Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths

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Summary

The involvement of telomerase in cellular immortalization and senescence has often been assessed by means of telomerase expression at the RNA level and quantification of telomerase activity by the telomeric repeat amplification protocol assay. However, these methods either neglected the existence of various telomerase splice variants, or ignored the nonconventional functions of telomerase independent of its ability to elongate and maintain telomere length. Immunodetection of telomerase is now being recognized as a necessary approach to precisely elucidate its roles in oncogenesis and senescence. A few antibodies directed against the catalytic subunit of the human telomerase (hTERT) are currently used but their specificity is not always demonstrated. A survey of the literature showed inconsistencies and led us to comparatively re-evaluate the most frequently used antibodies. Surprisingly, mass spectrometry, two-dimensional gel analysis and immunofluorescent experiments revealed that the most frequently used hTERT immunoprobe, a mouse monoclonal antibody that was claimed to be directed against an hTERT protein epitope, in fact recognizes nucleolin rather than telomerase. Our findings have interesting implications regarding the biology of nucleolin and telomerase in the context of pathophysiological investigations recently carried out.

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Key words: Telomerase, Nucleolin, Immunodetection

Introduction

Telomerase is a ribonucleoprotein complex that maintains chromosome stability and, ultimately, cell lifespan by telomere maintenance (reviewed in Counter et al., 1992; Harley and Villeponteau, 1995). The absence of telomerase is associated with shortening of telomeres and aging of somatic cells. The activity of this enzyme is normally downregulated in somatic cells, whereas it is highly upregulated in cells that are actively dividing, such as malignant cells, strongly suggesting a key role in tumourigenesis (Greider, 1998; Shay and Bacchetti, 1997). Given the difference in telomerase expression between normal and tumour cells, telomerase activity or telomerase components could be potentially useful as novel diagnostic markers for a wide range of cancers (reviewed by Hiyama and Hiyama, 2003; Ulaner, 2004). Extensive characterization of telomerase activity was made possible through the development of the highly sensitive telomeric repeat amplification protocol-polymerase chain reaction (TRAP-PCR) assay (Kim et al., 1994), which was the first method employed to estimate the abundance of telomerase in human normal and cancerous tissues. Detection of high levels of telomerase activity in tumour tissues usually suggests a less favourable prognosis and predicts unfavourable outcomes and shorter survival (Hiyama et al., 1995a; Hiyama et al., 1995b; Langford et al., 1997). Hence, inclusion of telomerase activity as a prognostic marker should be considered in future validation studies.

The cloning of the catalytic subunit of telomerase gene (hTERT) has allowed the evaluation of hTERT mRNA level and its correlation with telomerase activity (Counter et al., 1997; Lingner et al., 1997; Nakamura et al., 1997). Importantly, these studies have revealed that these two parameters were not always correlated (Counter et al., 1998; Klapper et al., 2001; Rohde et al., 2000). This is reminiscent of the existence of additional nontranscriptional mechanisms involved in the regulation of telomerase activity. Indeed, besides transcription, telomerase may be regulated at post-transcriptional (alternative splicing) and post-translational (phosphorylation) levels, and its intracellular localization may also play an important role (Cong et al., 2002; Kilian et al., 1997; Liu et al., 2001; Mergny et al., 2002). Furthermore, it is now well-recognized that telomerase is endowed with additional functions far from telomeres that are independent of the telomere elongating activity measured by the TRAP

assay (Dudognon et al., 2004; Fu et al., 1999; Stewart et al., 2002).

