

The BRCT domain of mammalian Pes1 is crucial for nucleolar localization and rRNA processing

Michael Hölzel*, Thomas Grimm, Michaela Rohmoser, Anastassia Malamoussi, Thomas Harasim, Anita Gruber-Eber, Elisabeth Kremmer¹ and Dirk Eick*

Institute of Clinical Molecular Biology and Tumour Genetics, GSF Research Centre, Marchioninistrasse 25, 81377 Munich, Germany and ¹Institute of Molecular Immunology, GSF Research Centre, Marchioninistrasse 25, 81377 Munich, Germany

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ABSTRACT

The nucleolar protein Pes1 interacts with Bop1 and WDR12 in a stable complex (PeBoW-complex) and its expression is tightly associated with cell proliferation. The yeast homologue Nop7p (Yph1p) functions in both, rRNA processing and cell cycle progression. The presence of a BRCT-domain (BRCA1 C-terminal) within Pes1 is quite unique for an rRNA processing factor, as this domain is normally found in factors involved in DNA-damage or repair pathways. Thus, the function of the BRCT-domain in Pes1 remains elusive. We established a conditional siRNA-based knock-down-knock-in system and analysed a panel of Pes1 truncation mutants for their functionality in ribosome synthesis in the absence of endogenous Pes1. Deletion of the BRCT-domain or single point mutations of highly conserved residues caused diffuse nucleoplasmic distribution and failure to replace endogenous Pes1 in rRNA processing. Further, the BRCT-mutants of Pes1 were less stable and not incorporated into the PeBoW-complex. Hence, the integrity of the BRCT-domain of Pes1 is crucial for nucleolar localization and its function in rRNA processing.

INTRODUCTION

Ribosome biogenesis is the major metabolic challenge of rapidly proliferating cells, in particular tumour cells. However, little is known about the molecular mechanisms that ensure the equilibrium between cell division and ribosome biogenesis required for balanced cell proliferation and thus a successful duplication of the translational machinery (1). Recently it has become evident that ribosome synthesis is cell cycle controlled and sensitive to growth factor and nutrient signalling, and inhibited upon stress signals (2–6). On the

other hand, disturbance of the structural and/or functional integrity of the nucleolus is linked to the induction of the tumour suppressor p53 (7–10). Several ribosomal proteins have been identified so far as crucial mediators of the ribosomal stress response. Impaired ribosome synthesis decreases the demand and thereby results in an accumulation of free ribosomal proteins, such as L11, L5 and L23 that have been shown to inhibit the function of Mdm2 (11–14). p53 is targeted by the E3 ubiquitin ligase Mdm2 for proteasomal degradation. Thus, ribosomal stress mediates the accumulation of p53 by blocking its degradation. A profound knowledge of the mammalian ribosome synthesis pathways is required to further unravel the ribosomal stress response, a promising target for non-genotoxic p53 induction in tumour cells. Fortunately, ribosome biogenesis is a highly evolutionary conserved process that has been intensively studied in yeast (15). In addition, the recent characterization of the human nucleolar proteome further facilitates the investigation of mammalian ribosome biogenesis (16–18).

The nucleolar protein Pes1 (Pescadillo) is evolutionarily highly conserved and essential for embryogenesis and nucleogenesis in mice (19). Its expression is tightly associated with cell proliferation and thus highly elevated in tumour cells (20). Two studies have addressed the function of Pes1 in cell proliferation and ribosome biogenesis (21,22). Either transposon mediated insertion mutants or truncations of the N-terminal or C-terminal region resulted in a dominant-negative phenotype, blocking processing of the 32S pre-rRNA and cell proliferation. Ribosomal RNA (rRNA) is synthesized as a large 47S precursor that is subsequently cleaved into the mature 5.8S, 18S and 28S rRNA. Pes1 interacts with two other nucleolar proteins Bop1 and WDR12 in a stable complex, termed PeBoW-complex (8). Both factors are also implicated in the processing of ribosomal RNA (8,23). In yeast, a stable trimeric complex of the respective homologues of Pes1 (Nop7p/Yph1p), Bop1 (Erb1p) and WDR12 (Ytm1p) has been identified (24–26). Further, it was shown that the yeast Pes1 homologue Nop7p (Yph1p) functions in DNA replication independently of ribosome biogenesis (27). In this

*To whom correspondence should be addressed. Tel: +49897099512; Fax: +49897099500; Email: hoelzel@gsf.de

*Correspondence may also be addressed to Dirk Eick. Tel: +49897099512; Fax: +49897099500; Email: eick@gsf.de

study, Nop7p was co-purified with the origin recognition proteins Orc1p and Orc2p and required for S-phase entry subsequently to the release from a hydroxyurea mediated cell cycle arrest. The presence of a BRCT-domain (BRCA1 C-terminal) within the central region of Pes1 is conserved throughout evolution and remarkable for several reasons. The BRCT-domain has been first identified as target of germline mutations in the BRCA1 protein, that predispose to the early development of breast and ovarian cancer (28). BRCT-domain containing factors, such as BRCA1, XRCC1 and 53BP1 play a role in DNA-damage or repair pathways. Based on recent structural studies, the BRCT-domain is thought to function as phosphopeptide recognition motif (29,30). A bioinformatic analysis of the nucleolar proteome reveals only a few BRCT-domain containing factors, but having all a confirmed role in DNA-damage or replication (<http://Lamondlab.com/NOPdb/>). Thus, the presence of a BRCT-domain within the Pes1 protein is therefore quite unusual if not unique for an rRNA processing factor. In this study, we established a conditional siRNA mediated knock-down-knock-in system to analyse the function of Pes1 truncation mutants in rRNA processing in the absence of a dominant-negative phenotype. An extended deletion the BRCT-domain resulted in aberrant nucleoplasmic localization, however only in the context of the full-length protein, and failed to reconstitute for the endogenous protein in rRNA processing assays. To study the role of the BRCT-domain more in detail, we inserted single point mutations of highly conserved residues. We found that the BRCT-domain of Pes1 is essential for rRNA processing and nucleolar localization in a context dependent manner. Moreover, point mutations of the BRCT-domain reduce the stability of Pes1 and abolish its interaction with the PeBoW-complex.

MATERIALS AND METHODS

Cloning/plasmids

Conditional Pes1 expression constructs and mutagenesis were described previously (21). The novel Pes1 mutants M9 and M10 were generated by the use of restriction sites. Single point mutations of the BRCT-domain were introduced by site directed mutagenesis. Successful mutagenesis was verified by sequencing. Pes1 mutants were then cloned into the pRTS-1 vector using the SfiI restriction site (31).

Tissue culture

H1299 lung carcinoma cells (non-small cell lung carcinoma) were cultured in DMEM with 10% FBS at 8% CO₂. Polyclonal cell lines were generated by transfection of the respective pRTS-1 plasmids using Polyfect (QIAGEN) and stably selected in the presence of 200 µg/ml hygromycin B for ~10 days. Conditional gene expression was induced with 0.5 µg/ml doxycycline and monitored by determining the percentage of eGFP expressing cells using FACS analysis.

Knock-down-knock-in assay

H1299 cells were treated with 0.5 µg/ml doxycycline to activate expression of Pes1wt and the respective mutants for 3 days. Depletion of endogenous Pes1 was performed by

two subsequent transfections of siRNA oligos specific for the 3'-untranslated region (3'-UTR) (5'-CCAGAGGACCU-AAGUGUGAtt-3') of the Pes1 mRNA using oligofectamine (QIAGEN). Cells were harvested for western blot analysis or metabolic labelling of nascent rRNA at 48 h after the last siRNA transfection.

