells. (A) Western blot for endogenous p53 levels. Stably transfected TGR-1 cells were induced at subconfluent density for 30 h with doxycycline. Cell lysates were prepared and analysed by immunoblotting for p53 accumulation using anti-p53 antibodies (Pab-240). n.s.: non specific. (B) Upper panel: western blot as described in (A), but the cells had been serum-starved for 72 h before induction. Lower panel: immunoblot against the HA-tag to confirm the expression of recombinant wt and mutant Pes1. (C) Analysis of the endogenous p53 response to expression of Pes1 wt, and mutants M1 and M5 by indirect immunofluorescence. Asynchronously proliferating and serum-starved (72 h) cells were treated with doxycycline for 24 h to induce the Pes1 constructs. Cells were fixed in methanol and stained against p53 with anti-p53 (Pab122). Cell nuclei were counterstained with DAPI. Representative images are shown. (D) Percentage of p53-positive nuclei in TGR-1 cells upon induction of Pes1 wt, and the dominant-negative mutants M1 and M5. Proliferating and serum-starved cells were analysed by immunofluorescence as described in (C). Numbers of examined cells are indicated in brackets. Error bars indicate SD of the percentage of p53-positive nuclei cells.

25S rRNA. Recent evidence suggests that the assembly of the trimeric yeast complex occurs in the nucleolus, with assembly of Nop7 and Erb1 into the pre-ribosomes prior to Ytm1 (18). Whether Nop7 and Erb1 assemble before binding to 66S pre-ribosomes is not known. A stable complex of Nop7 and Erb1 has not been demonstrated yet, neither in yeast, nor for the homologues Pes1 and Bop1 in mammalian cells. This is intriguing since the interaction of Pes1 and Bop1 can easily be demonstrated in GST-pull-down and yeast two hybrid experiments (15). This suggests that the interaction of endogenous Pes1 and Bop1 may be inhibited during their passage

to the nucleolus and occurs only after association with the pre-ribosome and/or the assembly of WDR12 in the complex.

Nucleolar transport and assembly of Pes1 into the PeBoW-complex

In this study we have constructed a set of deletion mutants to characterize the domains of Pes1 required for the nucleolar localization and assembly into the PeBoW-complex. The Pes1 protein has an evolutionary highly conserved N-terminal domain of pescadillo-like proteins of 250 amino acids