Nowadays, the current knowledge on telomerase expression and functions imposes a precise and reliable detection of telomerase protein by immunological techniques. Antibodies against epitope-tagged fusion hTERT proteins have been used for the detection of ectopic expression of hTERT and molecular applications in cell model systems (Etheridge et al., 2002; Khurts et al., 2004; Santos et al., 2004; Seimiya et al., 2000; Wong et al., 2002; Zhu et al., 2004). However, it cannot be applied to fresh tissues or cells to study hTERT endogenous expression and localization. An increasing number of reports based on immunological methods establish telomerase as a marker for tumour growth and progression. Unfortunately, to date, only a few antibodies directed against telomerase epitopes have been reported. However, difficulties in detecting the enzyme have been mentioned, related to the low abundance of the enzyme in cells (Frost et al., 2000; Wada et al., 2000) and to the lack of specificity of these antibodies, all features which are generally well-recognized although not actually discussed in the publications.

An overall investigation on the epitope specificity of commonly used anti-hTERT antibodies has revealed unexpected but interesting biological features of several of these reagents. The most salient points are herein reported. A new mouse monoclonal antibody (Novocastra, NCL-hTERT, clone 44F12) has been widely used, based on the assumption that this antibody correctly recognizes the protein peptide against which it has been claimed to be developed (Brustmann, 2005; Dalerba et al., 2005; Domont et al., 2005; Dutu et al., 2005; Elkak et al., 2005; Falchetti et al., 2003; Fullen et al., 2005; Gulmann et al., 2005a; Kraemer et al., 2003; Lantuejoul et al., 2005; Lantuejoul et al., 2004; Luzar et al., 2005a; Luzar et al., 2005b; Maes et al., 2005; Sabah et al., 2004; Sato et al., 2004; Smith et al., 2004; Uziel et al., 2005; Yan et al., 2004). Using this antibody, we confirmed on western blots, as previously shown, the labelling of a unique protein band in the 100 kDa area, which is generally thought consistent with the estimated molecular weight of telomerase of 127 kDa (Wick et al., 1999). Strikingly, control experiments using either telomerase-negative or hTERT-overexpressing cells cast some doubt on the nature of the protein recognized by this antibody. Starting from these observations, a detailed re-evaluation of the efficacy and specificity of this antibody was performed comparatively with other commercially available anti-hTERT antibodies.

Results

Expression of hTERT mRNA level in engineered human cell lines

Different sets of cell lines were investigated for their relative levels of hTERT mRNA expression (supplementary material Fig. S2) using fluorescence real-time PCR. HeLa cells were engineered to ectopically over-express either hTERT or haemagglutinin (HA)-tagged hTERT. This latter subline allows a precise evaluation of the specificity of any protein band detected on western blots. NB4-LR1 acute promyelocytic leukemia (APL) cells exhibited a high hTERT expression. This expression was further increased by ectopic hTERT expression in the NB4-LR1/hTERT cells. GM847 was described as a telomerase-negative expressing cell line which used alternative lengthening of telomeres (ALT) mechanisms to maintain telomere length (Bryan et al., 1995; Henson et al., 2002). In this cell line, hTERT mRNA expression was nearly undetectable. Ectopic hTERT mRNA in GM847 cells was detectable, although it remained at a much lower level than in any other ectopically hTERT-over-expressing cell lines tested.