Metabolic labelling of nascent rRNA

H1299 were incubated in phosphate-free DMEM/10% FBS for at least 30 min prior to the *in vivo* labelling. Cells were then incubated for 1 h with 15 µCi/ml ³²P-orthophosphate. Subsequently, the metabolic labelling medium was removed and cells were then further cultured in regular DMEM/10% FBS for 4–5 h. Total RNA was then isolated using the RNeasy Mini kit (QIAGEN). A total of 1.5 µg of total RNA was separated on a 1% agarose formaldehyde gel and subsequently dried on a whatman paper using a regular gel drier (BioRad) connected to a vacuum pump. Metabolically labelled RNA was visualized by autoradiography.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2× SDS-loading buffer (100 mM Tris-HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromphenol blue and 20% glycerol). Cell lysates were separated by SDS-PAGE and blotted on nitrocellulose membranes (Amersham). Immunodetection was performed with monoclonal antibodies directed against the HA-tag (3F10; Roche), Pes1 (8E9) and tubulin (Dianova). The monoclonal antibody against human Pes1 was described previously (8). The antibody against rodent Bop1 was produced by simultaneous immunization of guinea pigs with ovalbumin conjugated peptides specific for 6–30 and 541–566 amino acids of mouse Bop1. The polyclonal serum was only reactive against the N-terminal 6–30 amino acids. For immunofluorescence, cells were grown on cover slides, fixed with ice-cold methanol and air dried. Unspecific binding was blocked with phosphate-buffered saline (PBS)/10% FBS. HA-tagged forms of Pes1 and WDR12 were detected with the anti-HA (3F10) antibody. Primary antibodies were incubated over night at 4°C in a humidified chamber. Cy3 labelled secondary antibodies (Dianova) were incubated for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma-Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging) with a 63 (1.15) plan oil objective connected to a CCD-camera (Hamamatsu, ORCA-479).

Immunoprecipitation

For immunoprecipitations, H1299 cells were seeded at sub-confluent density and treated with 0.5 µg/ml doxycycline for 24 h. Cells were harvested by trypsination and washed three times with PBS. Subsequently, cells were resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, protease inhibitors and phosphatase inhibitors] and incubated on ice for 20 min and sonicated. In the meanwhile protein G beads were incubated either with antibodies against the HA-tag (3F10, Roche), WDR12 (1B8), Bop1 (6H11) or cytohesin for at least 1 h at 4°C. Monoclonal antibodies directed against WDR12, Bop1 and

cytohesin were described previously (8). After centrifugation, cell lysates were incubated with protein G beads at 4°C overnight. Finally, the beads were washed three times with lysis buffer and resuspended in SDS-loading buffer/lysis buffer (1:1). The resuspension volume was equal to the volume of the cell lysates initially used for the IP.

Native gel electrophoresis

A total of 3×10^6 cells were lysed in 100 μ l lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, phosphatase inhibitors and protease inhibitors] at 4°C for 20 min. Total cell lysates were briefly vortexed several times in between. Lysates were then cleared by centrifugation in an Eppendorf FA-24-11 rotor at 24 000g for 10 min at 4°C. 7.5 μ l of 2 \times sample buffer [125 mM Tris-HCl (pH 6.8), 30% glycerol and 0.02% bromophenol blue] was added to 7.5 μ l of total lysate and separated by PAGE (6.5%) in the absence of SDS at 4°C. Blotting was performed in the absence of methanol. Immunoblotting was performed as described above.

RESULTS

Functional analysis of Pes1 mutants by a knock-down-knock-in assay

Previously, we have analysed a set of truncation mutants of human Pes1 for dominant-negative phenotypes in rRNA processing and cell proliferation assays (Figure 1A). Thereby we identified two mutants, a N-terminal (M1) and a C-terminal (M5) truncation, that impaired rRNA processing and cell cycle progression (21). The respective regions were also found as hotspots for the generation of dominant-negative mutants using transposon mediated mutagenesis (22). In this study we extended the panel of mutants by two novel truncated forms of Pes1, namely M9 and M10. Nomenclature was preserved to facilitate crossreading with our previous study (21).

So far it remained unclear whether all the other deletion mutants were still functional in rRNA processing, in particular the ones that localized to nucleolus as the wild-type protein. Pes1 is composed of several distinct domains and little is known about their role in rRNA processing (Figure 1A). Therefore we established a conditional knock-down-knock-in system for mammalian cells (Figure 1B). Briefly, H1299 human lung carcinoma cells were stably transfected with pRTS-1 constructs, conditionally expressing the respective HA-tagged forms of human Pes1 in a doxycycline dependent manner (21,31). Exogenous gene expression was activated for three days and then maintained throughout the subsequent course of two siRNA transfections using siRNA oligos directed against the 3'-UTR of the Pes1 mRNA to specifically deplete the endogenous Pes1 protein. We used H1299 cells, because stable polyclonal cell cultures could be easily propagated following transfection of the pRTS-1 plasmids and conditional gene expression could be achieved in >95% of the cells (Supplementary Figure 1). A bidirectional promoter drives transcription of eGFP at the same time as the indicated forms of Pes1 and thus allowed easy monitoring by FACS analysis. Efficient knock-down of the endogenous Pes1 protein was verified by western blot analysis 2 days after

the last siRNA transfection (Figure 1C). The various Pes1 mutants were detected by antibodies directed against the HA-tag and the C-terminal region of Pes1. The mutants were detected at the expected size, however deletion of the acidic regions in M5, M6 and M10 facilitated faster migration. The Pes1 specific antibody did not recognize the mutants M5, M10 and M7, as they lacked the respective epitope. The enlarged panel at the bottom shows the size-dependent discrimination of the HA-tagged wild-type and the endogenous Pes1 protein. Including the 3 days of pre-activation, the various Pes1 mutants were expressed for 7 days when finally harvested. Thus, the different expression levels of the indicated forms of Pes1 reflect steady-state levels that depend on the respective protein stability. In particular, M2, M4 and M9 are poorly expressed despite identical induction rates as monitored by FACS analysis (Supplementary Figure 1). Interestingly, the steady-state expression of the HA-tagged wild-type Pes1 closely resembled the levels of the endogenous Pes1, thus providing a knock-down-knock-in system at physiological expression conditions.

The acidic domain of Pes1 is dispensable for rRNA processing

In parallel to the western blot analysis, rRNA processing was investigated by *in vivo* labelling of nascent rRNA using 32 P-orthophosphate (Figure 2). Ribosomal RNA is transcribed as a large 47S precursor that is subsequently cleaved into the mature rRNA forms. Figure 2A depicts an overview of mammalian rRNA processing pathways. 32 P-orthophosphate is incorporated into the nascent rRNA transcripts and thus allows an *in vivo* analysis of rRNA processing. The 45S and the 32S rRNA are the most abundant precursor rRNAs, besides the mature 18S and 28S forms. Expression of Pes1wt-HA but not WDR12wt-HA completely rescued the abrogated production of mature 28S rRNA mediated by depletion of endogenous Pes1 (Figure 2B, lanes 1–4). As the HA-tagged wild-type Pes1 was fully functional in terms of rRNA processing, we now tested the whole panel of Pes1 mutants. The majority of the mutants failed to compensate for depletion of endogenous Pes1. However, M6 and M8 fully restored the synthesis of the mature 28S rRNA (Figure 2B, lanes 17, 18 and 21, 22). As M6 lacks the C-terminal acidic region, this domain is dispensable for rRNA processing. This is intriguing, as the acidic domain of Pes1 is preserved throughout evolution and similar stretches of acidic residues are frequently found in nucleolar proteins, such as nucleolin and nucleophosmin. Several studies provided evidence that these acidic regions might be important for protein-protein interactions (32,33). M8 harbors a point mutation of the potential SUMOylation site of Pes1, however SUMOylation at this site has not been experimentally confirmed yet. Interestingly, all mutants lacking the BRCT-domain were incapable to substitute for endogenous Pes1.

