p53 localizes to intranucleolar regions distinct from the ribosomal production compartments

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Accepted 16 January 2010
Journal of Cell Science 123, 1203-1208
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doi:10.1242/jcs.062398

Summary
The tumor suppressor p53 has been implicated in the regulation of ribosome biogenesis based on its inhibitory effect on RNA polymerase I (pol I)-dependent transcription. Consistent with this, p53 has been described in nucleoli, albeit under specific experimental conditions. Since data on the intranucleolar localization of p53 are controversial, we have analyzed in detail its subnucleolar distribution. Our results show that p53 does not localize to one of the well-known structural components of the nucleolus involved in ribosome biogenesis, but rather occupies distinct intranucleolar regions that constitute nucleolar cavities. When cells were treated with the proteasome inhibitor MG132, the size and frequency of p53-containing nucleolar cavities increased, and the protein partially colocalized with inactivated proteasomes. Importantly, p53 did not colocalize with pol I at the transcription sites in fibrillar centers (FCs) as has previously been reported. The observed intranucleolar distribution and accumulation of p53 raises the question of how the protein influences rDNA transcription in vivo.

Key words: p53, Nucleolus, Nucleolar cavities, Proteasome, MG132

Introduction
The nucleolus has various functions in addition to its well known role in ribosome biogenesis; these include control of the cell cycle, cell proliferation, the stress response and biogenesis of various ribonucleoprotein particles (Boisvert et al., 2007; Pederson and Tsai, 2009). A prominent reaction to cellular stress is the activation of the cell-cycle regulator p53, which is controlled by the interaction of a nucleolar protein, p14ARF, with MDM2 (or HDM2 in humans), a p53-specific E3 ubiquitin ligase. In response to cellular stress, p14ARF expression is elevated, which prevents MDM2 from tagging p53 for proteasomal destruction. The resulting increase of p53 levels leads to cell-cycle arrest or apoptosis. p14ARF can rescue p53 from degradation either by direct inhibition of MDM2 in the nucleoplasm or by sequestration of MDM2 in the nucleolus (Kruse and Gu, 2009). MDM2 can also be inhibited by ribosomal proteins upon perturbation of nucleolar ribosome biogenesis and by defects in other nucleolar proteins (for reviews, see Ferreira-Cerca and Hurt, 2009; Warner and McIntosh, 2009).

Thus, nucleolar proteins can determine, directly or indirectly, the activity of p53 in the nucleoplasm. However, the nucleolar localization of the tumor suppressor itself might be important, because it is able to repress rDNA transcription (Budde and Grummt, 1999; Montanaro et al., 2005; Zhai and Comai, 2000). p53 can also bind to a sequence in the intergenic spacer of the tandemly repeated rRNA genes (Kern et al., 1991).

Localization of p53 to the nucleolus has only been shown under certain experimental conditions. Non-activated p53 is barely detectable by conventional immunofluorescence microscopy. However, when HepG2 cells were permeabilized with Triton X-100 before fixation, latent p53 was found at or near the sites of rRNA synthesis (Rubbi and Milner, 2000). In other studies, p53 was found to localize to nucleoli of specific human cell lines after treatment with the proteasome inhibitor MG132 (Karni-Schmidt et al., 2007; Karni-Schmidt et al., 2008; Klibanov et al., 2001; Latonen et al., 2003; Pokrovskaja et al., 2001). Notably, nucleolar sequestration of p53 was seen in only 10-20% of MG132-treated cells, following nucleoplasmic accumulation of the protein (Karni-Schmidt et al., 2007; Karni-Schmidt et al., 2008). In one of these studies, double-localization experiments were performed with antibodies against UBF or nucleolin to identify the subnucleolar location of p53 (Karni-Schmidt et al., 2008). The authors concluded that p53 is enriched in the FCs, the site of rDNA transcription. However, other authors reported that after MG132 treatment, p53 colocalized with either nucleolin (Klibanov et al., 2001) or B23/nucleophosmin (Pokrovskaja et al., 2001), which would place p53 either in the dense fibrillar component (DFC) or granular component (GC) of the nucleolus.