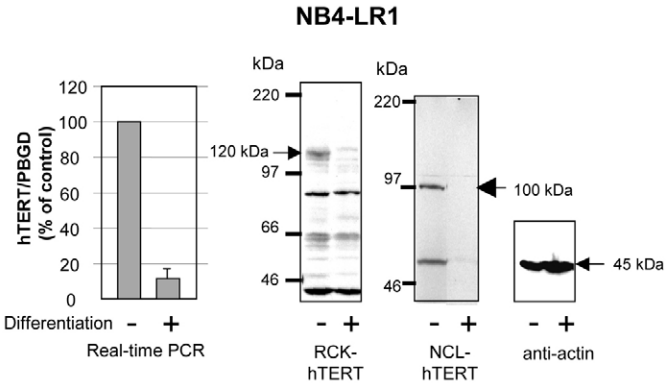

Fig. 1. hTERT is downregulated during NB4-LR1 differentiation. Differentiation (+) of NB4-LR1 cells was obtained by a 72-hour treatment with the combination of ATRA (1 μM) and 8-CPT-cAMP (200 μM). RNA and protein extracts were prepared as described in the Materials and Methods. hTERT mRNA expression was quantified by fluorescence real-time RT-PCR using the LightCycler® technology and the LightCycler Telo*TAGGG* hTERT Kit from Roche Diagnostics (Meylan, France). The hTERT level was normalized to the expression of the housekeeping gene porphobilinogen deaminase (PBGD) and expressed as a percentage of that detected in the untreated cells. Western blot analyses were performed using RCK-hTERT and NCL-hTERT antibodies. Positions of the molecular weight markers are indicated on the left. Note that the low molecular weight band on NCL-hTERT blot was not reproducibly detected.

hTERT protein expression in differentiating NB4-LR1 APL cells

As previously shown (Pendino et al., 2003; Pendino et al., 2001), differentiation of NB4-LR1 APL cells (induced by the combination of ATRA and 8-(4-chlorophenylthio)adenosine cyclic 3′,5′-monophosphate (8-CPT-cAMP) treatments at 1 μM and 200 μM, respectively) was associated with a decrease in hTERT mRNA level measured by real-time quantitative PCR (Fig. 1). Western blots were then performed using the corresponding protein extracts from control or differentiated NB4-LR1 cells and two commercially available anti-hTERT antibodies [(RCK-hTERT (Rockland) versus NCL-hTERT (Novocastra)]. In extracts from untreated cells, RCK-hTERT antibody revealed, among others, a band at 120 kDa, a molecular weight expected for hTERT protein. The intensity of labelling of this protein band is highly reduced during cell differentiation, concomitant with the decrease of hTERT mRNA level. NCL-hTERT antibody, one of the most frequently used (according to the literature) tested here in the same conditions on the same cell extracts, revealed a unique protein band within the 100 kDa area. Note, however, that, as previously shown on RCK-hTERT blots for the 120 kDa band, this 100 kDa protein band was no longer detected in differentiated NB4-LR1 cells. These large differences in migration velocity prompted us to re-evaluate the efficacy and

Table 1. Anti-hTERT antibodies used in the study

Supplier	Reference	Immunogen	Location*	Nature	Dilution†
Novocastra	NCL-hTERT	aa: 173-320	N-terminal domain	Mouse monoclonal (clone 44F12)	1:800
Calbiochem	581400 (Calb-hTERT)	aa: 568-581	Central domain	Rabbit polyclonal	1:1000
Rockland	600-401-252 (RCK-hTERT)	aa:1104-1123	C-terminal domain	Rabbit polyclonal	1:1000
Santa-Cruz TERT(L-20)	sc-7214 (SC-hTERT)	aa: 1092-1111	C-terminal domain	Goat polyclonal	1:1000
Novus Biologicals	NB 100-137 (NB-hTERT)	Full recombinant protein		Mouse monoclonal (clone 2C4)	1:1500

*For details see supplementary material Fig. S1; †dilution used in immunoblot experiments; aa, amino acid.

specificity of some commercially available anti-hTERT antibodies and compare them with RCK-hTERT and NCL-hTERT.

Immunodetection of hTERT in cell lines using commercial antibodies

NCL-hTERT detection of a single protein band irrelevant to hTERT by its SDS-PAGE migration velocity

The properties and specific recognition domains on hTERT protein by the various commercial antibodies used in this study are shown in Table 1 and Fig. S1 in supplementary material. HA-tagged hTERT and non-tagged hTERT-expressing HeLa cells and anti-HA antibodies were used as migration controls to precisely determine the position of the hTERT protein band detected on western blots using the various anti-hTERT antibodies. When lysates prepared from HA-tagged hTERT-expressing HeLa cells were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, anti-HA antibodies recognized a protein with a molecular weight of ~120 kDa as expected for the hTERT protein (Fig. 2A, lane 2 and Fig. S3 in supplementary material). No protein band was detected in extracts prepared from cells transfected either with p-Babe empty vector (Fig. 2, lane 1) or with non-tagged hTERT (supplementary material Fig. S3).