Role of the BRCT-domain for subcellular localization of Pes1

Analysis of the subcellular localization by indirect immunofluorescence revealed that M9, missing the BRCT-domain, was dispersed in the nucleoplasm (Figure 3A), as described previously (21). Further, deletions within the Pes1 protein

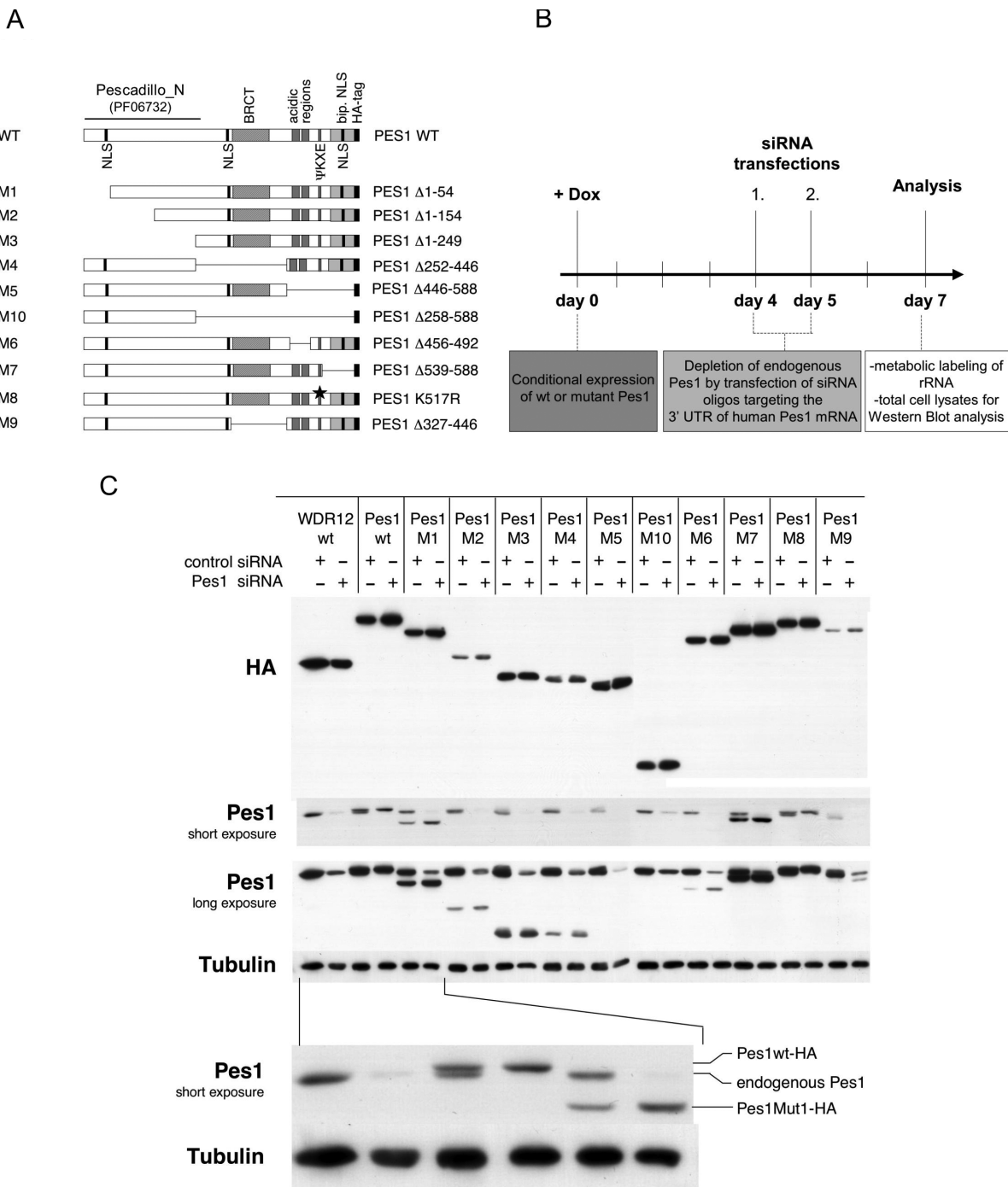
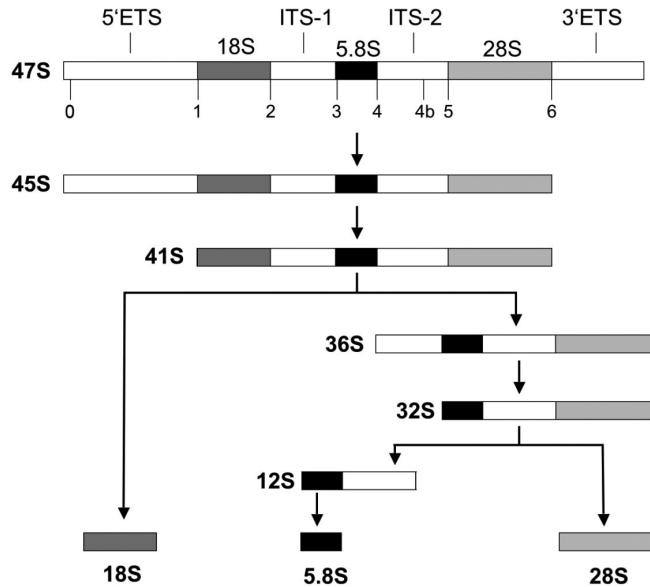


Figure 1. Pes1 mutagenesis, experimental approach of knock-down-knock-in assay and conditional expression of Pes1 mutants. (A) Panel of Pes1 deletion mutants harbouring a C-terminal HA-tag, as described previously. M9 and M10 are novel deletion mutants. NLS: classical nuclear localization signal; bip. NLS: bipartite NLS; Pescadillo_N: N-terminal region of Pes1 (Pfam-database: PF06732); BRCT: BRCT-domain; acidic regions: two glutamic acid rich regions; ΨKXE: consensus SUMOylation site; HA: hemagglutinin-tag. (B) Experimental approach of knock-down-knock-in assay. (C) Western blot analysis of knock-down-knock-in assay as described in (B). Expression of recombinant wt or mutant Pes1 proteins was analysed with the anti-HA antibody (3F10). Knock-down of endogenous Pes1 by siRNA was verified probing with the anti-Pes1 antibody (8E9). All mutants besides M5, M10 and M7 were also detected with the anti-Pes1 antibody. Equal loading was controlled by immunodetection of α -tubulin. Size-dependent discrimination of HA-tagged wt and endogenous Pes1 protein using the anti-Pes1 antibody is shown in the enlarged panel at the bottom.

(M4 and M10) partially or completely restored nucleolar localization [Figure 3A, Supplementary Figure 2 and (21)]. Thus, lack of the BRCT-domain results in a diffuse nucleoplasmic distribution in a context dependent manner. However,

the isolated BRCT-domain of Pes1 (Pes1 BRCT) was diffusely distributed in the cytoplasm and therefore insufficient for nuclear/nucleolar localization (Figure 3A). M6, harbouring the deletion of the acidic region, localized to the nucleolus

A



B

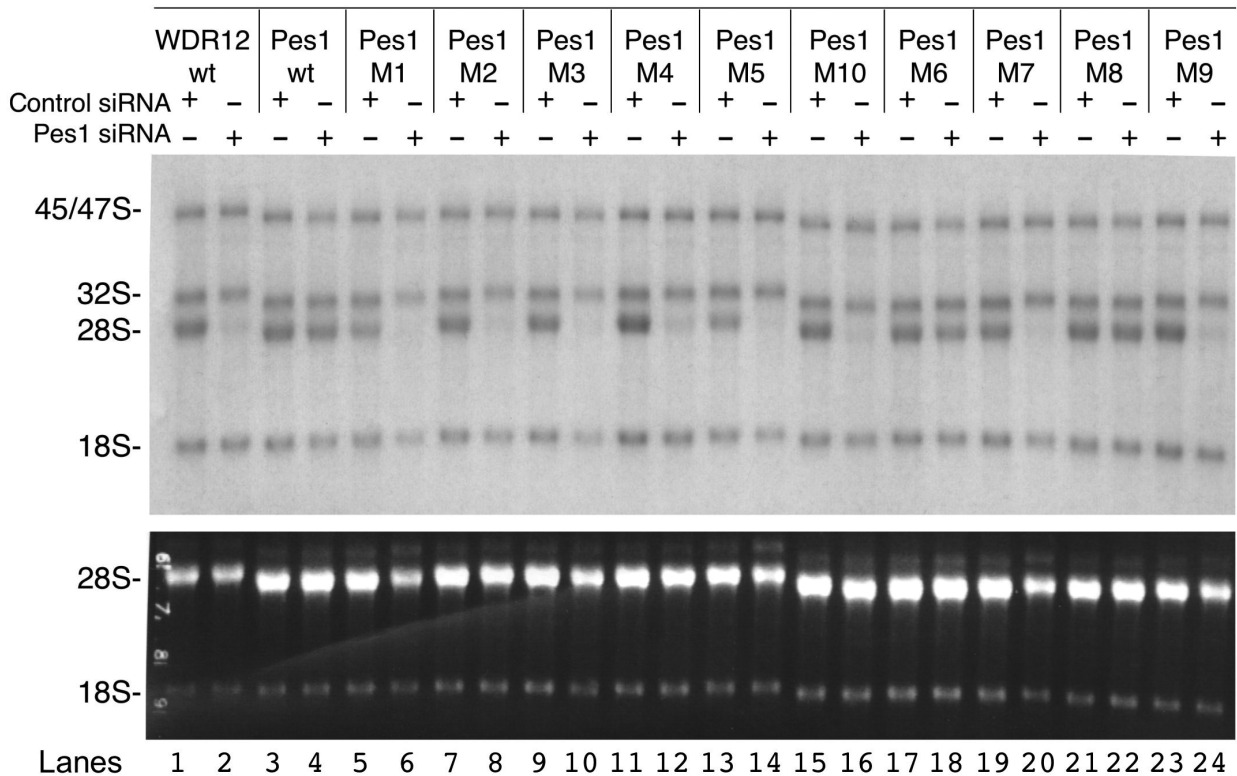
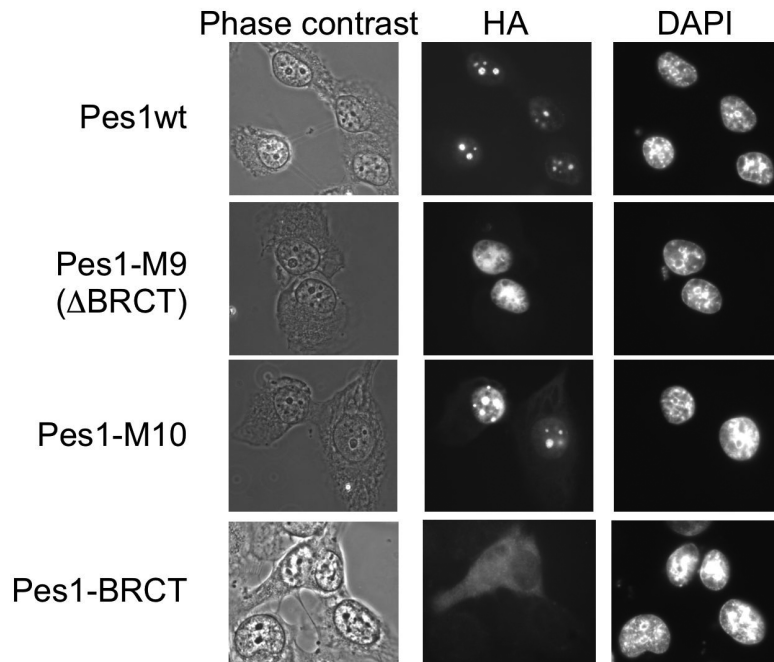
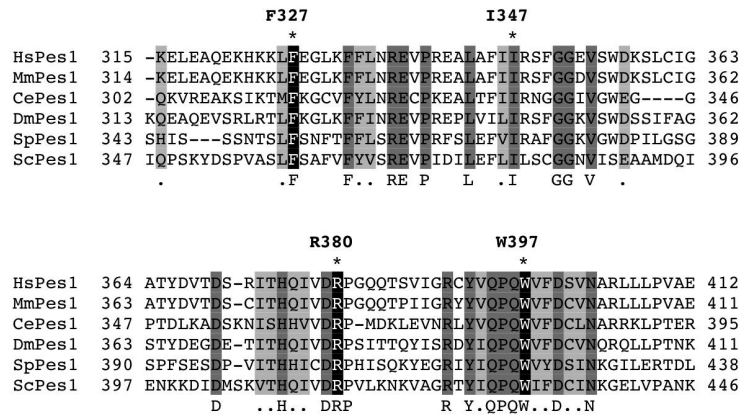


Figure 2. Functionality of Pes1 mutants in rRNA processing in the absence of endogenous Pes1. (A) Simplified schematic view of mammalian rRNA processing pathways. The primary 47S transcript is processed into subsequent precursor rRNAs. Cleavage sites are indicated by the corresponding numbers. 5'ETS and 3'ETS (external transcribed spacers), ITS-1 and ITS-2 (internal transcribed spacers) depict the regions that are cleaved off the primary transcript without being used in the mature ribosomes. The 45S and the 32S rRNA are the most abundant rRNA precursors that can be easily detected by the metabolic labelling. (B) Autoradiography of metabolic labelling of nascent ribosomal RNA using ³²P-orthophosphate. Knock-down-knock-in assay was performed as summarized in Figure 1B. Stable polyclonal H1299 cell lines expressing the indicated HA-tagged proteins were either treated with control or Pes1 (3'-UTR) specific siRNA. Cells were then incubated in phosphate-free medium for 30 min, labelled with ³²P-orthophosphate for 1 h and subsequently cultured in regular medium for 4.5 h. Total RNA was separated by formaldehyde-agarose gel electrophoresis. The gel was dried before autoradiography. The ethidium bromide stained gel is shown as loading control.

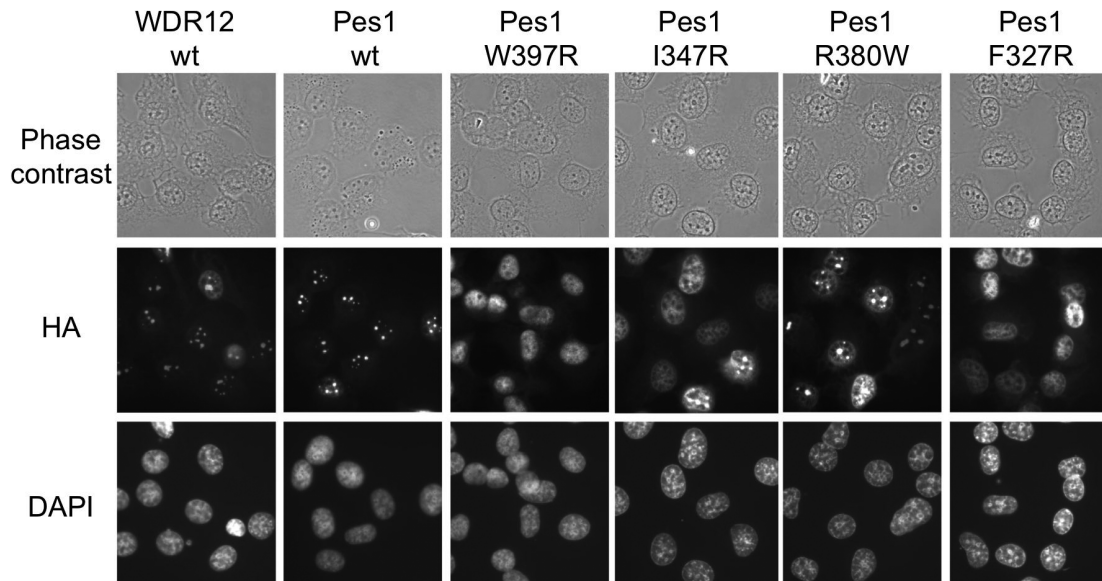
A



B



C



as the wild-type protein [Supplementary Figure 2 and (21)]. This observation is in line with its full functionality in the rRNA processing assay. The presence of the BRCT-domain within the Pes1 protein is conserved throughout evolution, from yeast to human. In Figure 3 we have shown that it is crucial for the nucleolar localization of Pes1, but only in the context of the full-length protein, as the highly conserved N-terminal region (M10) was sufficient for nucleolar localization. However, a gross deletion of the BRCT-domain including adjacent residues might affect overall stability and protein folding. Therefore, we generated a set of single point mutations of highly conserved residues within the BRCT-domain (Figure 3B). W397 is one of the most conserved amino acids corresponding to the missense mutation W1837R in the BRCT-domain of BRCA1. Analogous exchanges of W397R, I347R and F327R in the yeast Pes1 homologue Nop7p (Yph1p) resulted in temperature sensitive strains (20,27). So far, no temperature sensitive alleles were isolated in yeast that would correspond to the R380W exchange.

We then investigated the subcellular localization of the respective mutant forms by indirect immunofluorescence (Figure 3C). Pes1-W397R and -F327R exhibited a diffuse nucleoplasmic distribution. Pes1-I347R was also dispersed in the nucleoplasm, however a small percentage of the cells also displayed nucleolar enrichment. Pes1-R380W predominantly localized in the nucleolus, but the intensity of the nucleoplasmic staining was slightly increased in comparison to Pes1wt. Thus, the integrity of the BRCT-domain is crucial for proper nucleolar localization of Pes1, and notably, either deletion or critical point mutations of the BRCT-domain resulted in aberrant nucleoplasmic distribution.

Point mutations within the BRCT-domain of Pes1 abrogate its functionality in rRNA processing

Subsequently, we analysed the functionality of the BRCT-mutants in terms of rRNA processing. We depleted the cells for endogenous Pes1 and performed *in vivo* labelling of nascent ribosomal RNA to test the capacity of the respective mutants to sustain production of the mature 28S rRNA. In analogy to Figure 2, the HA-tagged Pes1wt was fully functional in our assay (Figure 4A, lanes 1–4). In contrast, the predominantly nucleoplasmic BRCT-mutants Pes1-W397R, -I347R and F327R failed to substitute for depletion of endogenous Pes1 (Figure 4A, lanes 5–8 and 11–12). Only the nucleolar Pes1-R380W mutant rescued maturation of the 28S rRNA. For western blot analysis, cells were harvested in parallel to the *in vivo* labelling (Figure 4B). Noteworthy, the non-functional BRCT-domain mutants Pes1-W397R, -I347R, -F327R displayed lower steady-state expression levels than Pes1wt or

the functional Pes1-R380W mutant, even though the percentage of ectopic Pes1 expressing cells was equal as determined by detection of eGFP using flowcytometry (Supplementary Figure 3). Successful knock-down of the endogenous Pes1 was verified by using an antibody directed against Pes1 (Figure 4B). As this antibody recognizes the endogenous and exogenous forms of Pes1, the expression of the respective mutants can be analysed in the context of the endogenous Pes1 levels. The non-functional nucleoplasmic BRCT-domain mutants were expressed at significant lower levels than the endogenous Pes1, whereas Pes1wt and Pes1-R380W closely mimicked the physiological expression levels.

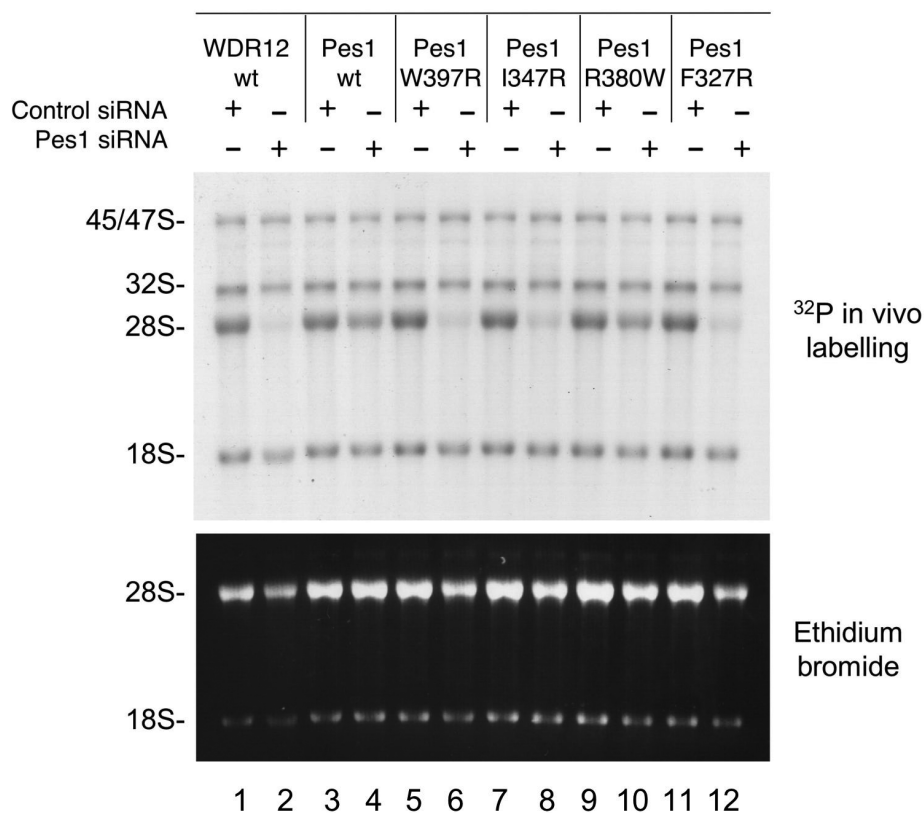
Point mutations within the BRCT-domain of Pes1 negatively affect protein stability

Thus, we reasoned that the lower protein expression levels of the non-functional BRCT-domain mutants might result from decreased protein stability. Therefore we inhibited proteasomal degradation by treating the cells with MG-132 for 8 h. Indeed, the non-functional BRCT-domain mutants exhibited a more pronounced accumulation than Pes1wt and Pes1-R380W (Figure 5A). Further, proteasome inhibition resulted in the occurrence of multiple bands with higher molecular weight strongly suggesting that Pes1 and the respective BRCT-domain mutants become polyubiquitinated. We also analysed the panel of Pes1 truncation mutants for the occurrence of polyubiquitination to further map the ubiquitination site. However, all mutants tested so far exhibited signs of polyubiquitination (data not shown). Thus, we concluded that several lysines within different regions of Pes1 might serve for ubiquitination to mediate subsequent proteasomal degradation. Incubation with ALLM, an inhibitor of calpain and cathepsin proteases, had no effect on the protein levels of the BRCT-domain mutants (Figure 5A). Our attempts to analyse whether proteasome inhibition mediated accumulation of the BRCT-domain mutants restored functionality were hampered by the fact that proteasome inhibitors itself potentially abrogated rRNA processing (Supplementary Figure 4).

Next, we analysed the stability of the Pes1 BRCT-domain mutants by treating the cells with cyclohexamide to block *de novo* protein synthesis (Figure 5B). Expression levels of Pes1wt and Pes1-R380W remained unchanged over 12 h in the presence of cyclohexamide. However, the non-functional mutants Pes1-W397R, -I347R and -F327R rapidly decreased within several hours in the absence of *de novo* protein synthesis. In conclusion, mutations of highly conserved residues within the BRCT-domain of Pes1 negatively affect protein stability and cause aberrant nucleoplasmic localization. Thus it might be that incorporation of Pes1 mutants into

Figure 3. Mutagenesis of the BRCT-domain of Pes1 and subcellular localization of Pes1 mutants carrying single point mutations within the BRCT-domain. (A) Subcellular localization of HA-tagged Pes1wt and mutant M9 (BRCT-domain deletion mutant) and M10 (lacking the BRCT-domain and the C-terminus). Stable polyclonal H1299 cell lines conditionally expressing the indicated Pes1 mutants were grown on cover slips and fixed with ice-cold methanol. Cells expressing HA-tagged wt WDR12 are shown as a control. Nuclei were counterstained with DAPI. Subcellular localization of the full-set of Pes1 mutants is shown in Supplementary Figure 2. Additionally, H1299 cells were transiently transfected with the isolated HA-tagged BRCT-domain of Pes1 (Pes1 BRCT). (B) Alignment of the Pes1 BRCT-domain of diverse species: HsPes1: *Homo sapiens* Pes1 (NP_055118); MmPes1: *Mus musculus* Pes1 (NP_075027); CePes1: *Caenorhabditis elegans* Pes1 (NP_498661); DmPes1: *Drosophila melanogaster* Pes1 (NP_609305); SpPes1: *Schizosaccharomyces pombe* Pes1 (NP_596543); ScPes1: *Saccharomyces cerevisiae* Pes1 (Nop7p, Yph1p, NP_011617). Highly conserved residues are highlighted in dark grey and similar exchanges in light grey. Conserved residues used for mutagenesis are shown in black and marked by asterisks (*). (C) Subcellular localization of Pes1wt and Pes1 BRCT-domain mutants by indirect immunofluorescence using the anti-HA antibody (3F10). Stable polyclonal H1299 cell lines conditionally expressing the indicated proteins were grown on cover slips and fixed with ice-cold methanol. Nuclei were counterstained with DAPI.

A



B

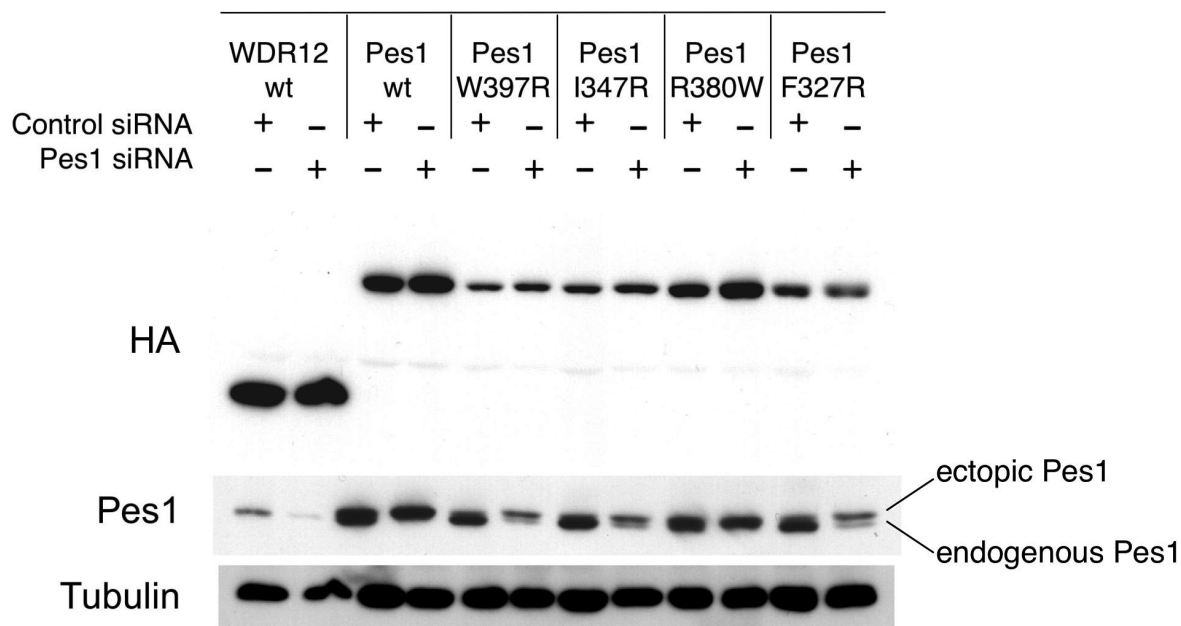


Figure 4. Knock-down-knock-in assay of H1299 cells conditionally expressing wt WDR12, wt Pes1 and the indicated BRCT-domain mutants of Pes1. Experimental procedure was performed as summarized in Figure 1B. (A) Metabolic labelling of nascent rRNA. Cells were incubated in phosphate-free medium for 30 min, labelled with ³²P-orthophosphate for 1 h and subsequently cultured in regular medium for 4.5 h. Total RNA was separated by formaldehyde-agarose gel electrophoresis. The gel was dried before autoradiography. The ethidium bromide stained gel is shown as loading control. (B) Expression of recombinant wt WDR12, wt or mutant Pes1 proteins was analysed using the anti-HA antibody (3F10). Efficient knock-down of endogenous Pes1 by siRNA was verified with the anti-Pes1 antibody (8E9). Equal loading was verified by immunodetection of α -tubulin.

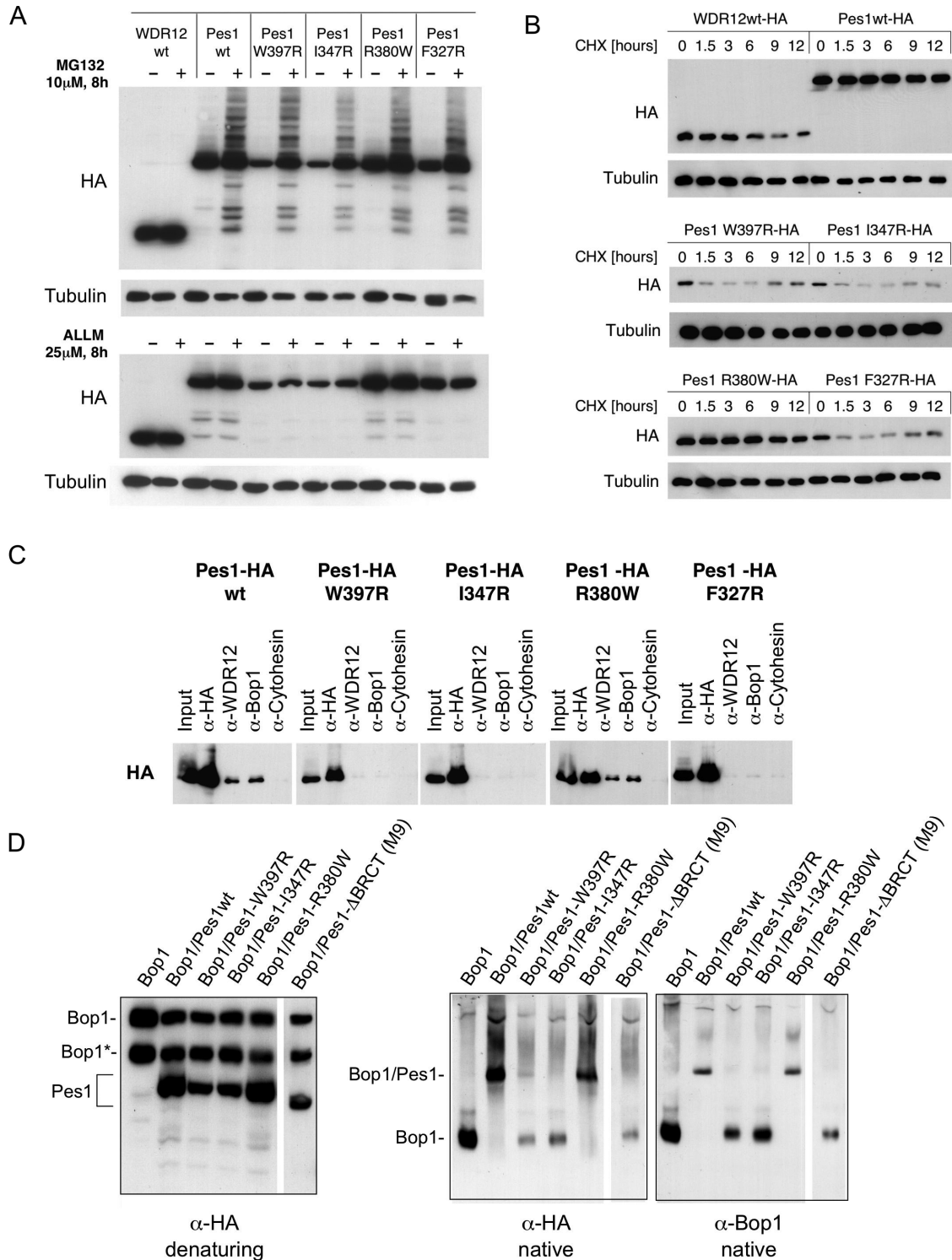


Figure 5. Stability of Pes1 BRCT-domain mutants and incorporation into the PeBoW-complex. (A) Stable polyclonal H1299 cells conditionally expressing wt WDR12, wt Pes1 and the indicated Pes1 BRCT-domain mutants were either left untreated or incubated with MG-132 or ALLM for 8 h. Total cell lysates were harvested and analysed by western blot analysis using the anti-HA antibody (3F10). Equal loading was verified by immunodetection for α -tubulin. (B) Cells were treated with 50 μ g/ml cycloheximide for the indicated time points to block *de novo* protein synthesis. Western blot analysis of total cell lysates was performed using the anti-HA antibody (3F10). Immunodetection of α -tubulin is shown as loading control. (C) Incorporation of Pes1 BRCT-domain mutants into the PeBoW-complex. H1299 cells stably transfected with the indicated constructs were treated with doxycycline for 48 h to induce expression of the respective proteins. Cell lysates were subjected to immunoprecipitations with antibodies either directed against the HA-tag (3F10), WDR12 (1B8), Bop1 (6H11) or the isotype control (cytohesin). Equivalent amounts of total lysates used for immunoprecipitations were loaded in each lane or 20% thereof for the input. Immunodetection was performed using the anti-HA antibody. (D) Total cell lysates of stably transfected H1299 cells expressing HA-tagged wild-type Pes1 or the indicated mutant together with HA-tagged rodent Bop1 were separated by native gel electrophoresis. Immunodetection was performed using antibodies specific for the HA-tag and rodent Bop1. Denaturing gel electrophoresis and HA-tag immunodetection was performed to control expression levels.

the nucleolar PeBoW-complex (Pes1, Bop1 and WDR12) is crucial for their stability.

Point mutations within the BRCT-domain of Pes1 prevent its incorporation into the PeBoW-complex

Therefore, we performed co-immunoprecipitation studies using antibodies specific for the HA-tag, WDR12, Bop1 and cytohesin, as an isotype control (8,21). Immunodetection was carried out using the HA-tag antibody to test whether the Pes1 BRCT-domain mutants could be co-precipitated with antibodies directed against the two other endogenous PeBoW-components, Bop1 and WDR12 (Figure 5C). Indeed, Pes1wt and Pes1-R380W efficiently co-precipitated with Bop1 and WDR12. However, the nucleoplasmic mutants Pes1-W397R, -I347R and -F327R were not detected in the respective immunoprecipitations. In a previous study, the region of Pes1 required for the interaction with Bop1 was identified by a yeast-two-hybrid assay and localized in the N-terminal region (~200 amino acids) and therefore distant from the BRCT-domain (22). A recent study also demonstrated that the N-terminal region of Pes1 is sufficient and essential for *in vitro* interaction with Bop1 (34). Importantly, a Pes1 mutant harbouring a deletion of the BRCT-domain (corresponding to the nucleoplasmic mutant M9 of our study) precipitated *in vitro* translated Bop1 as efficiently as wild-type Pes1 in glutathione *S*-transferase (GST)-pull down assays. These experiments suggested that deletion or point mutations of the BRCT-domain only play a critical role for the Pes1–Bop1 interaction under *in vivo* conditions. Aberrant nucleoplasmic localization is one possible mechanism that may prevent *in vivo* interaction with endogenous Bop1. However, additional mechanisms may contribute *in vivo* as well. As endogenous Bop1 might be rate limiting for Pes1–Bop1 complex formation we analysed complex assembly by native gel electrophoresis upon enforced expression of Pes1 and Bop1. Wild-type Pes1 and Pes1-R380W efficiently formed a stable complex with Bop1 under these conditions. However, either deletion or the other point mutations of the BRCT-domain abrogated complex formation (Figure 5D). Thus, deletion or critical point mutations of the BRCT-domain of Pes1 prevent its interaction with the endogenous PeBoW-components *in vivo*, whereas its physical interaction with Bop1 under *in vitro* conditions apparently remains unaffected, as recently shown.

DISCUSSION

Several recent studies have underlined the importance of the nucleolus in sensing cellular stress (2,7,11,14). The tremendous metabolic challenge of ongoing ribosome biogenesis is controlled by growth factor and stress signalling cascades, as well as the cell cycle machinery (3). Likewise, disturbed ribosome synthesis impairs cell cycle progression by activating the p53 pathway (8–10). Studying the coordination of cell cycle progression and ribosome biogenesis requires profound knowledge of both processes. The conveniences of yeast genetics, such as the generation of temperature sensitive mutants have provided deep insides into the complex eukaryotic ribosome synthesis machinery (15). However, classical genetic knock-out approaches in mammalian cells are laborious, in particular, if required in a conditional manner for

essential genes, such as many ribosome synthesis factors like Pes1 presumably might be (19). The siRNA mediated knock-down technique now provides a rapid and powerful tool for the analysis of factors involved in mammalian ribosome biogenesis. In this study we showed that the combination of conditional gene expression and siRNA mediated knock-down techniques enabled the functional analysis of mutant Pes1 proteins in the absence of a dominant-negative phenotype. First, we noticed that exogenous wt Pes1 closely resembled the endogenous expression levels after the prolonged incubation (7 days) with doxycycline and substituted for endogenous Pes1 in rRNA processing. Interestingly, exogenous wt Pes1 exceeds the endogenous expression levels, when cells were harvested one day after the addition of doxycycline (data not shown). Hence, the Pes1 protein level apparently is tightly controlled in mammalian cells and excess amounts of Pes1 might be subject to proteasomal degradation. Interestingly, polyubiquitination bands were virtually not detected with the endogenous Pes1 upon treatment with proteasome inhibitors (data not shown), however easily with the overexpressed exogenous Pes1 when incubated with doxycycline for one day.

Next, we found that M6, lacking the acidic regions, reconstituted for depletion of endogenous Pes1 in rRNA processing. Acidic domains are frequently found in nucleolar proteins, such as nucleophosmin and nucleostemin, but so far a common function remains elusive. Deletion of the acidic domain of nucleostemin did not interfere with its nucleolar localization (35). In nucleophosmin, the acidic regions were found to be crucial for its interaction with the tumour suppressor p19ARF (32). Hence, the acidic domain of Pes1 might mediate unknown protein–protein interactions, as its deletion does not interfere with incorporation into the PeBoW-complex (21). Interestingly, truncation of the acidic domain of Pes1 in conjunction with the very C-terminal region (M5) results in a nucleolar localized dominant-negative mutant that blocks rRNA processing. However, removal of the C-terminal region only, is insufficient to elicit a dominant-negative phenotype (21). Thus, additional removal of the acidic regions apparently contributes to the inhibitory effects of M5. Nevertheless, further investigations are required to explore the role of the acidic domains of Pes1. We cannot exclude that the acidic regions of Pes1 have any impact on ribosome maturation and may influence overall ribosomal stability, but they are not required for the processing of the 32S rRNA precursor into the mature 28S rRNA. Deletion of the BRCT-domain of Pes1 caused diffuse nucleoplasmic localization and failure to sustain rRNA processing in Pes1 depleted cells. This is remarkable, as the mutant M10, consisting of the Pes1 N-terminal domain only and hence lacking the BRCT-domain, localizes properly to the nucleolus. The Pes1 N-terminal domain (Pfam-domain: PF06732) exhibits the highest degree of evolutionary conservation and apparently is sufficient to direct itself into the nucleolus. Thus, the BRCT-domain is required for nucleolar localization only in the context of the full-length Pes1 protein. These observations are reminiscent of the ones reported for the nucleolar GTP-binding protein nucleostemin. Single residue exchanges within the GTP-binding domain resulted in diffuse nucleoplasmic distribution. Additional removal of an extended region, termed internal-domain (I-domain),

restored nucleolar localization and relieved the nucleoplasmic holdback (35). It will be interesting to explore whether so far unknown regions within the Pes1 protein cooperate with the BRCT-domain in the control of the nucleolar localization.

BRCT-domains are frequently found in factors involved in DNA-damage and repair pathways and are thought to mediate phosphorylation dependent protein–protein interactions (29). Occasionally, the BRCT-repeats specifically contact BRCT-repeats in other proteins, as it has been reported for the interactions of XRCC1 with DNA ligase III (36). Most frequently, however, BRCT-repeats interact with protein domains of different structure. For example, the tandem BRCT-repeats of 53BP1 contact the DNA-binding domain of p53 (37). Only a few BRCT-repeat containing proteins were identified in the nucleolar proteome (<http://Lamondlab.com/NOPdb/>), such as XRCC1 and TOPBP1 (topoisomerase II binding protein 1). XRCC1 functions as scaffold protein in the base excision response and is therefore directly linked to DNA repair. To our knowledge, Pes1 is the only rRNA processing factor harbouring a BRCT-domain. Yeast strains with point mutations in the BRCT-domain of Nop7p (Yph1p), the respective homologue of Pes1, exhibited a temperature sensitive growth (20,27). However, subcellular localization of Nop7p and rRNA processing were not investigated in these strains. Therefore, we inserted analogous and novel point mutations in highly conserved residues of the BRCT-domain of Pes1 and analysed the subcellular localization and functionality in rRNA processing of the respective mutants. The W397R exchange was performed in analogy to the W1837R cancer predisposing mutation in BRCA1 (27). The BRCT-repeat of Pes1 exhibits a higher homology to the second BRCT-repeat of BRCA1 than to the first repeat. Based on structural analysis W1837 is located within the $\alpha 3'$ α -helix of the second BRCT-repeat of BRCA1 and destabilizes the BRCT-repeat (29,38,39). ClustalW-alignment algorithm suggests an identical position for W397 of Pes1 within the $\alpha 3$ α -helix of the BRCT-domain (Supplementary Figure 5). Thus, the W397R mutation of Pes1 might also result in specific BRCT folding defects. The I347R substitution is predicted to be located within the $\alpha 1$ α -helix and might affect the secondary structure in a similar way. No point mutation in BRCA1 corresponding to I347R in Pes1 has been described so far, besides an adjacent M1783T exchange. Further, a BIC-database (Breast Cancer Information Core Database) research reveals a F1761S mutation in BRCA1 that correlates with the F327R exchange in Pes1. In conclusion, a comparison of structural elements and missense mutations in BRCA1 with the BRCT-repeat of Pes1 suggests a crucial role for the residues W397, I347 and F327. No such correlations were found for the Pes1-R380W mutant.

Disturbing the integrity of the BRCT-domain by deletion or by point mutations of critical residues resulted in aberrant diffuse nucleoplasmic distribution of Pes1. Further, the nucleoplasmic BRCT-mutants (Pes1-M9, -W397R, -I347R and F327R) exhibited no obvious accumulation in the cytoplasm or formation of precipitates that would be suggestive of general defects in protein folding. Thus, we suppose that the mutations within the BRCT-repeat do not affect the overall structural integrity but rather impose specific conformational changes of the BRCT-domain or defects in protein–protein interactions mediated by the BRCT-domain.

Therefore we favour the view of a specific regulatory mechanism that requires an intact BRCT-domain for proper nucleolar localization of Pes1. The nucleoplasmic BRCT-mutants also failed to interact with the endogenous PeBoW-components Bop1 and WDR12. Only Pes1wt and Pes1-R380W were efficiently co-immunoprecipitated. In line with these observations Pes1-W397R, -I347R and F327R could not compensate for depletion of endogenous Pes1 in rRNA processing assays. Bop1 interacts with Pes1 via a region (~200 amino acids) located in the Pes1 N-terminal domain and thus quite distant from the BRCT-domain (22). Therefore, disabling the nucleolar localization of Pes1 by deletion or point mutations within the BRCT-domain precludes its interaction with endogenous Bop1 and WDR12, even though the interaction site with Bop1 is not affected by the mutagenesis. Importantly, a recent study demonstrated that deletion of the BRCT-domain of Pes1 (corresponding to our Δ BRCT-mutant M9) did not interfere with its capacity to interact with Bop1 *in vitro* (34). Consistent with previous studies, the N-terminal domain of Pes1 was essential and sufficient for *in vitro* interaction with Bop1 (21,22). Thus it is unlikely, that a general folding defect of the Δ BRCT-mutant impairs Bop1 interaction and nucleolar localization *in vivo*, as demonstrated in our study. But nucleoplasmic sequestration may not be the only mechanism that prevents the interaction of the Δ BRCT-mutant (M9) or the respective BRCT point mutants with Bop1 under *in vivo* conditions. Coordinated overexpression of the Pes1 mutants together with Bop1 failed to recapitulate the *in vitro* results, even though we were able to efficiently produce stable complexes of ectopic wild-type Pes1 and Bop1. In principal, providing excess amounts of the Pes1 BRCT-mutant and Bop1 should have at least partially circumvented the regulatory impact of subcellular compartmentalization. It will be interesting to further investigate the role of the BRCT-domain of Pes1 for PeBoW-complex assembly and to explore the mechanism that prevents Pes1 BRCT-mutant interaction with Bop1 *in vivo*. Nevertheless, failure to interact with the endogenous PeBoW-complex might then negatively affect the stability of the BRCT-mutants. Indeed, the stability of PeBoW-proteins is interdependent, and knock-down of Bop1 reduces the level and stability of Pes1 (M. Rohrmoser *et al.*, manuscript submitted). This argues for a highly controlled assembly of Pes1 with the remaining PeBoW-members.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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