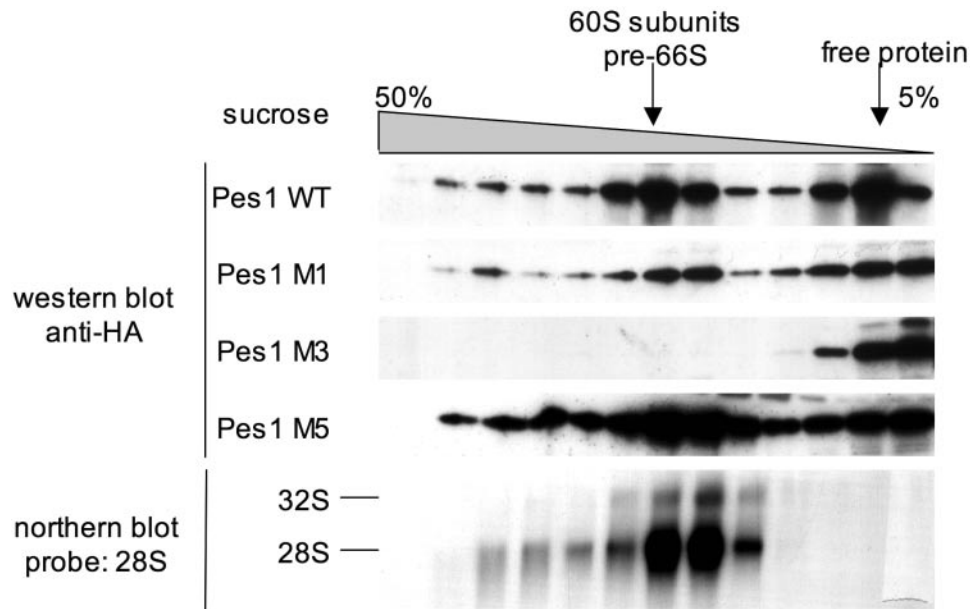


Figure 7. Dominant-negative mutants M1 and M5 associate with large pre-ribosomal particles. Western-blot analysis of sucrose gradient fractions of TGR-1 cells expressing Pes1-HA, and Pes1-HA mutants M1, M3 and M5 with an HA-specific antibody. RNA of each fraction was analysed by northern analysis with 28S rRNA specific probe (lower panel).

followed by a distal BRCT domain (27). While the NPLP-domain is present in all eukaryotes, the BRCT domain is missing in some simple organisms as *Plasmodium falciparum*. Three of our deletions mutants, M1, M2 and M3, successively lacked parts of the NPLP-domain. The removal of 54 N-terminal amino acids of Pes1 in mutant M1 resulted in a strong dominant-negative phenotype. Sucrose gradient centrifugation, co-immunoprecipitation experiments and native gel-electrophoresis experiments (data not shown) revealed proper incorporation of mutant M1 protein into the PeBoW-complex. Hence, the N-terminal 54 amino acids of the NPLP-domain are neither required for the assembly of the PeBoW-complex nor for the transport of Pes1 to the nucleolus. It is more likely that the N-terminal domain of Pes1 fulfils other essential tasks after the assembly of the complex by recruitment of further factors, or by catalysing specific steps in the maturation of the 32S pre-rRNA precursor. However, an enzymatic activity of the complex has not been demonstrated yet, neither in mammals nor in yeast. The mutants M2 and M3 lacked larger parts of the NPLP-domain. Proteins of both mutants were still transported into the nucleus. A transport into the nucleolus, however, was inhibited. This is in line with the observation that mutant M2 and M3 proteins did not co-immunoprecipitate Bop1 and WDR12, because the assembly of the PeBoW-complex in the nucleolus could not take place. The absence of a dominant-negative phenotype of both mutants also supports the model that incorporation of Pes1 mutants into the PeBoW-complex is a prerequisite for a dominant-negative phenotype. The region deleted in M2 and M3 has been reported to interact directly with Bop1 (15). The interaction domain with Bop1 has been identified in murine Pes1 after transposon insertion mutagenesis of 19 extra amino acids downstream of amino acid 198 (Pes1-tn2), amino acid 202 (Pes1-tn11) and amino acid 204 (Pes1-tn12) (Figure 9) in yeast two hybrid experiments

(15). The failure of mutants M2 and M3 proteins to immunoprecipitate complexes containing Bop1 and WDR12 supports this notion. Anyway, incorporation of both mutant proteins into the PeBoW-complex cannot take place, since they do not localize to the nucleolus. In conclusion, the NPLP-domain is critical for nucleolar localization and assembly of the PeBoW-complex.

The Pes1 mutant M4 harbours a deletion of the BRCT (BRCA1 C-terminal) domain that was first described as an essential domain of the tumour suppressor function of the BRCA1 protein. Similar repeat sequences have been identified in many proteins that function in DNA damage, repair and replication. Many BRCT-containing proteins have phospho-peptide binding activity suggesting that BRCT repeats might mediate phosphorylation-dependent protein-protein interactions in processes that are central to cell cycle checkpoint and DNA repair functions. It is tempting to speculate that the BRCT domain in Pes1 might fulfil similar tasks and links nucleolar processes to cell cycle control and DNA synthesis. Interestingly, a temperature sensitive mutant of yeast Nop7, a homologue of Pes1, can specifically inhibit S phase entry in yeast cells (17). This points to additional cell cycle control mechanisms by Pes1, which may act independently of ribosome biogenesis. The mutant M4 protein did not co-immunoprecipitate Bop1 and WDR12 from cellular lysates. This lack of interaction suggests that the NPLP-domain in Pes1 is not sufficient for binding of Bop1 in the nucleoplasm, or that the interaction of nucleoplasmic Pes1 and Bop1 is negatively controlled by a yet unknown mechanism. Importantly, in addition to the Bop1 interacting domain, the BRCT domain also plays an important role in nucleolar localization.