Numerous non-specific bands were generally seen in immunoblotting experiments using various anti-hTERT antibodies, except for NCL-hTERT antibody (Fig. 2A, lanes 3 and 4). Note that in this study we make a point of showing the blots in full, in order to visualize both the specific and the non-

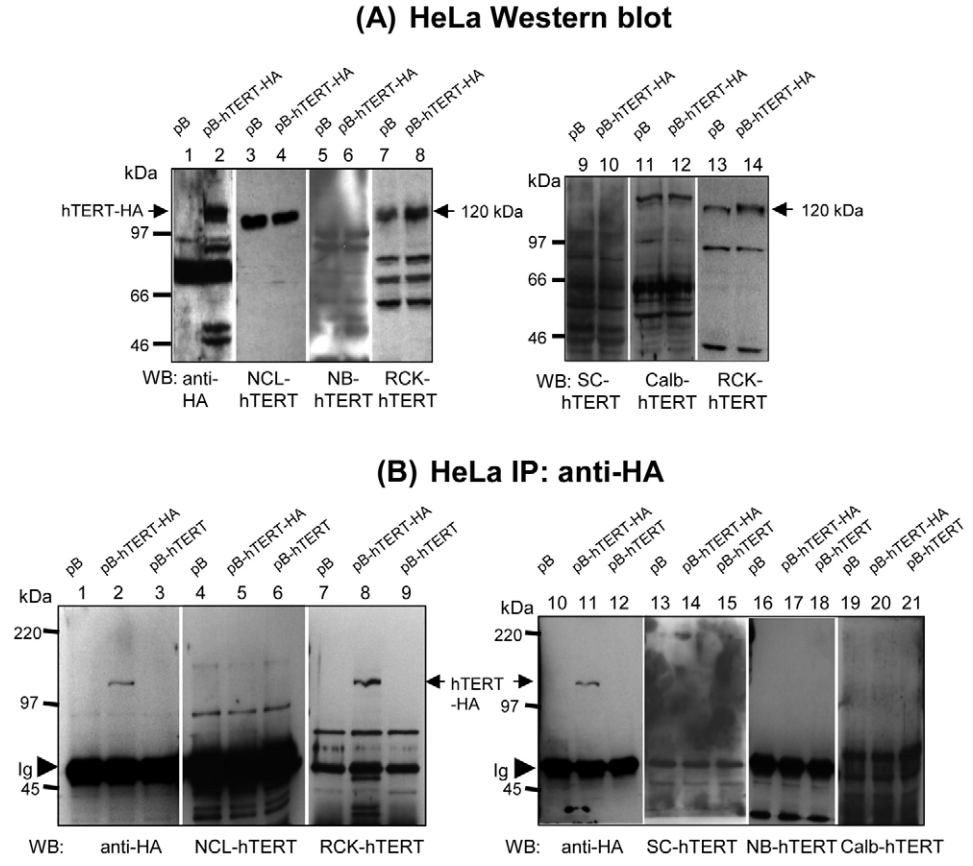

Fig. 2. Comparative study for the detection of hTERT protein in HeLa cells by immunoblotting and immunoprecipitation using commercially available antibodies. (A) Western blot analysis of total cell lysates from HeLa cells transfected with either HA-tagged hTERT (pB-hTERT-HA) or p-Babe empty vector alone (pB) using an anti-HA antibody and various anti-hTERT antibodies. (B) Immunoprecipitation (IP) of lysates from HeLa cells transfected with HA-tagged hTERT (pB-hTERT-HA), the non-tagged hTERT protein (pB-hTERT), or p-Babe empty vector alone (pB) with anti-HA antibody and immunoblotting with either anti-HA antibody or various anti-hTERT antibodies as indicated. Positions of the molecular weight markers are indicated on the left. Positions of the proteins of interest are indicated by arrows. Position of the heavy chain (Ig) of the anti-HA antibody is shown.