In view of these contrasting results and the lack of in vivo localization data, we decided to perform a detailed analysis of p53 subnucleolar distribution. When analyzing putative nucleolar proteins, the question generally arises as to whether they are spatially integrated into the ribosome production machinery or are located in nucleolar regions devoid of preribosomes, termed ‘nonribosomal’ areas (Politz et al., 2005). Furthermore, the nucleolar body often contains interstices of variable size, which appear as regions of low density in electron microscopic thin sections and have been described as nucleolar vacuoles or cavities (Busch and Smetana, 1974; Thiry and Goessen, 1996).

In the present study, we analyzed the distribution of nucleolar marker proteins involved in ribosome biogenesis in living cells in relation to the localization of p53 and p53-GFP in untreated and proteasome-inhibited MCF-7 (wild-type p53) cells. We show that p53 does not colocalize with pol I in FCs, nor does it localize to other nucleolar compartments involved in ribosome biogenesis; however, p53 does accumulate with proteasomes in nucleolar cavities.
Results and Discussion

After treatment of human cells with the proteasome inhibitor MG132, p53 was reported to localize to nucleolar FCs using immunofluorescence analyses (Karni-Schmidt et al., 2008). We sought to study the relationship between p53 and the rDNA transcription sites by live-cell imaging, which allows for optimal visualization of nucleolar substructure by expression of fluorescent protein (FP)-tagged marker proteins (Krüger et al., 2007). We therefore transiently expressed p53 fused to GFP in MCF-7 cells (a human breast adenocarcinoma cell line, p53 wild type). In untreated cells, p53-GFP was detected mainly in the nucleoplasm and excluded from the nucleoli, as was previously shown by immunofluorescence for ectopically expressed untagged p53 (Karni-Schmidt et al., 2008). Interestingly, in a few untreated cells (~0.4%), we also detected p53-GFP within the nucleolus in the form of one or a few distinct spots (Fig. 1A1). This distribution matched that of coexpressed monomeric RFP (mRFP), which diffuses freely through the cytoplasm and nucleoplasm. Similarly to p53-GFP, mRFP was excluded from nucleoli, except for p53-GFP-positive dots (Fig. 1A2,A3). In differential interference contrast (DIC) microscopy, the p53-GFP- and mRFP-containing nucleolar regions appeared as cavities, indicating a lower refractive index than the surrounding nucleolar regions (Fig. 1A4).

After treatment with 10 μM MG132 for 8 hours, p53-GFP was localized to distinct intranucleolar foci in around 10% of transfected cells. As in untreated cells, these foci also contained mRFP and appeared in DIC as low-density cavities of varying size, the larger of which typically assumed an appearance resembling vacuoles (Fig. 1B). On average, the cavities were significantly larger than in untreated cells. After 16 hours of MG132 treatment, p53-GFP accumulated in the nucleoli of around 20% of the transfected cells, where nucleolar fluorescence was often significantly concentrated in comparison with the nucleoplasmic signal. In most cases, the p53-GFP-containing nucleolar regions no longer exhibited the vacuolar appearance of nucleolar cavities in DIC, but were more irregularly shaped and took up the greater part of the nucleolar volume. Furthermore, mRFP no longer colocalized with nucleolar p53 in these cells (Fig. 1C).

To assess whether nucleolar p53-GFP was localized in FCs, we visualized pol I in untreated p53-GFP-expressing cells by immunofluorescence. The pol I fluorescence pattern was characterized by several intranucleolar dots – the FCs. Notably, they never corresponded to the typically less numerous p53-GFP loci (Fig. 1D). After treatment of the cells with MG132 for 8 hours, the p53-GFP-filled cavities were still clearly separate from FCs (Fig. 1E) and after 16 hours of MG132 treatment, the FCs were exclusively distributed around the p53-GFP aggregates at the periphery of the deformed nucleoli (Fig. 1F).

These results showed that the distribution pattern of p53-GFP in fixed cells was comparable to the in vivo status and that the fusion protein did not localize to pol-I-containing FCs. This is in direct contrast to the results of Karni-Schmidt and co-workers (Karni-Schmidt et al., 2008), who reported a virtually identical distribution pattern of ectopic p53 and UBF, another FC marker. However, the data in question showed a limited resolution of nucleolar structure, leaving room for interpretation; in other images, the authors clearly showed localization of p53 in nucleolar cavities by DIC microscopy (Karni-Schmidt et al., 2008), which is consistent with our results. In fact, the localization of p53 in intranucleolar cavities, which are visible by DIC imaging and distinct from FCs, seems to be a general feature of mammalian cell lines (see below).

![Fig. 1. p53-GFP localizes in nucleolar cavities of MCF-7 cells before and after proteasome inhibition.](image)

Next, we examined the distribution of endogenous p53 after MG132 treatment. In untreated MCF-7 cells, p53 was almost undetectable (not shown) (see also Rubbi and Milner, 2000). After MG132 treatment, endogenous p53 behaved essentially identically to p53-GFP, accumulating in the nucleoplasm and, after 16 hours,
In around 20% of the nucleoli (Fig. 2). In the nucleolus, p53 was predominantly localized to cavities, which were not pol-I-containing FCs, after an 8 hour treatment (Fig. 2A). After 16 hours of treatment, p53 was mostly found in large aggregates, which appeared to distort nucleolar organization (Fig. 2B). Again, we did not observe any colocalization of p53 with pol I.

Our results point to a time-dependent accumulation of p53 after proteasome inhibition in nucleolar subregions not associated with rDNA transcription. The distribution of mRFP, a freely diffusible protein, suggested that the nucleolar cavities might correspond to nucleoplasmic spaces enclosed within the nucleolus. We therefore analyzed the nucleolar ultrastructure of untreated and MG132-treated cells by electron microscopy. In untreated cells, nucleolar cavities were clearly visible as lightly stained roundish regions, distinct from the FCs, DFC and GC (Fig. 3A). We also found nucleolar cavities of varying size in other mammalian cell lines, such as Hep-2 and Cos-7 cells (Fig. 3B). In some cases, the cavities appeared to be continuous with the nucleoplasm (Fig. 3B,D). The interior of the intranucleolar cavities contained a loose fibrillar granular meshwork, and on occasion, also clusters or linear arrays of several distinct ~40 nm particles of relatively high contrast (Fig. 3A, inset). The size and electron density of these particles resembled perichromatin granules, which normally occur as individual particles in nucleoplasmic regions and have been implicated in storage and transport of pre-mRNAs (Puvion and Moyne, 1981). Since the molecular composition of perichromatin granules is as yet unknown and specific markers are unavailable (Charlier et al., 2009), it is not possible to examine the relationship between them and the observed particles in the nucleolar cavities in more detail using immunocytochemical approaches. After MG132 treatment for 8 hours, the nucleolar cavities of MCF-7 cells appeared larger than those in control cells and were often filled with numerous ~40-nm-diameter granules (Fig. 3C). After 16 hours of treatment, the cavities increased further in size to expand over the whole nucleolar interior. They contained considerable numbers of granules with a thickness ranging from 45 to 55 nm and were often arranged as linear arrays (Fig. 3D). The development from small cavities to large nucleolus-spanning regions with increasing duration of drug treatment parallels the progressive enlargement of the p53-containing intranucleolar regions as observed by light microscopy, and we assume the structures to be equivalent. To confirm that the nucleolar cavities were not part of the compartments of ribosome biogenesis, we compared their spatial arrangement relative to the distribution of various FP-tagged nucleolar marker proteins in Hep2 cells by live-cell imaging. Hep2 cells were used because they generally showed more prominent nucleolar cavities than MCF-7 cells by DIC microscopy. Although