The second mutant with a strong dominant-negative phenotype on cell proliferation and rRNA processing was M5. Similar to M1, mutant M5 protein localized to the

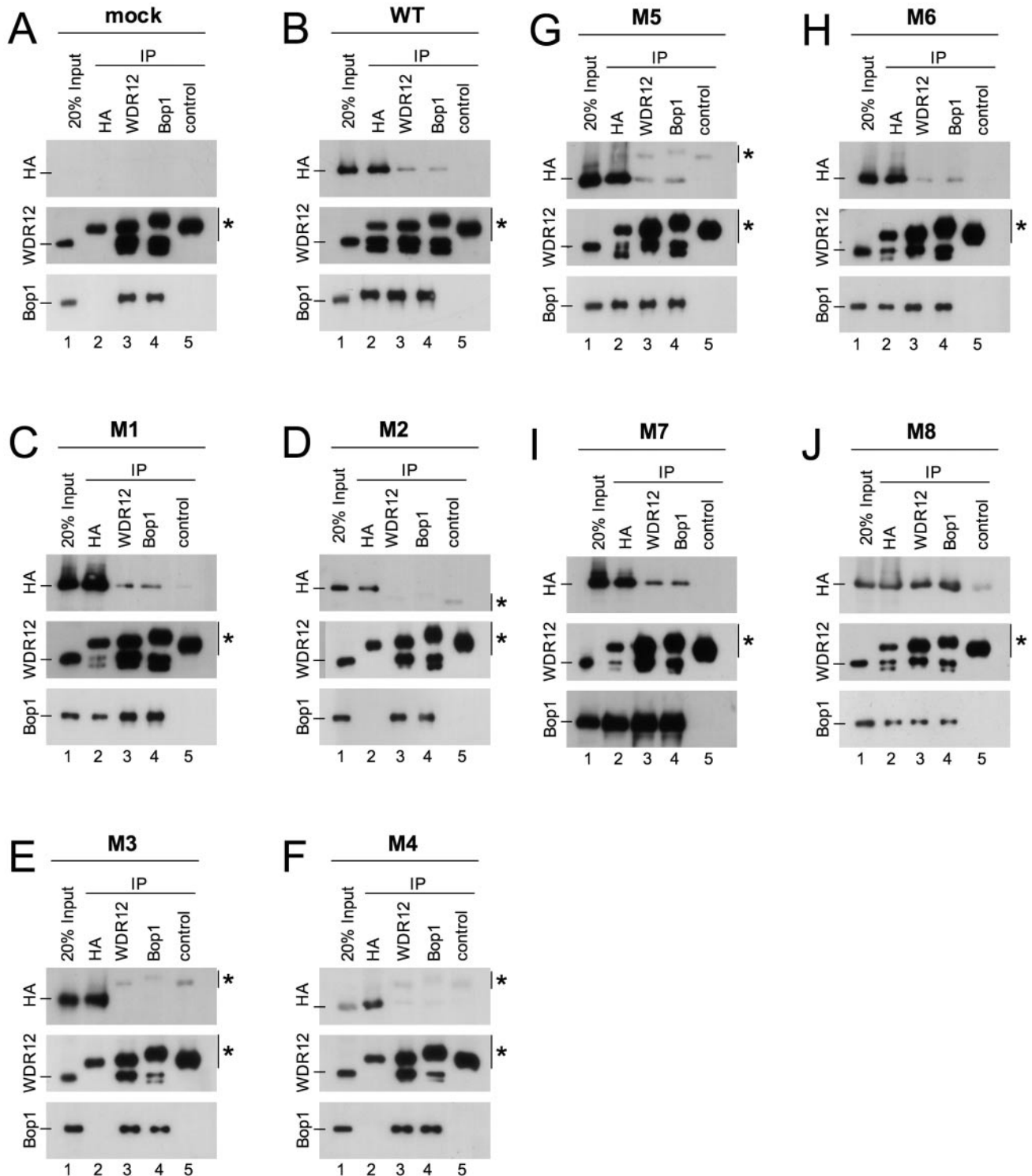


Figure 8. Incorporation of Pes1 mutants into the PeBoW-complex. U2OS cells stably transfected with the indicated constructs (A–J) were seeded at subconfluent density and treated with doxycycline for 20 h. The lysates were subjected to immunoprecipitation with antibodies either against the HA-tag (3F10), WDR12 (1B8), Bop1 (6H11) or the isotype control coupled to protein G Sepharose beads. The amount of protein loaded in each lane resembles an equivalent amount of total lysate or 20% thereof for the input. * indicates cross-reactivity to Ig molecules.