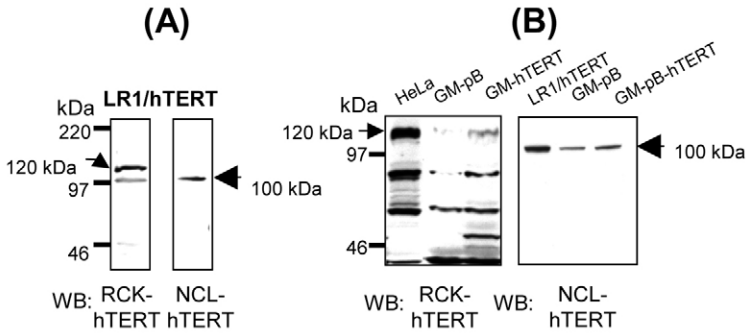

Fig. 3. Detection of hTERT protein in NB4-LR1/hTERT (A) and GM847 cell extracts (B) using NCL-hTERT or RCK-hTERT antibodies. Western blot analyses of total cell lysates from NB4-LR1/hTERT (A) and from GM847 (B) cells transfected with either hTERT (GM-pB-hTERT) or pBabe empty vector alone (GM-pB). Positions of the molecular weight markers are indicated on the left. Positions of the proteins of interest are indicated by arrows.

specific bands. Among others, both Calb-hTERT and RCK-hTERT antibodies recognized a protein that migrated in the area of the expected molecular weight for hTERT (~120 kDa), although at a different velocity (Fig. 2A, lanes 11, 12 and 7, 8, 13, 14, respectively). In contrast, and as previously noticed (Lantuejoul et al., 2004) a clear and unique protein band was observed on immunoblots using NCL-hTERT antibody (Fig. 2A, lanes 3 and 4). However, as previously shown (Fig. 1), this protein migrated within a band with an apparent molecular weight lower than expected for hTERT. Furthermore, this antibody failed to recognize hTERT in hTERT-HA immunoprecipitates (Fig. 2B, lane 5). Note that among all the anti-hTERT antibodies used in this study, only RCK-hTERT antibody was able to recognize the hTERT-HA fusion protein in HA-immunoprecipitates (Fig. 2B, lane 8). No protein was detected by either anti-HA antibody or RCK-hTERT antibody in HA-immunoprecipitates from cells transfected by non-tagged hTERT (Fig. 2B, lanes 3, 9 and 12) or p-Babe vector alone (Fig. 2B, lanes 1, 7 and 10), confirming the specificity of recognition of these antibodies.

These distinct patterns of recognition were also observed when RCK-hTERT and NCL-hTERT antibodies were used to label blots from NB4-LR1/hTERT extracts (Fig. 3A). Note that RCK-hTERT antibody detected a protein band that migrated at the same velocity as the protein recognized by NCL-hTERT. Although it is possible that these proteins are identical, the nature of this protein band remained to be elucidated.

NCL-hTERT detection of a single protein band in the GM847 telomerase-negative cells

In extracts from the GM847 cell line, RCK-hTERT antibody detected a faint protein band in the 120 kDa area, as expected for this ALT cell line type found to be negative for telomerase activity (Fig. 3B, left panel). In contrast, in the hTERT over-expressing GM847, the same antibody detected a clear band at 120 kDa, likely to be hTERT. As an additional surprise, NCL-hTERT antibody detected a single band in GM847 cells, at the same 100 kDa molecular weight as in HeLa and NB4-LR1 cell variants, and no label was found at ~120 kDa in the hTERT-expressing GM847 cells (Fig. 3B, right panel).