![Fig. 2. The nucleolar distribution of endogenous p53 differs from that of pol I. (A,B) MCF-7 cells were exposed to MG132 for the indicated times and double-stained with antibodies against p53 (green) and pol I (red). After prolonged MG132 treatment, nucleolar cavities are transformed into a large p53-positive aggregate, which is surrounded by numerous pol I foci (row B). Scale bars: 10 μm.](image1)

![Fig. 3. Electron microscopic visualization of nucleolar cavities before and after proteasome inhibition. (A) Typically, a number of small nucleolar cavities (*) are seen in untreated MCF-7 cells, often in proximity to the electron-dense DFC. The light nucleolar cavities are clearly distinguished from the FCs. The inset shows the presence of distinct ~40 nm particles in a nucleolar cavity. (B) A nucleolus of a COS-7 cell with two prominent cavities. Note the apparent continuity of a cavity with the nucleoplasm (arrow). (C) In MCF-7 cells treated with MG132, the nucleolar cavities are typically enlarged and enclose a larger amount of ~40 nm granules. (D) Cells treated for 16 hours with MG132 often show cavities occupying a large central space of the nucleolus and containing masses of enlarged granules with diameters ranging from 45 to 55 nm. Scale bars: 0.5 μm and 0.1 μm (inset in A).](image2)
p53 is inactivated by the viral oncogene E6 in Hep2 cells, the results of the p53 and p53-GFP localizations were essentially the same in both cell lines (not shown). As already shown for mRFP (Fig. 1), the nucleolar cavities were accessible to free GFP in transfected Hep-2 cells – the GFP was equally as concentrated there as it was in the nucleoplasm – whereas the remaining nucleolar body was mostly devoid of GFP (Fig. 4A1). The cell shown in Fig. 4A1 was cotransfected with fibrillarin-mRFP, an established marker for the DFC, where early processing events and modifications of the pre-rRNAs take place (Boisvert et al., 2007; Krüger et al., 2007). Fibrillarin was confined to the nucleolar body surrounding the GFP-positive cavity (Fig. 4A). To visualize all compartments of ribosome biogenesis, we simultaneously expressed marker proteins for the FC, DFC and GC. A subunit of pol I (RPAl94-GFP) labeled the FCs (Fig. 4B1). This GFP fusion of a pol I subunit has been shown to be functional in rDNA transcription (Dundr et al., 2002). Fibrillarin-CFP stained the DFCs (Fig. 4B2) and B23-mRFP was specific for the GC (Fig. 4B3), where later pre-rRNA processing and assembly of the ribosomal subunits take place. As can be seen in Fig. 4 (row B), the nucleolar cavities were clearly separate from all components of the nucleolus involved in ribosome production. The images of a Hep-2 cell expressing pol I-GFP and fib-mRFP in Fig. 4C resolve the sites of nucleolar transcription centers in more detail. The numerous FCs with surrounding DFCs revealed no colocalization with the nucleolar cavities seen in DIC.

The compartmentalization of the nucleolar GC into subdomains with nascent ribosomes next to rRNA-deficient regions has been described at the EM level (Politz et al., 2005). To find out whether nucleolar cavities can be considered to be expanded ‘nonribosomal’ GC regions, we co-expressed the marker protein nucleostemin (Politz et al., 2005) with fibrillarin and the ribosomal protein S6 (Fig. 4, row D). Nucleostemin-mRFP (Fig. 4D3) and S6-GFP (Fig. 4D1) showed a similar distribution, and together accounted for the labeling of the GC, which in turn surrounded the DFC as defined by the presence of fibrillarin-CFP (Fig. 4D2). The nucleolar cavities observed in DIC images were consistently free of any fluorescence signal (Fig. 4D4, D5). Thus, the nucleolar cavities described in the present study are distinct from the nucleostemin-containing nonribosomal regions and might represent yet another example of nucleolar subcompartimentalization.