nucleolus and co-immunoprecipitated Bop1 and WDR12. However, we noticed a specific difference in the processing of the 5.8S rRNA for mutants M1 and M5. While the amount of 12S pre-rRNA was reduced for mutant M1, the levels of

12S pre-rRNA were unchanged for the mutant M5. This suggests an inhibition of 12S pre-rRNA processing by mutant M5 and an accumulation of this precursor, whereas 12S pre-rRNA processing proceeds in the presence of mutant M1.

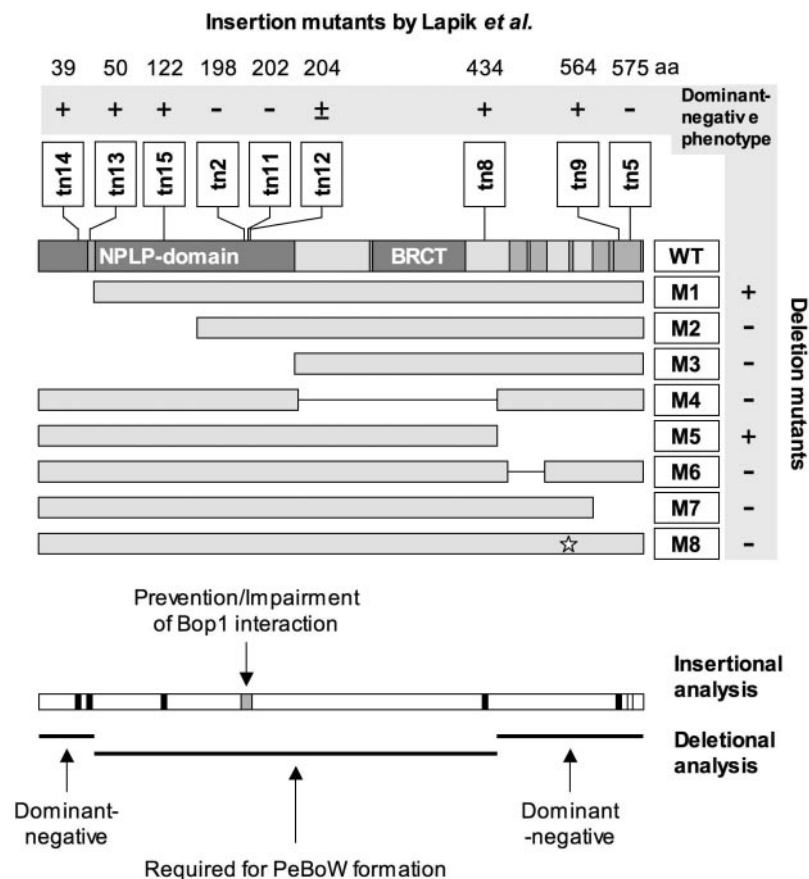


Figure 9. Boundaries and domains important for Pes1 function and PeBoW formation. Pes1 deletion mutants of this work were compared with transposon insertion mutants published by Lapik *et al.* (15). Deletion mutants conferring a dominant-negative phenotype or affecting formation of PeBoW are indicated.