Two distinct protein entities are recognized in a single molecular complex by cross-immunoprecipitation

By co-immunoprecipitation experiments, we found that NCL-hTERT antibody recognized the single 100 kDa protein band in RCK-hTERT immunoprecipitates (Fig. 4A, lane 3) and conversely, in a reverse immunoprecipitation experiment, RCK-hTERT antibody recognized the 120 kDa hTERT protein band in NCL-hTERT immunoprecipitates (Fig. 4B, lane 1). Note that this antibody also recognized a ~100 kDa band in NB4-LR1 whole extract (see in Fig. 3A).

Altogether these results demonstrate that the band detected on western blots by NCL-hTERT antibody is most likely not the expected hTERT protein but a protein entity associated with hTERT since it co-immunoprecipitates with a protein which was further detected as being hTERT on western blot using RCK-hTERT. As NCL-hTERT antibody is so far, among the commercially available antibodies, one of the most reported in the literature for correlation studies between hTERT mRNA level or telomerase activity and for quantitative evaluation of the protein, we were eager to identify the actual protein detected by this antibody and to know whether it was related to telomerase.

Protein identification by mass spectrometry after immunoprecipitation with RCK-hTERT and NCL-hTERT antibodies

Separate immunoprecipitations from NB4-LR1/hTERT cell lysates using either RCK-hTERT or NCL-hTERT antibodies

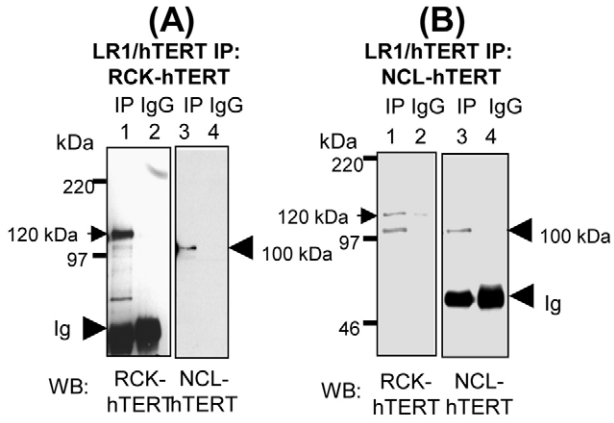

Fig. 4. Immunoprecipitations of lysates from NB4-LR1/hTERT cells with RCK-hTERT (A) or NCL-hTERT (B) antibodies and immunoblotting with both antibodies. Positions of the molecular weight markers are indicated on the left. Positions of the proteins of interest are indicated by arrows. Position of the heavy chain (Ig) of the NCL-hTERT antibody is shown. IgG, control for nonspecific binding by performing immunoprecipitation reaction using normal IgG. No immunoprecipitation was observed with control IgG.

Table 2. Identification of the proteins following data searching with Mascot software (http://matrixscience.com)

Immunoprecipitating antibody*	Protein band (kDa)	Identification
RCK-hTERT (600-401-252)	120	Human telomerase catalytic subunit
	100	Heat shock protein Hsp90
	100	Nucleolin
NCL-hTERT (clone 44F12)	100	Nucleolin
	100	Alpha-actinin-1 and -4

*Immunoprecipitates prepared from NB4-LR1 cell extracts using the indicated antibody were fractionated by SDS-PAGE. The stained protein bands of interest were cut from the gel in the corresponding region of interest deduced from immunoblotting analysis, and subjected to mass spectrometry (see Materials and Methods).

were performed. Immunoprecipitated materials were then separated by SDS-PAGE and proteins visualized by Coomassie Blue G-250 staining. Protein bands in the region of interest (at 100 kDa for NCL-hTERT and RCK-hTERT immunoprecipitates and at 120 kDa for RCK-hTERT immunoprecipitates) were cut and subjected to mass spectrometry (see Materials and Methods). The protein identification data are summarized in Table 2.