Because p53 accumulates in nucleolar cavities in response to the exposure to a proteasome inhibitor, we were interested in the behavior of the affected proteasomes. We analyzed the distribution of p27, a protein of the 26S proteasome core particle, by immunofluorescence after MG132 treatment. p53 and p27 revealed a very similar distribution pattern in MCF-7 cells. Both proteins localized diffusely in the nucleoplasm and additionally, in nucleoplasmic aggregates and nucleolar cavities (supplementary material Fig. S1A, B). Comparison of the distribution of p53 and p27 after MG132 treatment suggests that a fraction of nucleolar p53 is associated with drug-inhibited proteasomes (supplementary material Fig. S1C). The proteasomal antigen was also detected in the nucleolar cavities of untreated cells. In all cases, the p27-positive nucleolar cavities did not correspond to the FCs labelled with pol I (supplementary material Fig. S1D). These results are consistent with a recent report in which the authors provide evidence that nucleoplasmic invaginations into nucleoli of Hep-2 cells might lead to false-positive nucleolar signals of proteasomes (Scharf et al., 2007).

It is tempting to speculate that nucleolar cavities are actually nucleoplasmic regions embedded in the nucleolar body. Several results support this view: (1) the similarity of texture between cavities and nucleoplasmic spaces as seen in electron micrographs, (2) the sometimes visible direct connections between cavities and nucleoplasm, and (3) the similar concentration of free FPs in both spaces. However, the specific induction of more frequent and larger displacements of nucleolar cavities in response to MG132 treatment is an area for further study.

**Fig. 4. Live-cell imaging of nucleolar components.** (A) Hep2 cells coexpressing GFP (A1) and fibrillarin-mRFP (A2). The GFP-filled central nucleolar cavity, which is clearly identified in the DIC image (A5) is surrounded by fibrillarin, a marker protein of the DFC. (B) A triple-transfected Hep2 cell expressing differentially tagged marker proteins for the three nucleolar components FC (pol I-GFP; B1), DFC (fib-CFP; B2) and GC (B23-mRFP; B3) shows that the nucleolar cavities are distinct from these regions. (C) High magnification reveals the absence of pol I and fibrillarin from the nucleolar cavities. In this case, the cells were cotransfected with pol I-GFP (C1) and fib-mRFP (C2). Note that the nucleolar cavities seen with DIC optics (C5) are clearly distinct from the similarly sized FCs (arrows) by the absence of pol I. (D) Hep2 cell triple-transfected with ribosomal protein S6-GFP (D1), Fib-CFP (D2) and nucleostemin-mRFP (D3) shows that nucleolar cavities do not overlap with the GC, with ribosome-containing regions or with RNA-deficient regions labelled with nucleostemin. Scale bars: 10 μm (A), 5 μm (B–D).
cavities by the proteasome inhibitor MG132 and the concurrent accumulation of p53 to these nucleolar regions, which has been shown to be energy-dependent (Karni-Schmidt et al., 2007; Karni-Schmidt et al., 2008), points to a specific sequestration function of the nucleolar cavity. The presence of conspicuous arrays of perichromatin-granule-like particles in the nucleolar cavities and, after prolonged MG132 treatment, of larger granules not seen in extranucleolar regions, favors the idea of a specific nucleolar compartment that differs from the nucleoplasmic space. This view is further corroborated by analyses of plant nucleoli, which are often characterized by the presence of a prominent central cavity where snRNAs, snoRNAs and proteins of the post-spooling exon-junction complex (EJC), but no ribosomal RNPs, have been found (Pendle et al., 2005; Shaw and Brown, 2004). Interestingly, a nucleolar RNA-processing center has recently been described in plants, where components of the nuclear siRNA pathway involved in chromatin modification were localized. These proteins and siRNAs did not colocalize with rRNA precursor transcripts and could correspond to nucleolar cavities in Arabidopsis (Pontes et al., 2006). Notably, the nucleolar cavities of mammalian cells described in the present report resemble those of Arabidopsis nucleoli as visualized by DIC microscopy (supplementary material Fig. S2). It will be interesting to find out whether the EJC proteins, which are contained in the human nucleolar proteome (Andersen et al., 2005), and the components of the siRNA-processing pathway can also be sequestered in animal nucleolar cavities.

In summary, we have shown that the tumor suppressor p53 can localize to nucleolar cavities in a number of cultured human cells. After proteosomal inhibition by treatment of cells with the drug MG132, the frequency and size of p53-containing nucleolar cavities increases. Importantly, the nucleolar cavities are intranucleolar regions that are distinct from the nucleolar compartments where ribosome biogenesis takes place. In particular, we have shown that, contrary to a previous report (Karni-Schmidt et al., 2008), p53 is not localized to FCs. Thus, our results do not support the view that p53 represses rDNA transcription by direct binding to the pol-I-specific transcription factor SL1 as proposed by Zhai and Comai (Zhai and Comai, 2000). The components of the SL1 complex, i.e. the TATA-binding protein (TBP) and the TBP-associated factors (TAF), colocalize with pol I to the rDNA transcription sites in the FCs (Dundr et al., 2002; Jordan et al., 1996), and are thus spatially distinct from the p53 foci of the nucleolar cavities. Rather, our results are compatible with the finding that p53 downregulates rDNA transcription by an indirect mechanism (Budde and Grummt, 1999). How exactly p53 exerts its repressive function on pol I transcription in the living cell remains an important topic for future studies.

Materials and Methods

Antibodies, plasmids and transfection

Antibodies used were: the autoimmune serum S18 against pol I (Reimer et al., 1987) and mAb p27 (Progen, Heidelberg). Plasmids used were: pEGFP, pDsRed-Monomer and pEGFP (Clontech); Fbs-EF1, Fbs-EF2, B23-MRF and S6-EF2 (Krüger et al., 2007). RPA-194 (pol I)-EGFP was kindly provided by Miroslav Dundr (Rosalind Franklin University of Medicine and Science, North Chicago, IL). P53-EGFP was a kind gift from Wolfgang Deppert (Heinrich-Pette-Institut, Hamburg, Germany). Nucleostemin-MRF was produced by RT-PCR amplification of Nucleostemin cDNA (GenBank accession no. 6593129) from total RNA isolated from Hep2 cells and ligation into the vector pDsRed-Monomer-C1 (Clontech). Transfections were performed using Effectene (Qiagen) or Fugene (Roche) according to the manufacturer’s instructions. Transiently transfected cells were analyzed 24–72 hours after transfection.

Indirect immunofluorescence and live-cell microscopy

MCF-7 and Hep2 cells grown on coverslips were fixed with 2% formaldehyde in PBS and permeabilized with 0.2% Triton in PBS, incubated for 0.5–1 hour with the primary antibodies and 15 minutes with the appropriate Texas-Red- or Cy2-conjugated secondary antibodies (Dianova). To inhibit proteasomal pathways, cells were treated with 10 μM MG132 (Calbiochem) for 8–16 hours before fixation or live-cell imaging. For live-cell microscopy, cells were grown on glass-bottomed dishes (WPI, Berlin). Images were taken with a Leica TCS-SP2 CLSM equipped with a ×63/1.4 NA oil-immersion objective and a 37°C, 5% CO2 incubation chamber.

Electron microscopy

Cells grown on coverslips were fixed at 4°C with 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) for 30 minutes, postfixed with 1% osmium tetroxide, dehydrated in an ascending ethanol series and flat embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM101a electron microscope operating at 80 kV.

We are grateful to Wolfgang Deppert for p53-EGFP, Miroslav Dundr for RPA194-EGFP and Rosalía Deeken for A. thaliana suspension cells. We thank Kattrin Eberhardt, Silke Braune and Elisabeth Hegewer for technical assistance.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/8/1203/DC1

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