The data suggests that the PeBoW-complex is required for consecutive rRNA processing steps during maturation of the 60S ribosomal subunit and that mutant M5 can block the processing of the 12S pre-rRNA in addition to the 32S pre-rRNA. Accordingly, the C-terminal domain of Pes1 is required for processing of the 32S and 12S pre-rRNA precursors, whereas the N-terminal domain appears to be dispensable for processing of the latter.

Role of the PeBoW-complex in cell cycle control

The impaired function of the PeBoW-complex evokes a p53 response in proliferating but not in serum-starved cells. All three components of the PeBoW-complex are encoded by Myc target genes and usually are not expressed in quiescent cells (14,28,29). This suggests that expression of dominant-negative mutants of Pes1 (this paper), or WDR12 (14) may be not sufficient for the induction of p53, because they cannot assemble into the PeBoW-complex and block its function. However, a recent study showed that human WI-38 fibroblasts accumulated p53 under growth-restricting conditions, such as serum starvation or confluency (30).

Whether the PeBoW-complex is directly involved in the degradation of p53 remains unclear. Since dominant-negative Pes1 mutants act only in the presence of ongoing ribosome biogenesis it is conceivable that signalling of p53 degradation should be linked to this process. Two non-mutually exclusive

mechanisms have been proposed for p53 degradation: nucleolar sequestration of Mdm2, and the blockage of a postulated nucleolar route of p53 export for cytoplasmic degradation (13,31). The Mdm2 sequestration model is supported by the recent finding that several ribosomal proteins, L5, L11 and L23, can bind and inhibit the function of Mdm2. Inhibition of ribosome biogenesis leads to accumulation of free L5, L11 and L23 proteins, which bind to and inhibit Mdm2-mediated p53 ubiquitination and degradation. Inhibition of Mdm2 restores p53-mediated transactivation, accumulation of p21 protein levels, and induction of cell cycle arrest (5–9,27). The second model postulates a nucleolar export route for Mdm2–p53 complexes and subsequent cytoplasmic degradation of p53, termed by Sherr and Weber (13) as Mdm2–p53 complexes ‘riding the ribosome’. This model is supported by the observation that p53 can be covalently linked to 5.8S rRNA, and associates with a subset of ribosomes (32–34). Further support comes from the finding that inhibition of nuclear export leads to accumulation of p53 in the nucleus. If p53 has to enter the nucleolus for the second model, or is loaded on ribosomal subunits during the passage through the nucleoplasm, is currently an open question. Evidence for the presence of p53 in the nucleolus has recently been shown following cell permeabilization, where most soluble nucleoplasmic p53 was eliminated, but nuclear-bound p53 remained readily detectable in the nucleoli (6). Accumulation of nucleolar p53 has also been observed