RCK-hTERT immunoprecipitates contained a protein at the expected molecular weight (120 kDa) identified as hTERT by mass spectrometry. This result validates, without any ambiguity, the hTERT specificity of this antibody. Heat shock protein (Hsp)90β and nucleolin were identified in two protein bands of lower molecular weights (90 and 100 kDa, respectively). This observation is in agreement with earlier reports (Forsythe et al., 2001; Holt et al., 1999; Khurts et al., 2004) indicating that these two proteins belong to the telomerase complex. We further confirmed the presence of these two proteins (Hsp90β and nucleolin) in the telomerase complex by co-immunoprecipitation experiments on NB4-LR1/hTERT extracts and immunoblotting using RCK-hTERT, Hsp90β and nucleolin antibodies, respectively (Fig. S4A in supplementary material).

Two proteins, nucleolin and alpha-actinin, were identified by mass spectrometry analysis as components of the 100 kDa band immunoprecipitated by the NCL-hTERT antibody. Intriguingly, no band was identified as an hTERT-related protein.

Co-immunoprecipitation experiments using extracts from NB4-LR1/hTERT, followed by western blot analysis using NCL-hTERT, RCK-hTERT, anti-nucleolin and anti-alpha-actinin antibodies were performed. Nucleolin was found in NCL-hTERT immunoprecipitates (Fig. 5, lane 1). A reverse immunoprecipitation carried out with anti-nucleolin as the precipitating antibody, pulled down a protein recognized by NCL-hTERT that migrates at the same molecular weight (100 kDa) as nucleolin (Fig. 5, compare lane 5 with 7). Similarly, alpha-actinin was found in both NCL-hTERT and nucleolin immunoprecipitates (Fig. S4B in supplementary material).

Altogether these results suggest that nucleolin or alpha-actinin are directly or indirectly immunoprecipitated by NCL-hTERT. Of note, the fact that mass spectrometry failed to identify any hTERT peptides in immunoprecipitates performed with NCL-hTERT antibody supports the hypothesis that this antibody does not recognize any related or truncated hTERT protein.

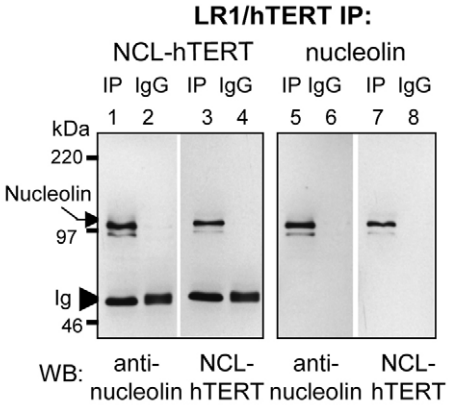

Fig. 5. Immunoprecipitations of lysates from NB4-LR1/hTERT cells with NCL-hTERT or nucleolin antibodies and immunoblotting with both antibodies. Positions of the molecular weight markers are indicated on the left. Positions of the proteins of interest are indicated by arrows. Position of the heavy chain (Ig) of antibodies is shown. IgG, control for nonspecific binding by performing immunoprecipitation reaction using normal mouse or rabbit IgG.

Two-dimensional gel electrophoresis (2-DGE) shows that NCL-hTERT recognizes nucleolin

Cell extracts from NB4-LR1 cells were subjected to two-dimensional gel electrophoresis (2-DGE) and western blots were developed with NCL-hTERT, anti-nucleolin and anti-alpha-actinin antibodies (Fig. 6). Using anti-nucleolin antibody, immunoreactive spots were detected at isoelectric point (pI) 5.5-6.1, characteristic for nucleolin and its isoforms. The nature of the spots detected at pI 4.5-4.8 has not yet been identified. Surprisingly, using NCL-hTERT, no immunoreactive protein spot was detected in the pI range 9.0-9.6 that has been predicted for telomerase (data not shown). However, it recognized spots of about 100 kDa ranging from pI 4.5-4.8 and pI 5.5-6.0 that precisely corresponded to those recognized by anti-nucleolin antibody. