

Redundant role of DEAD box proteins p68 (Ddx5) and p72/p82 (Ddx17) in ribosome biogenesis and cell proliferation

Carolyn Jalal, Heike Uhlmann-Schiffler and Hans Stahl*

FR 2.3 Medical Biochemistry and Molecular Biology, Theoretical Medicine, University of the Saarland, D-66421 Homburg, Germany

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ABSTRACT

The DEAD box proteins encoded by the genes *ddx5* (p68) and *ddx17* (isoforms p72 and p82) are more closely related to each other than to any other member of their family. We found that p68 negatively controls p72/p82 gene expression but not vice versa. Knocking down of either gene does not affect cell proliferation, in case of p68 suppression, however, only on condition that p72/p82 over-expression was granted. In contrast, co-silencing of both genes causes perturbation of nucleolar structure and cell death. In mutant studies, the apparently redundant role(s) of p68 and p72/p82 correspond to their ability to catalyze RNA rearrangement rather than RNA unwinding reactions. In search for possible physiological targets of this RNA rearrangement activity it is shown that the nucleolytic cleavage of 32S pre-rRNA is reduced after p68 subfamily knock-down, most probably due to a failure in the structural rearrangement process within the pre-60S ribosomal subunit preceding the processing of 32S pre-rRNA.

INTRODUCTION

p68 and p72/p82 are prototypic members of the DEAD box protein family (1,2) with the isoforms p72 and p82 arising from the *ddx17* gene through the use of different in-frame translation initiation codons (3). The three proteins form a subfamily with similar biochemical activities, including an ATP-dependent RNA helicase (4,5), and possibly also similar or even redundant cellular functions. The products of both genes are important transcriptional regulators, functioning as co-activators (6,7) and/or co-repressors (8) depending on the context of the promoter and the transcriptional complex in which they occur. They also seem to be involved in alternative splicing (9,10) where their target genes differ, however.

It is unclear, if/how the RNA-specific biochemical activities of p68 and p72/p82 are involved in these processes as well as in DNA deglycosylation (11), though at least some of the functional complexes formed contain small RNAs (6,11). Different specific functions of isoforms p82 and p72 are not known.

In *Saccharomyces cerevisiae*, the (only) homolog of the p68 subfamily proteins, Dbp2p (12), functions in the nonsense-mediated mRNA decay pathway and plays a role in ribosomal RNA (rRNA) processing (13). Ribosome assembly starts in the nucleolus by formation of a 90S particle from non-ribosomal and ribosomal proteins and a single pre-rRNA primary transcript (35S in yeast cells, 47S in mammals). Within the 90S pre-ribosome the primary transcript undergoes rapid cleavage steps, which separate the precursors to the large and small subunits. Within the precursors to the large subunit (60S pre-ribosomal particles) the pre-rRNA is further matured to the large rRNA (25S RNA in yeast, 28S RNA in mammals) and 5.8S rRNA. The early pre-60S particles are restricted to nucleoli, but later maturing forms are released into the nucleoplasm and are eventually exported to the cytoplasm (14–16). From this, a nucleoplasmic role of Dbp2, and possibly also p68, in rRNA processing must be envisaged, since both proteins are excluded from nucleoli in interphase cells (17) and have recently been shown to be associated with (at least in part) nucleoplasmic forms of 60S pre-ribosomes (18, for a review see also 19). Only in late mitosis, are the p68 subfamily proteins detected in the reorganizing nucleoli (prenucleolar bodies) where they may additionally be involved in the structural (re)organization process (17).

DEAD box proteins are believed to act as ATP-dependent modulators of RNA structure, and, not surprisingly, several family members seem to assist the structural rearrangement of pre-rRNA in the course of its correct processing and folding within pre-ribosomes (20,21). However, an understanding of their exact roles in ribosome biogenesis is still lacking. At least some of the protein-catalyzed rRNA structural rearrangements may

*To whom correspondence should be addressed. Tel: +49 6841 16 26020; Fax: +49 6841 16 26521; Email: bchsta@uniklinikum-saarland.de

proceed via a branch migration type of mechanism in which portions of two competing secondary structures transiently coexist (22,23) and which has been shown to be used in RNA rearrangement reactions catalyzed by p68 and p72 (5) or by the cyanobacterial DEAD box RNA helicase CrhR (24).

Here, we report that either p68 or p72/p82 is essential for pre-rRNA maturation and that knock-down of both genes induces cell death. Additional data suggest a molecular mechanism by which the p68 subfamily proteins, with the participation of U8 small nucleolar RNA (snoRNA), may promote an RNA structural rearrangement within the pre-60S ribosomal subunit, indispensable to the timed endonucleolytic cleavage of pre-28S rRNA.

MATERIALS AND METHODS

Plasmids, siRNAs and transfections

To generate expression plasmid pCMVp68-wt, the coding sequence of pUHDp68KT (25) was amplified by use of primers pCIneo-fwd and His-KT3-rev (see Table 1) and cloned into the XbaI/BamHI-restriction site of pCMV (3). p68-mutant plasmids pCMVp68DQAD and pCMVp68GNT were generated from pCMVp68-wt using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Plasmids pGEM-U8 and pGEM-U14 were constructed by insertion of the U8- (26) or U14- (27) cDNA in 3' to 5'-orientation into the MCS of vector pGEM7zf(-) (Promega). All DNA constructs were confirmed by direct DNA sequencing. For protein expression, cells were transfected twice at an interval of 24 h by use of FuGENE6 (Roche) as described in the user manual.

siRNAs with 3'-dTdT-overhangs were produced by Qiagen and covered the sequences shown in Table 1. siRNA transfections were carried out using HiPerFect (Qiagen) as a transfection reagent with 10–20 nM siRNA following the manufacturer's protocol.

Table 1. siRNAs and primers used

(a) siRNAs (sense strand 5' → 3')	
Control (Qiagen)	UUCUCCGAACGUGUCACGU
p68	CCGCAACCAUUGACGCCAU
p68 _{alt}	CUCUAAUGUGGAGUGCGAC
subfamily	GCCUAGAUGUGGAAGAUGU
p72/p82	GAGGCCAAUCAGGCCUAUCA
p72/p82 _{alt}	UACACCUAUGGUCAAGGCA
(b) Primers	
pCIneo-fwd	GGGGTCTAGACTGCAGGTGAG GCACTGGGCAGGTAA
HIS-KT3-rev	ACAGGGATGCCACCCGGGATCCTTATG TCTCAGGCTCTGGTGGGTGATGGTGAT GATGATGTTGGGAATATCCTGTTGGCA CAAACATGGAAAAGCTCCTATT
p68-fwd	CTCTGTCTTCGACCAACTGA
p68-rev	TATGTGTATCCATGGAGACAAGAGTC
p72/p82-fwd	CTGCATCAGTTTTGGATTGAT
p72/p82-rev	GCTGGCTGCTCCAGTTCGAT
i11-p68-fwd	GCACATGTCTGTATAATCA
i11-p68-rev	CAATCCCAGCAGTAGAGAGAA
i11-p72/p82-fwd	GTCAATGATGAGTCAGTGTGTA
i11-p72/p82-rev	

Cell proliferation and FACS analysis

Cells were cultured at 37°C and 5% CO₂ in DMEM with 10% FCS. For colony formation assays, 5 × 10² or 10³ cells were plated in 10-cm dishes the day after transfection and after 7–14 days the resulting colonies were fixed in 5% glutaraldehyde and stained with 1% crystal violet in PBS (137 mM NaCl, 2.6 mM KCl, 6.5 mM Na₂HPO₄, 1.4 mM KH₂PO₄ pH 7.4). Incorporation of 5-bromo-2'-deoxy-uridine (BrdU) was measured using the respective Roche Kit I. Photographs are representative of the whole slide. For FACS analysis, HeLa cells were fixed with ice cold 70% ethanol for 30 min at –20°C 5 days after siRNA transfection followed by an incubation with 1/10 vol. RNase A (1 mg/ml) and 1/10 vol. propidium iodide (500 µg/ml) for 30 min at 37°C and analyzed with the FACScan (Becton Dickinson). Growth kinetics were determined for three independent cultures of each siRNA transfection and counted in duplicate. The resulting mean values are given.

RT-PCR and northern analysis

Total RNA was prepared using the RNA Kit II (Invitex), and cDNA was synthesized with RevertAidTM M-MuLV Reverse Transcriptase (Fermentas) with either oligo-dT or gene-specific 3'-primers. For relative semiquantitative PCR analyses, Taq-DNA polymerase (Fermentas) and a serial dilution of RT-generated cDNA first strand were used to produce amplified DNA products in the linear range. The amplified products were normalized to those specific for host genes β-actin or histone 2A and analyzed by agarose gel electrophoresis. Primer pairs were used as shown in Table 1. For GAPDH, β-actin and histone primers see (28). Northern blot analysis was performed as described elsewhere (3) with digoxigenin-labeled negative strand full-length U8 or U14 snoRNA as a probe (obtained by *in-vitro* transcription of plasmids pGEM-U8 and -U14 and detected according to the Roche DIG Northern Starter Kit manual) or with ³²P end-labeled DNA oligonucleotides specific for human Second internal transcribed spacer (ITS2), 18S, 28S or 5,8S rRNA (29).

Analysis of rRNA processing

rRNA processing was monitored by pulse-chase experiments. HeLa cells were starved of methionine for 60 min and then pulse labeled with 2.22 MBq/ml L-(methyl-³H)-methionine (Amersham) for 60 min. Thereafter, cold methionine (15 µg/ml) was added in order to chase the label for 60 or 120 min. From all aliquots, total RNA was extracted as described above and the incorporated radioactivity measured by liquid scintillation counting. Equal amounts of radioactivity were loaded onto a 1% agarose denaturing gel. The RNA was fractionated and transferred onto a nylon membrane (Roche). The membrane was dried, sprayed with EN³HANCE (Perkin Elmer) and exposed to Fuji medical X-ray films at –80°C for 2 days.

Antibodies, protein analyses and RNA structural rearrangement reactions

For monoclonal antibody C10, see (5), for rabbit polyclonal α -human p72/p82 antibodies, see (3) and for monoclonal PAb421 see (30). Monoclonal α -fibrillarin antibody 72B9 was a gift of Prof. U. Scheer, University of Würzburg, and rabbit α -p19^{ARF} antibodies were provided by Prof. M. Montenarh, University of the Saarland. α - β -actin antibodies were from SIGMA, α -B23 antibodies as well as α -p53 antibodies DO-1 from Santa Cruz, [α -PARP] antibodies from Pharmingen and α -His antibodies from Qiagen. FITC-conjugated as well as TRITC-conjugated secondary antibodies were from Molecular Probes and horseradish peroxidase-conjugated ones from SIGMA.

Western blotting experiments were performed as described by (3) using ECL (Roche) for detection. For indirect immunofluorescence, cells were grown on coverslips, fixed in 3.7% formaldehyde in PBS for 7 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS + 1% BSA for 6 min on ice and stained with the indicated antibodies. FITC- or TRITC-conjugated secondary antibodies were added for 60 min at room temperature at 1:1000 dilution. Samples were analyzed with a fluorescence microscope (Zeiss Axioscop).

For isolation of wt-p68 and its mutants, COS cells (1×10^8), transfected with the respective expression plasmids for 4 days, were extracted as described by (5) except that the nuclear extraction buffer contained 4 mM EDTA, which was subsequently removed by dialysis before the recombinant proteins were purified by affinity chromatography on Ni²⁺ NTA-cellulose and ssDNA-cellulose as described (5). Purified proteins were stored at -70°C . RNA structural rearrangement reactions and preparation of the used RNA substrates were performed exactly as described previously (5).

ATP binding by wild-type (wt) or mutant p68 was analyzed by UV-induced photo-cross-linking as described (31). Briefly, p68 or one of its mutants (300 nM) was incubated in a buffer containing 20 mM Tris-HCl (pH 7.5), 70 mM KCl, 5 mM magnesium acetate, 0.11 MBq of [α -³²P] ATP (110 TBq/mmol), 10% glycerol, 1.5 mM dithiothreitol (DTT) for 10 min at 37°C. Samples were placed on ice and brought under the UV cross-linker about 4 cm under the light source. The samples were irradiated for 4 min, boiled in SDS-PAGE sample buffer, and after addition of unlabeled ATP (final concentration of 4 mM) subjected to SDS-PAGE (10% polyacrylamide). After electrophoresis, the gel was stained with Coomassie blue, dried and processed for autoradiography.

ATPase assays were performed as described previously (32). Briefly, p68 (or one of its mutants; 30 nM) was incubated in ATPase assay buffer containing 25 mM HEPES pH 7.8, 5 mM MgCl₂, 100 mM NaCl, 1 mM DTT, 0.01% albumin and 10% glycerol and 20 μM [γ -³²P] ATP (13 Bq/pmol) for 20 min at 37°C. The unreacted ATP was precipitated by acid-washed charcoal (20% in 0.25 M HCl, 0.25 M H₃PO₄), and after centrifugation the free ³²PO₄³⁻ in the supernatant was counted in Aquasol (Dupont). ATPase activities were determined

three times for each protein. The resulting mean values are given.

RNA helicase assays were performed in ATPase assay buffer containing 10 U/sample RNase inhibitor, 4 mM ATP, 35 nM (in nucleotides) helicase substrate [17 bp RNA; (4)] and 10 nM wt or mutant p68 in a final volume of 20 μl as previously described (5).

Analysis of the profile of ribosomal subunits and U8 snoRNPs

For profiling of ribosomal subunits, HeLa cells were sonicated 2×15 s in 40 mM Tris-HCl pH 8.5, 150 mM NaCl, 0.1% NP40. After centrifugation at 10 000 r.c.f. for 10 min, the supernatant was adjusted to 30 mM EDTA, layered onto a linear 10–30% sucrose gradient (w/v) in 20 mM Tris-HCl pH 7.6, 30 mM KCl, 1 mM MgCl₂, 6 mM β -mercaptoethanol and centrifuged in a Beckman L-60 ultracentrifuge for 200 min at 37 000 r.p.m. with a Beckman SW41Ti-rotor. The absorbance of each fraction was measured at 260 nm.

For sedimentation analysis of U8 snoRNPs, equal absorption units (OD₂₆₀) of HeLa cell extracts were analyzed on sucrose gradients as described above except that the sonication buffer contained 300 mM NaCl instead of 150 mM. From each fraction RNA was prepared and analyzed for U8 snoRNA by northern blot analysis.

RESULTS

Knock-down experiments reveal a negative expression control of p68 over p72 and p82 and no essential function of p68 subfamily proteins in general splicing

Different siRNAs were designed to knock-down the p68 subfamily proteins by RNA interference (RNAi; Table 1). In HeLa cells, the p68 level was reduced effectively and specifically (by almost 95% compared to control siRNA) after transfection of a siRNA (p68 siRNA) complementary to the translational start site of the p68 mRNA (Figure 1A). The absence of p68 led to a remarkable increase in cellular p72 and p82 protein and mRNA levels (but not vice versa), while that of other genes, like β -actin, remained constant (Figure 1A and B). Therefore, the possibility that p68 negatively controls the expression of p72 and p82, like that of its own, at the level of splicing was considered (3,12,25). As previously reported, we found an accumulation of partially spliced p72/p82 transcripts in HeLa cells (3,25) that may result from this negative expression control. In fact, by p68 depletion the level of these transcripts could be reduced (Figure 1B, lanes 6 and 7).