after inhibition of the proteasome (35). Alternatively, p53 associate with large pre-ribosomal subunits, if they leave the nucleolus. Comparable to processing of the 27S pre-rRNA in yeast (25), processing of the 32S pre-rRNA in mammalian cells probably occurs at the transition of the large pre-ribosomal subunit from the nucleolus to the nucleoplasm. At this moment, the functionality of the PeBoW-complex is required. Successful processing of the 32S rRNA would cause the release of the PeBoW-complex and loading of Mdm2 and p53. A non-functional PeBoW-complex would inhibit processing of the 32S pre-rRNA and thereby nuclear export of p53/Mdm2.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Warner,J.R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.*, **24**, 437–440.
- Brooks,C.L. and Gu,W. (2006) p53 Ubiquitination: Mdm2 and beyond. *Mol. Cell*, **21**, 307–315.
- Latonen,L. and Laiho,M. (2005) Cellular UV damage responses—functions of tumor suppressor p53. *Biochim.Biophys. Acta*, **1755**, 71–89.
- Falini,B., Mecucci,C., Tiacci,E., Alcalay,M., Rosati,R., Pasqualucci,L., La Starza,R., Diverio,D., Colombo,E., Santucci,A. *et al.* (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N. Engl. J. Med.*, **352**, 254–266.
- Grisendi,S., Bernardi,R., Rossi,M., Cheng,K., Khandker,L., Manova,K. and Pandolfi,P.P. (2005) Role of nucleophosmin in embryonic development and tumorigenesis. *Nature*, **437**, 147–153.
- Rubbi,C.P. and Milner,J. (2003) Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J.*, **22**, 6068–6077.
- Yuan,X., Zhou,Y., Casanova,E., Chai,M., Kiss,E., Grone,H.J., Schutz,G. and Grummt,I. (2005) Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. *Mol. Cell*, **19**, 77–87.
- Dai,M.S. and Lu,H. (2004) Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J. Biol. Chem.*, **279**, 44475–44482.
- Dai,M.S., Zeng,S.X., Jin,Y., Sun,X.X., David,L. and Lu,H. (2004) Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. *Mol. Cell Biol.*, **24**, 7654–7668.
- Jin,A., Itahana,K., O'Keefe,K. and Zhang,Y. (2004) Inhibition of HDM2 and activation of p53 by ribosomal protein L23. *Mol. Cell Biol.*, **24**, 7669–7680.
- Lohrum,M.A., Ludwig,R.L., Kubbutat,M.H., Hanlon,M. and Vousden,K.H. (2003) Regulation of HDM2 activity by the ribosomal protein L11. *Cancer Cell*, **3**, 577–587.
- Zhang,Y., Wolf,G.W., Bhat,K., Jin,A., Allio,T., Burkhart,W.A. and Xiong,Y. (2003) Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol. Cell Biol.*, **23**, 8902–8912.
- Sherr,C.J. and Weber,J.D. (2000) The ARF/p53 pathway. *Curr. Opin. Genet. Dev.*, **10**, 94–99.
- Holzel,M., Rohmoser,M., Schlee,M., Grimm,T., Harasim,T., Malamoussi,A., Gruber-Eber,A., Kremmer,E., Hiddemann,W., Bornkamm,G.W., Kremmer,E. and Eick,D. (2005) Mammalian WDR12 is a novel member of the Pes1-Bop1 complex and is required for ribosome biogenesis and cell proliferation. *J. Cell. Biol.*, **170**, 367–378.
- Lapik,Y.R., Fernandes,C.J., Lau,L.F. and Pestov,D.G. (2004) Physical and functional interaction between Pes1 and Bop1 in mammalian ribosome biogenesis. *Mol. Cell*, **15**, 17–29.
- Strezoska,Z., Pestov,D.G. and Lau,L.F. (2002) Functional inactivation of the mouse nucleolar protein Bop1 inhibits multiple steps in pre-rRNA processing and blocks cell cycle progression. *J. Biol. Chem.*, **277**, 29617–29625.
- Du,Y.C. and Stillman,B. (2002) Yph1p, an ORC-interacting protein: potential links between cell proliferation control, DNA replication, and ribosome biogenesis. *Cell*, **109**, 835–848.