In contrast to such a rather specific effect, Lin *et al.* (28) reported that p68 is a basic human splicing factor (essential e.g. for the splicing of β -actin and GAPDH pre-mRNA) and these authors also speculated on functional redundancy of p72 and p68 in this function. Thus, we checked for deficiencies of essential mRNAs in HeLa cells after knock-down of one or the other as well as both DEAD box genes at once. However, relative quantitative RT-PCR analyses did not reveal any reduction in the β -actin and GAPDH mRNA after siRNA transfection for 2 days (Figure 1B) or longer (e.g. 3, 4 and 5 days;

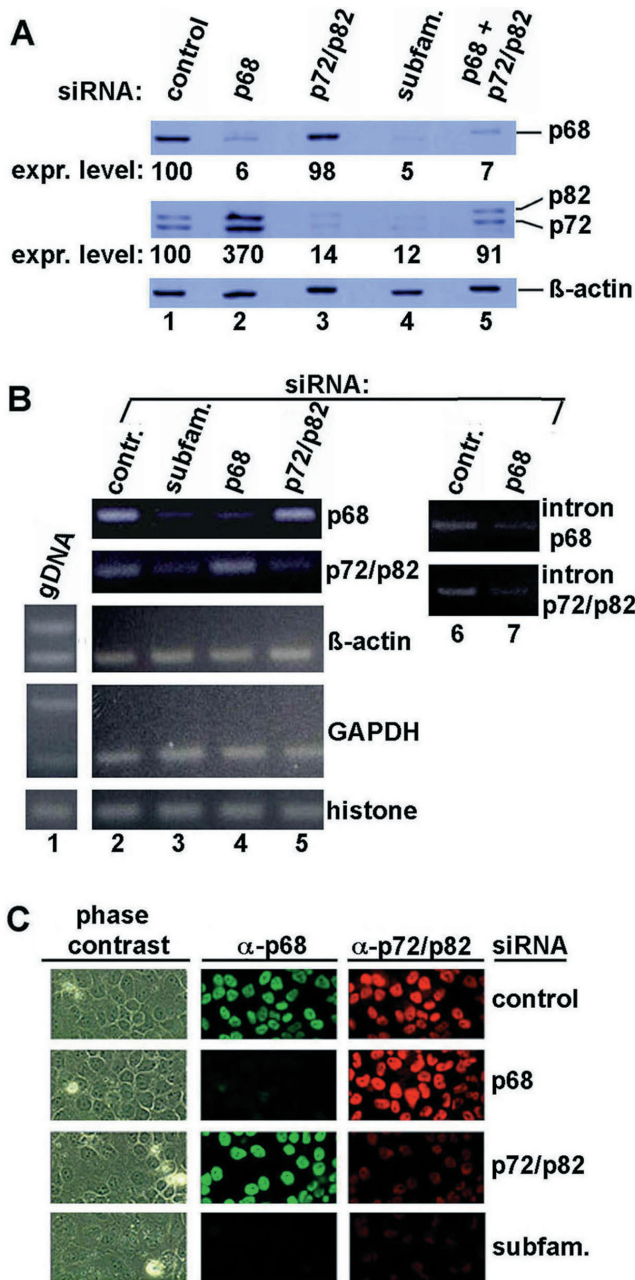


Figure 1. p68 negatively controls p72/p82 expression but is not involved in general splicing. (A) Down-regulation of p68 subfamily proteins by RNAi. HeLa cells were transfected with the indicated siRNAs, harvested 55h post-transfection and analyzed by western blotting with β -actin used as a loading control. Protein bands were quantitated by a Molecular Dynamics densitometer and expression levels of the individual proteins are given relative (%) to control siRNA-transfected cells (control). (B) Influence of down-regulation of p68 subfamily proteins on mRNA expression. Total RNA extracted from HeLa cells transfected with the indicated siRNAs for 45h was analyzed by semiquantitative RT-PCR and agarose gel electrophoresis. siRNAs used for transfection are indicated on top and genes analyzed by the respective primers are indicated on the right. For β -actin and GAPDH essentially the same results as those shown were obtained with RNA prepared 3 and 5 days after transfection (data not shown). As a control, PCRs were performed with genomic DNA (gDNA) as a template, which also contains respective pseudogenes that give rise to the same signals as their mRNAs. (C) Immunofluorescence of siRNA-transfected HeLa cells. Cells were double-immunostained with the indicated antibodies 55h post-transfection with the shown siRNAs.

data not shown), when the intronless histone H2A.X mRNA, not expected to be affected by possible splicing defects, was used to normalize the RT-PCR products. Furthermore, no DNA fragments representing unspliced transcripts of β -actin or GAPDH genes were detected by the same RT-PCR analysis, though the primers span an intron in both genes (Figure 1B). Those PCR signals were obtained, however, in control reactions performed with genomic DNA. Taken together, these results do not hint at an essential function of p68 and/or p72/p82 in general splicing.

We note that the knock-down experiments reported above and also those described below were confirmed by use of another p68- and p72/p82 siRNA (siRNAs_{alt}, see Table 1) as well as of other cell lines (HaCaT, MCF7; data not shown). The high rates of protein suppression obtained reflect similar or even higher siRNA transfection efficiency, confirmed by immunofluorescence analysis of respective cells (Figure 1C).

Some sequence identity at the cDNA level of p68 and p72/p82 enabled us to find a siRNA directed to both p68 and p72/p82 (called here as subfamily siRNA), which efficiently co-suppressed all three proteins (Figure 1A and B, lanes 4 and 3, respectively). On the other hand, mixing of a p68- and p72/p82 siRNA in co-transfection experiments made it possible to titrate the absolute cellular protein levels and to prevent, for example, an increase in p72 and p82 while simultaneously maximally suppressing p68 (Figure 1A, lane 5). Notably, the ratio of p72 to p82, translated at roughly similar rates from one mRNA (3), was unaffected by any siRNA used.

Effects of p68 subfamily proteins on cell proliferation

Though it has been speculated that p68 is essential for cell proliferation (33,34), its efficient knock-down as well as that of p72/p82 (to ~5 and 15% of the control, respectively; see Figure 1A) did only slightly alter the proliferation rate of HeLa cells (Figure 2A and B) and their distribution in G₁, S and G₂ phases as revealed by flow cytometry (FACS) analysis (Figure 2C). In contrast, after co-suppression of all three DEAD box proteins, the proliferation of HeLa cells stopped (Figure 2A) and their DNA replication was blocked (data not shown). Eventually, they became abnormally flat resembling serum-starved cells, and the size of their nucleoli decreased (see Figure 3A). A release of the nucleolar proteins fibrillarin, B23 and p19^{ARF} into the nucleoplasm was observed (through which the staining is softened down due to the distribution of the proteins within the whole nucleus; Figure 3A), and the cellular levels of fibrillarin and B23 were moderately reduced (by ~15%) most probably due to their mislocation (35) whereas that of p19^{ARF} remained unchanged (Figure 3B). At the same time, a clear decrease of cells in G₁ phase and an increase of the sub-G₁ population were observed, the latter indicating cell death (Figure 2C). Accordingly, co-depletion of all p68 subfamily proteins strongly reduced the clonogenic survival of HeLa, MCF7 and COS cells by >95% as compared to the control, while most cells (about 80%) with suppressed p68 or p72/p82 survived in this

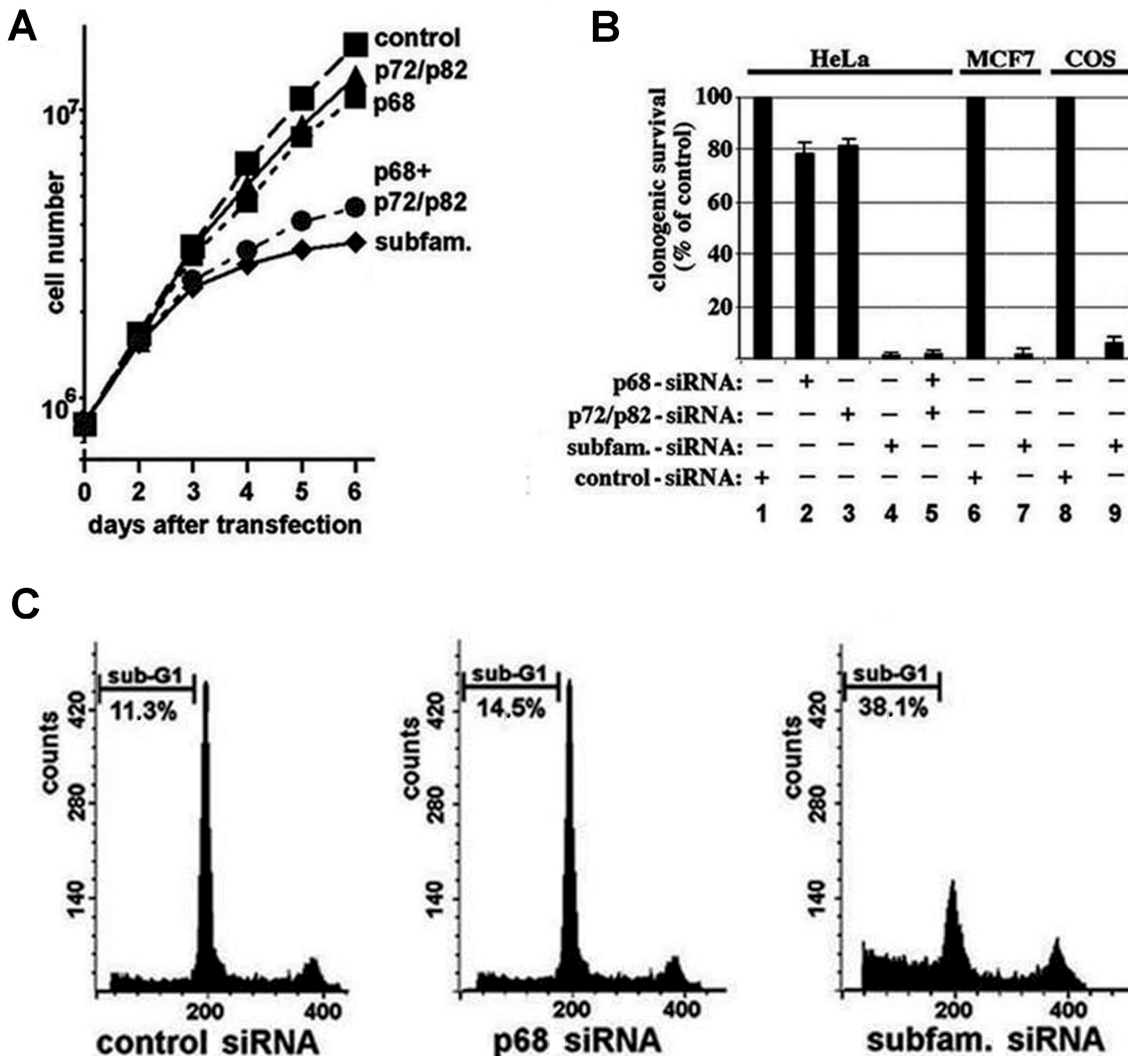


Figure 2. Effects of p68 subfamily proteins on cell proliferation and viability. (A) Growth curves of HeLa cells after transfection with the indicated siRNAs. Numbers of respective cells were counted at the indicated time points. Representative data of three independent experiments are shown. (B) Clonogenic survival of HeLa, MCF7 and COS cells after depletion of p68 and/or p72/p82 relative to control siRNA-transfected cells (% of control). The mean and standard deviation of three experiments are shown. (C) Cell death caused by simultaneous down-regulation of p68 and p72/p82. HeLa cells were transfected twice with the indicated siRNAs, harvested 5 days after the first transfection and stained with propidium iodide. The cell cycle distribution was determined by flow cytometry. Percentage of cells in sub-G₁ phase is indicated.

assay (Figure 2B). Taken together, these results indicate that p68 and p72/p82 have a redundant function which is essential for cell proliferation. Notably, the proliferation of the cells was also strongly reduced by co-transfection of individual p68- and p72/p82 siRNAs (Figure 2A and B), and even at a ratio that caused maximal suppression of p68, but kept a nearly constant level of p72/p82 (preventing its increase, data not shown, but see Figure 1A, lane 5). Therefore, the 'normal' (physiological) cellular level of p72/p82 does not preserve cell proliferation in the absence of p68, whereas that of p68, vice versa, clearly does.

Co-depletion of p68 and p72/p82 does not alter the level of p53 in HeLa cells (Figure 3C). Thus, the function of the human papilloma virus oncogene E6, which acts as an antiapoptotic factor by inducing degradation of p53 in

HeLa cells (36), seems not to be affected by our cell manipulations. Accordingly, no induction of PARP cleavage was observed (Figure 3C), confirming a p53-independent way of cell death in the absence of p68 and p72/p82. On the other hand, human breast cancer cells MCF7 (expressing wt p53), which displayed a similar low clonogenic survival upon co-suppression of p68 and p72/p82 (Figure 2B, lane 7), showed an increase in their p53 level, apparently resulting in apoptotic death as evidenced by PARP cleavage indicative of caspase 3 activity (Figure 3C). Thus, our results confirm that inhibition of ribosome biogenesis can induce cell death in a p53-dependent and -independent manner as has been shown before by depletion, e.g. of transcription factor TIF-IA (37) and B23 (38), respectively.

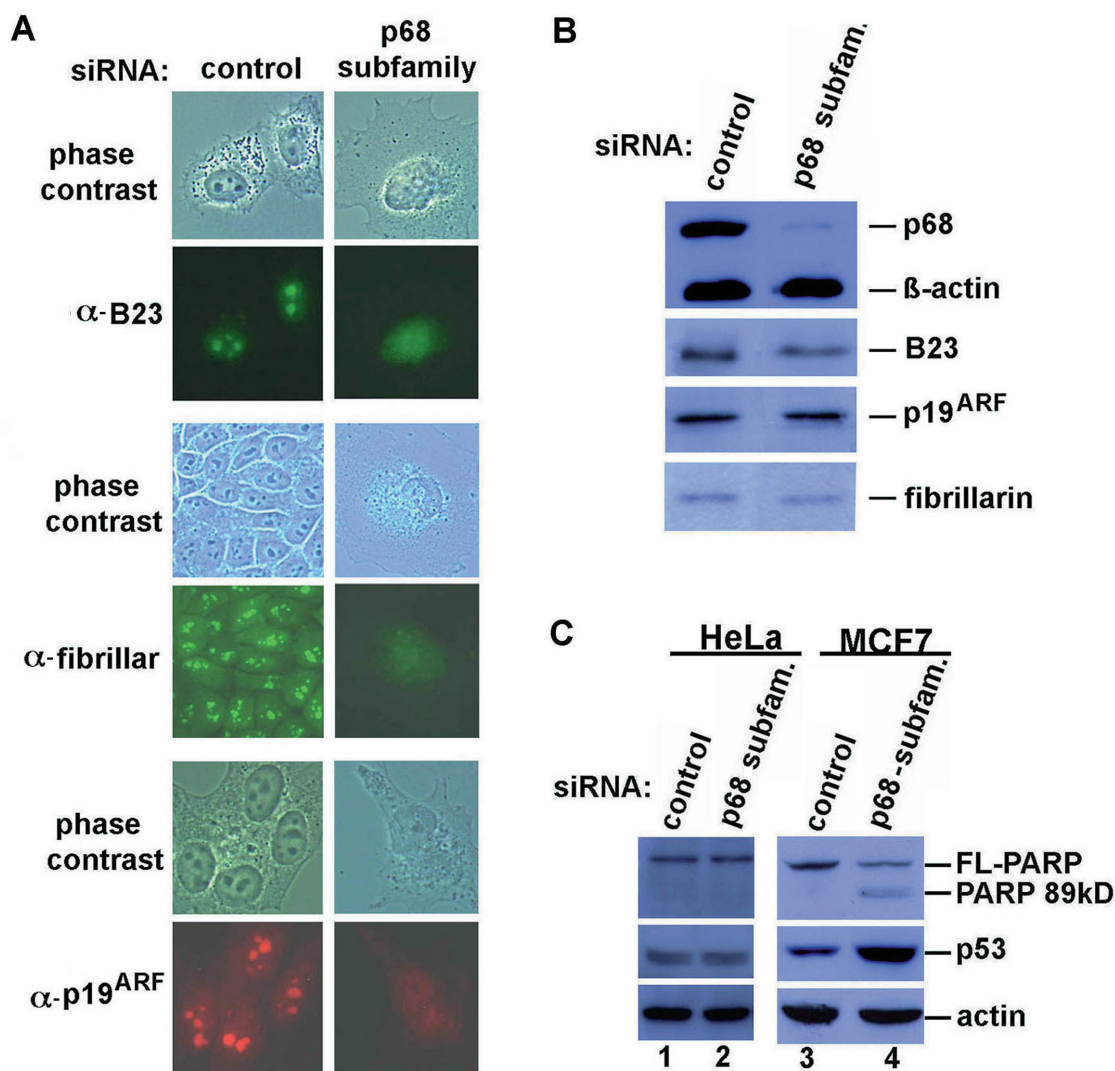


Figure 3. Destruction of nucleolar structure and induction of death signals after co-knock-down of p68 and p72/p82. (A) Nucleolar proteins are released into the nucleoplasm after p68 and p72/p82 co-knock-down. HeLa cells were transfected with control siRNA (control) or p68 subfamily-siRNA and the cellular localization of B23, fibrillarin and p19^{ARF} was visualized by indirect immunofluorescence 5 days after transfection. Phase contrast images are shown as indicated. (B) Levels of B23, fibrillarin and p19^{ARF} in HeLa cells were analyzed by Western blotting 55 h co-knock-down of p68 and p72/p82. (C) Western blot of HeLa and MCF7 cells with antibodies against p53 and PARP after transfection for 4 days with control siRNA (control) or p68 subfamily siRNA. The position of full length (FL) PARP and its 89-kD degradation product (PARP 89kD) is indicated.

A p68 mutant without ATP-binding activity shows a dominant negative phenotype

Mutant and wt p68 were transiently overexpressed in COS cells from expression plasmids containing a simian virus 40 origin of DNA replication, which reproducibly showed high transfection (90%, revealed by GFP cloned into the same vector; data not shown) and high protein expression efficiencies (Figure 4A), the latter being essential to override endogenous p68 subfamily proteins. A single amino acid substitution (Lys to Asn) in the ATP-binding (Walker A) motif of p68 (GNT-p68) had a drastic effect on COS cell proliferation and, given that 10% of the cells resisted the transfection, nearly completely abolished their clonogenic survival (Figure 4B). This effect was specific to GNT-p68, as DQAD-p68, a mutant with a single amino

acid substitution (Glu to Gln) in the DEAD box (Walker B) motif as well as exogenously expressed wt-p68 only moderately affected cell proliferation. Expression of the mutants apparently does not interfere with the negative expression control of p72/p82 by endogenous p68 (Figure 4A), and thus we can exclude that the still low amounts of p72/p82 can substitute for the p68 function in GNT-p68- or DQAD-p68-expressing cells. Notably, both mutants have no ATPase (Figure 4D) and RNA helicase (Figure 4E) activity, whereas ATP-binding activity is lost only in GNT-p68 (Figure 4C, see also 31,39). Thus, exogenous GNT-p68 is able to compete with the endogenous protein for function resulting in a dominant negative phenotype, and it has to be tested in the absence of endogenous protein whether only ATP

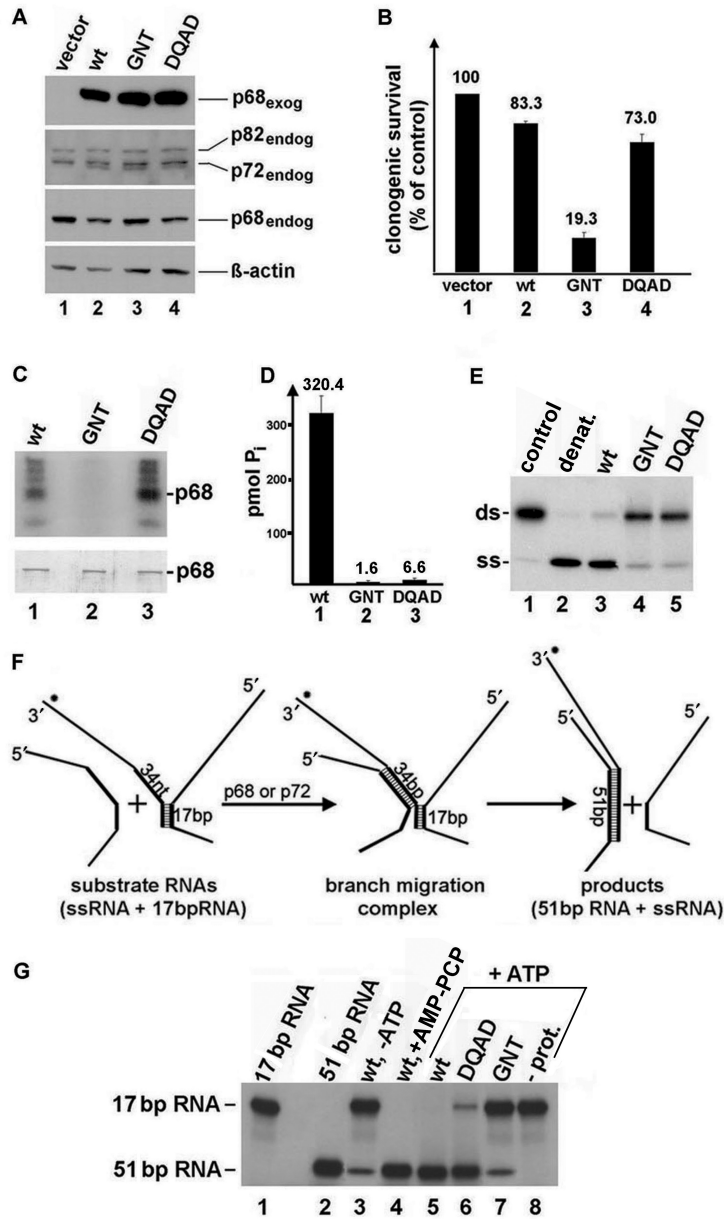


Figure 4. ATP binding is required for the function of p68 essential for cell proliferation and structural RNA rearrangement *in vitro*. (A) Transient overexpression of p68 polypeptides in COS cells. Cells were transfected twice with the indicated expression plasmid (pCMVp68-wt, wt; CMVp68-DQAD, DQAD or CMVp68-GNT, GNT) or with an empty vector (vector), harvested 5 days after the first transfection and cell lysates were analyzed by western blotting for indicated proteins (endog=proteins expressed from cellular genes and analyzed with the same antibodies as in Figure 1; p68_{exog}=plasmid-born p68, analyzed with monoclonal His-antibody). (B) Clonogenic survival of COS cells after transfection of the indicated plasmids (abbreviations as in A) relative to control (empty vector) transfected cells (% of control). The mean and standard deviation of three experiments are shown. (C) ATP-binding activity of p68 mutants. [α -³²P]ATP cross-linking reaction mixtures containing wt or mutant p68 (abbreviations as in A) were UV irradiated and processed for SDS-PAGE. The gel was stained with Coomassie blue, dried (lower panel) and processed for autoradiography (upper panel). (D) Effect of p68 mutations on ATPase activity. Reaction mixtures containing wt or mutant p68 (abbreviations as in A) plus 500ng of total RNA from HeLa cells were incubated in ATPase assay buffer in a total volume of 50 μ l. After precipitation of unreacted ATP, the free ³²PO₄³⁻ in the supernatant was counted. (E) RNA helicase activity of wt and mutant p68. RNA helicase assay samples were separated by SDS-PAGE and autoradiographed. The position of dsRNA and ssRNA is indicated. Lane 1 shows a control reaction without p68, lane 2 the denatured substrate (denat.), lane 3 an unwinding reaction with wt-p68 (wt), lane 4 with GNT-p68 (GNT) and lane 5 with DQAD-p68 (DQAD). (F) A scheme of the performed RNA structural rearrangement reaction. The reaction, designed here as an intermolecular process, may similarly proceed intramolecular. Thick lines indicate homologous regions and asterisks the labeled RNA strand in the reactions shown below. Notably, the branch migration complex shown as a reaction intermediate is unstable due to the small (17 bp) homologous double-stranded part. (G) RNA structural rearrangement catalyzed by wt- and mutant-p68. Reaction mixtures contained the partially ds 17-bp RNA (12.5 nM) plus the respective homologous ssRNA (37.5 nM) and were incubated at 37°C for 30 min without protein (lane 8, -prot.) or with 10 nM of wt-p68 (lanes 3–5) or 10 nM of DQAD-p68 (lane 6) or 10 nM GNT-p68 (lane 7). Standard reactions contained 4 mM Mg-ATP (lanes 5–8), which was omitted in the reaction shown in lane 3 or replaced by 4 mM adenylyl-(β , γ -methylene)-diphosphonate, a non-hydrolyzable analog of ATP (AMP-PCP, lane 4). For comparison, the ³²P-labeled 17- (lane 1) and 51-bp (lane 2) RNA, prepared by hybridization of the corresponding RNA strands in 80% formamide at 50°C, were run in parallel.

binding but not ATP hydrolysis or RNA unwinding of p68 is essential for cell proliferation. Nevertheless, a possible correlation of the ATP-binding activity of p68 with the capacity to catalyze rRNA rearrangement processes with an RNA branch migration complex as an intermediate product (see Figure 4F) was considered. In fact, p68 and p72 have recently been shown to catalyze similar reactions *in vitro* (5), and as it is shown in Figure 4G, isolated GNT-p68 is mostly inactive in such an *in-vitro* assay, while the same function of DQAD-p68 in comparison to wt-p68 was nearly unaffected (compare lines 5–7). In contrast to previous work (5), a dependence of RNA rearrangement on ATP binding, but not hydrolysis, became obvious here most probably because p68 was prepared in the presence of high concentrations of EDTA in the extraction buffer to efficiently remove protein-bound nucleotides. Notably, RNA rearrangement is catalyzed *in vitro* by isolated p72 as well (5).

Co-suppression of p68 and p72/p82 inhibits rRNA processing

The overall rate of protein synthesis decreased in p68 and p72/p82 co-suppressed cells, apparently due to a reduction in 60S (but not in 40S) ribosomal subunits (data not shown). This and the destruction of nucleoli after subfamily siRNA transfection prompted us to look for a possible role of p68 and/or p72/p82 in ribosome biogenesis (Figure 5). To determine the dynamic processing of rRNA precursors and intermediates, we carried out a pulse-chase labeling analysis of newly synthesized rRNA in HeLa cells after transfection of respective siRNAs for 70 h (Figure 5B, left panel). The pulse-labeled 47S rRNA precursor was readily detected in all experiments after a 60-min pulse, indicating that transcription of rRNA genes was not blocked by any siRNA used. In the chase process, the amount of mature 28S rRNA readily increased in cells transfected with control- or p68 siRNA and, after a 120-min chase, was roughly five times that of the 32S intermediate (with a 28S:32S ratio of 5.32 and 4.66, respectively; see Figure 5C). Similar results were obtained, when p72/p82 was knocked down (data not shown), indicating an effective processing of the 32S precursor in the absence of either p68 or p72/p82. Cells transfected with subfamily siRNA, in contrast, showed a decreased level of labeled 28S rRNA, and the ratio of 28S:32S was reduced to 0.53 after the 120-min chase (Figure 5C), whereas the maturation of 18S rRNA seemed not to be significantly affected in any case. These results were confirmed when we investigated the cellular levels of the different rRNA species by Northern blot analysis 4 days after siRNA transfection. For this, total cellular RNA was probed with sequences specific for ITS2-containing rRNA intermediates, 28S rRNA, 18S rRNA or 5.8S rRNA (Figure 5B, right panel). p68 and p72/p82 co-suppressed cells accumulated the 32S rRNA precursor whereas 12S, 5.8S and 28S rRNA levels were diminished. Thus, the simultaneous down-regulation of the p68 subfamily proteins inhibits

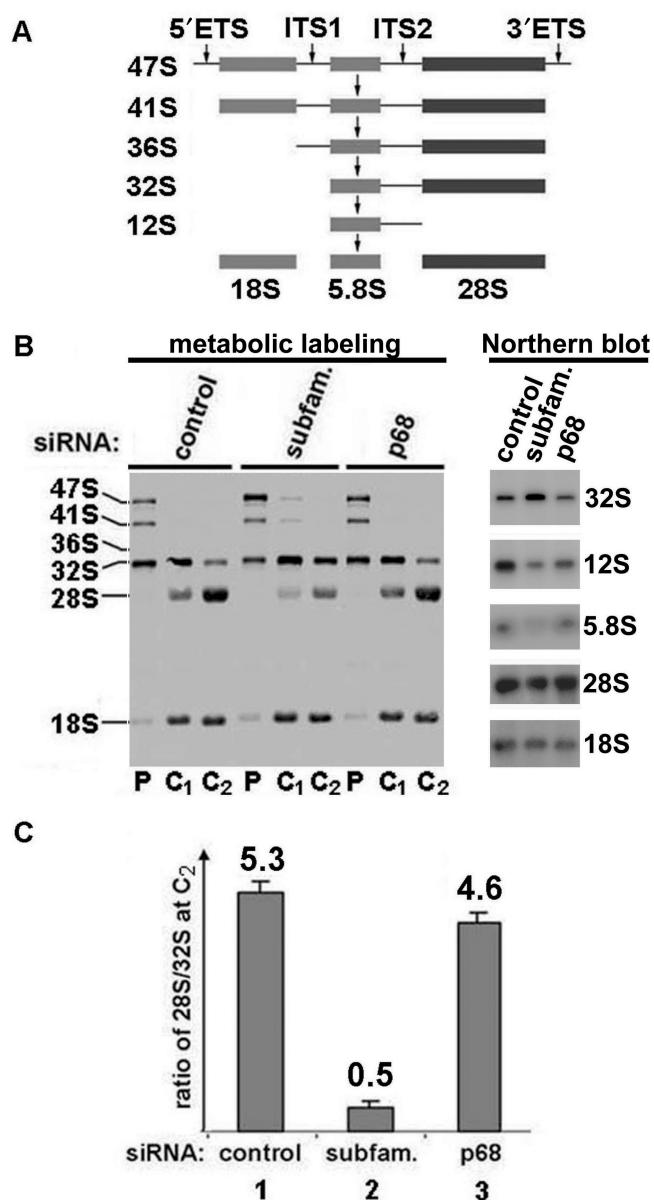


Figure 5. A redundant function of p68 and p72/p82 in the processing of pre-rRNA. (A) A diagram of rRNA processing (38). (B) Inhibition of 28S rRNA maturation by co-knock-down of p68 and p72/p82. Left panel: pulse-chase labeling analysis of newly synthesized rRNA. HeLa cells were transfected with the indicated siRNA for 70 h, pulse labeled with L-(methyl-³H)-methionine for 60 min (P) and chased for 60 min (C₁) or 120 min (C₂). An equal amount of radioactivity was loaded onto each lane. The positions of rRNA processing intermediates are indicated. The 36S rRNA band is visible only after overexposure of the film. Irrespective of poor RNA marking, experiments with shorter pulses (30–45 min) showed the same results (data not shown). Right panel: Northern blot analysis of rRNA species. Equal amounts of total RNA, isolated from cells transfected for 4 days with the indicated siRNAs, were successively hybridized with probes specific for the shown rRNA species. The 32S and 12S rRNA intermediates were analyzed with the same (ITS2-specific) probe, but with a 3-fold exposure time of the film for detection of 12S rRNA. Down-regulation of p68 subfamily proteins was controlled by western blotting and was identical to that shown in Figure 1A. (C) Ratios of 28S/32S rRNA at 120 min after metabolic labeling with ³H-methionine as described in B.

processing of the 32S intermediate into the mature 28S rRNA. Since we also noticed an increase in the pulse-labeled 47S precursor rRNA, this may hint to a defect also in very early steps of pre-rRNA processing in the absence of p68 and p72/p82.

A role of p68 and p72/p82 in the structural rearrangement of 32S pre-rRNA

Though U8 snoRNA also has some other function in rRNA processing, it is certainly essential for the accumulation of mature 28S and 5.8S rRNA (40). Its 5' end (outer 15 nucleotides) has the capacity to base pair with sequences at the 5' end of 28S in pre-rRNA (23). This interaction seems to transiently inhibit a premature 5.8S interaction at the same site of 28S that is essential for processing later on and still present in the mature ribosome. Thus, it has been proposed that U8 snoRNA is displaced from 28S by hybridization of the 3' end of 5.8S rRNA. Such an RNA rearrangement may be accomplished by the protein-controlled formation of an RNA branch migration complex, which is then resolved by branch migration to dissociate the U8 snoRNA (23,41; see also Figure 6A). U8 belongs to the class of fibrillar-associated snoRNAs (26), and a direct interaction of fibrillar with p68 and p72 has been demonstrated (42,43). Therefore, we checked the cellular level of U8 snoRNA after suppression of either one or both DEAD box genes. Northern blot analysis of total RNA from HeLa cells revealed an increase of U8 (2.2-fold) 2 days after both genes were co-suppressed, whereas U14, another processing snoRNP required for pre-rRNA cleavage on the pathway of 18S rRNA synthesis (21,44), was not affected (Figure 6B; we stress that at this time point cell proliferation was not yet diminished, see Figure 2A). When p68 or p72/p82 were depleted individually, almost no increase in the level of U8 was observed, confirming a redundant function in this step of rRNA processing. Sedimentation analysis of extracts from subfamily siRNA-transfected cells showed that the increase in U8 snoRNA appeared in both free U8 snoRNAs (ribonucleoproteins; 11S) and the 60S/90S pre-ribosomal subunit fractions (Figure 6C). Thus, in the absence of p68 and p72/p82, the displacement of U8 snoRNA from the pre-ribosomes seems to be hampered, and the cell tries to overcome this block by an overproduction of U8 snoRNA, which is usually limiting in ribosome biogenesis (45).

DISCUSSION

We have shown here that p68 and p72/p82 fulfill a redundant function in cell proliferation and viability. Our results are based on mutant studies and knock-down experiments with siRNAs, targeting either unique sequences in the individual mRNAs or a sequence common to both mRNAs and co-suppressing all three subfamily proteins at once. The diversity of the used siRNAs should exclude off-target effects due to, for example, partial sequence complementation. We point out that p72 and p82 could not functionally be discerned

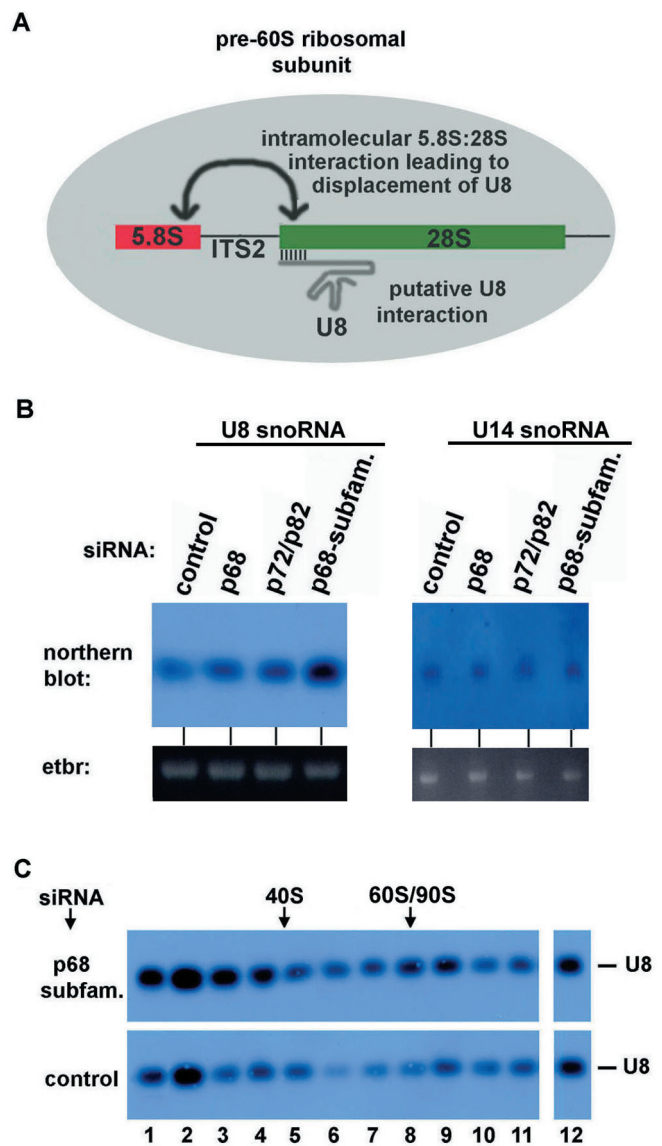


Figure 6. The level of U8 snoRNA is induced in the absence of p68 subfamily proteins. (A) Scheme of a 60S pre-ribosomal particle with 32S pre-rRNA, encoding 5.8S rRNA, ITS2 and 28S rRNA. The putative U8 interaction site (with U8 in its base-paired orientation) and the intramolecular 5.8S:28S interactions are indicated (61). (B) Total RNA probes from HeLa cells transfected with the indicated siRNAs for 3 days were analyzed by northern blotting with a probe against U8 or U14 snoRNA. Ethidium bromide staining of 28S rRNA is shown below. (C) Co-knock-down of p68 and p72/p82 leads to U8 accumulation in high molecular weight complexes. Cell lysates (prepared in the presence of 1 mM MgCl₂) from HeLa cells transfected with control siRNA or p68 subfamily siRNA were fractionated by centrifugation in 10–30% sucrose gradients. RNA was isolated from each fraction and analyzed by northern blotting with a probe against U8 snoRNA. To the right, total RNA (5 μg each) from untreated HeLa cells was loaded onto the gel and used as a transfer and hybridization control. Fractions containing the 40S and 60S/90S ribosomal complexes are indicated.

here, which will, however, be an interesting future task. Cells show only a moderately reduced proliferation rate when p72/p82 is suppressed on a normal p68 background and the same is true, vice versa, for p68 knocked-down

cells when p72 and p82 are overexpressed due to the cessation of the p68 control. This moderate reduction in cell proliferation in the absence of only p68 or p72/p82 may be attributed to other most probably individual function(s) of these proteins not analyzed here.

The negative expression control of p68 over p72/p82 seems to function, at least in part, at the level of splicing as the nuclear pool of partially spliced p72/p82 (as well as p68) pre-mRNA is nearly completely cleared at low p68 while that of the mature mRNA increased. Thus, p68, like yeast Dbp2p (12), seems to regulate its own expression and, in addition, that of its closest relative most probably by an intron-mediated mechanism, though an indirect effect cannot yet be excluded. The expression of less-related DEAD box genes such as Ddx20 (Gemin3, DP103; 46) and Ddx42 (47) was not found to be affected (C.J. and H.S., unpublished data). Selective effects on pre-mRNA splicing, including alternative splicing (9,10), correspond with the previously demonstrated association of p68 with spliceosomes (48–50) but seem to contradict a recently reported essential splicing function of p68 (28). Accordingly, we could not detect any impairment in the splicing of the housekeeping genes β -actin and GAPDH by efficient knock-down of p68. In addition, various pre-mRNA substrates with different *cis*-acting elements spliced equally well in mock- and p68-depleted HeLa nuclear extracts (H.U.-S., unpublished data). So far, we cannot explain this discrepancy, especially since a possible rescue of the implicated p68 splicing function by p72/p82, as discussed by (28), is excluded here by the co-suppression experiments. We note, however, that our data are in agreement with very recently published results on *Drosophila* p68 (51).

Co-suppression of p68 and p72/p82 by RNAi leads to disintegration of the nucleolar structure, cell cycle arrest and cell death. Concomitantly, we observed a decrease in 60S ribosomal subunits by a partial inhibition of the processing of pre-rRNA, more precisely the cleavage of the ITS2 in the 32S rRNA precursor most probably performed by B23 (52,53). Such a defect may also be deduced from the demonstrated increase in the cellular U8 snoRNA level and in the fraction of U8 associated with 60S pre-ribosomal subunits, as has been reported for U14 snoRNA after depletion of the putative helicase Dbp4p (21). This view is underscored by the interaction of p68 with fibrillarin (40,41), a known component of U8 snoRNPs (26). The inhibition of rRNA processing seems to lead to a disintegration of the nucleolar structure, cell cycle arrest and eventually cell death, as has been reported for the disturbance of other steps of rRNA synthesis or maturation (29,37,38,54). In MCF7, cell destruction seems to be executed via the induction of the p53 tumor suppressor protein, and thus our observation confirms the idea that some sort of stress sensor monitors the nucleolar structure and function and regulates p53 levels (55). How this monitoring, however, is translated into action in cells without functional p53 such as HeLa cells, is unknown (38).

For the processing of pre-28S RNA to proceed, U8 snoRNA must be displaced from 28S rRNA most probably by competitive hybridization of 5.8S rRNA via

an intermediate RNA branch migration complex (23), reminiscent of reactions previously shown to be catalyzed by p68 and p72 *in vitro* (5). We have shown that p68 ATP binding but not hydrolysis is essential *in vitro* for the formation of similar branch migration complexes, which in case of low stability as described here, spontaneously disintegrate at 37°C (5). Mutant p68, defective in ATP binding (GNT-p68), is incompetent to induce such a structural rearrangement *in vitro* and also drastically reduces cell proliferation in contrast to another mutant without RNA helicase activity (DQAD-p68), which can still bind ATP.

The moderate reduction in the clonogenic survival of DQAD-p68 and also wt-p68 overexpressing cells may be attributable to the mislocation of p68 proteins to the nucleolus due to overexpression (see also 34) or inhibition of RNA polymerase II activity as reported by Andersen *et al.* (56). Under normal conditions, p68 subfamily proteins are excluded from the nucleoli in interphase cells most probably by binding partners in the nucleoplasm, such as transcription factors, splicing complexes (see above) and the A-Kinase anchoring protein AKAP95, a nuclear matrix protein not occurring in nucleoli (57). AKAP95 is exposed to cytoplasmic components in mitosis and reenters the forming nucleus in telophase (58). It is conceivable that it then recruits p68 and p72/p82 from prenucleolar bodies, where they are transiently localized just at that time (17). Therefore, if these DEAD box proteins are involved in the displacement of U8 snoRNA by 5.8S rRNA, as implied from our experiments, this process may proceed in the nucleoplasm of interphase cells where, however, it could be difficult to detect because of potential short half-lives of respective intermediates (59). The same may halt for U8 snoRNA, which after its displacement from the 32S rRNA immediately may return to the nucleoli, where it had been located exclusively so far (26). In fact, it is not precisely known in which nuclear compartment ITS2 cleavage proceeds, but if the timing of ribosome biogenesis is modulated by hybridization of U8 to 32S as proposed by others (23), then the exclusion of p68 and p72/p82 from the nucleolus could provide a means on how premature interaction of 5.8S and 28S rRNA sequences may be prevented.

In *S. cerevisiae*, 18 putative RNA helicases have been implicated in ribosome biosynthesis (20,21), among them Dbp2p (13), which all do not seem to have redundant functions. Dbp2p-depleted cells exhibit slow growth and cold-sensitive phenotypes but do not die (12,13) most probably because the necessary structural alterations in rRNA, catalyzed by Dbp2p, can proceed spontaneously at normal growth temperature, though less efficiently. In fact, yeast apparently does not possess a U8 snoRNA homolog, instead ITS2 elements seem to provide *in cis* some of the functions proposed above for vertebrate U8 snoRNA, thereby possibly interacting with 25S rRNA sequences less stably and allowing spontaneous formation of the 5.8S–25S hybrid structure as a prerequisite for further processing (60). Nonetheless, the ribosome biogenesis (in contrast to the mRNA decay) phenotype of *dbp2 Δ* cells is complemented by heterologous expression

of human p68 (13). In mammals, displacement of U8 and formation of the 5.8S–28S structure by a spontaneous rearrangement reaction may also be possible to a certain extent. This and some rest protein always left by the RNAi method may explain the residual 32S pre-rRNA processing observed under p68 subfamily knock-down conditions.

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