- Miles,T.D., Jakovljevic,J., Horsey,E.W., Harnpicharnchai,P., Tang,L. and Woolford,J.L.,Jr (2005) Ytm1, Nop7, and Erb1 form a complex necessary for maturation of yeast 66S preribosomes. *Mol. Cell Biol.*, **25**, 10419–10432.
- Leich-Gaggl,A., Haque,J., Li,J., Ning,G., Traktman,P. and Duncan,S.A. (2002) Pescadillo is essential for nucleolar assembly, ribosome biogenesis, and mammalian cell proliferation. *J. Biol. Chem.*, **277**, 45347–45355.
- Maiorana,A., Tu,X., Cheng,G. and Baserga,R. (2004) Role of pescadillo in the transformation and immortalization of mammalian cells. *Oncogene*, **23**, 7116–7124.
- Killian,A., Le Meur,N., Sesboue,R., Bourguignon,J., Bougeard,G., Gautherot,J., Bastard,C., Frebourg,T. and Flaman,J.M. (2004) Inactivation of the RRB1-Pescadillo pathway involved in ribosome biogenesis induces chromosomal instability. *Oncogene*, **23**, 8597–8602.
- Bornkamm,G.W., Berens,C., Kuklik-Roos,C., Bechet,J.M., Laux,G., Bachl,J., Korndorfer,M., Schlee,M., Holzel,M., Malamoussi,A. *et al.* (2005) Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. *Nucleic Acids Res.*, **33**, e137.
- Pestov,D.G. and Lau,L.F. (1994) Genetic selection of growth-inhibitory sequences in mammalian cells. *Proc. Natl Acad. Sci. USA*, **91**, 12549–12553.
- Haque,J., Boger,S., Li,J. and Duncan,S.A. (2000) The murine Pes1 gene encodes a nuclear protein containing a BRCT domain. *Genomics*, **70**, 201–210.
- Kinoshita,Y., Jarell,A.D., Flaman,J.M., Foltz,G., Schuster,J., Sopher,B.L., Irvin,D.K., Kanning,K., Kornblum,H.I., Nelson,P.S. *et al.* (2001) Pescadillo, a novel cell cycle regulatory protein abnormally expressed in malignant cells. *J. Biol. Chem.*, **276**, 6656–6665.
- Dez,C. and Tollervey,D. (2004) Ribosome synthesis meets the cell cycle. *Curr. Opin. Microbiol.*, **7**, 631–637.
- Staub,E., Fizev,P., Rosenthal,A. and Hinzmann,B. (2004) Insights into the evolution of the nucleolus by an analysis of its protein domain repertoire. *Bioessays*, **26**, 567–581.
- Schlosser,I., Holzel,M., Murnseer,M., Burtscher,H., Weidle,U.H. and Eick,D. (2003) A role for c-Myc in the regulation of ribosomal RNA processing. *Nucleic Acids Res.*, **31**, 6148–6156.
- Schlosser,I., Holzel,M., Hoffmann,R., Burtscher,H., Kohlhuber,F., Schuhmacher,M., Chapman,R., Weidle,U.H. and Eick,D. (2005) Dissection of transcriptional programmes in response to serum and c-Myc in a human B-cell line. *Oncogene*, **24**, 520–524.
- Bhat,K.P., Itahana,K., Jin,A. and Zhang,Y. (2004) Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation. *EMBO J.*, **23**, 2402–2412.
- Tao,W. and Levine,A.J. (1999) Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. *Proc. Natl Acad. Sci. USA*, **96**, 3077–3080.
- Fontoura,B.M., Atienza,C.A., Sorokina,E.A., Morimoto,T. and Carroll,R.B. (1997) Cytoplasmic p53 polypeptide is associated with ribosomes. *Mol. Cell Biol.*, **17**, 3146–3154.
- Fontoura,B.M., Sorokina,E.A., David,E. and Carroll,R.B. (1992) p53 is covalently linked to 5.8S rRNA. *Mol. Cell Biol.*, **12**, 5145–5151.
- Samad,A. and Carroll,R.B. (1991) The tumor suppressor p53 is bound to RNA by a stable covalent linkage. *Mol. Cell Biol.*, **11**, 1598–1606.
- Klibanov,S.A., O'Hagan,H.M. and Ljungman,M. (2001) Accumulation of soluble and nucleolar-associated p53 proteins following cellular stress. *J. Cell. Sci.*, **114**, 1867–1873.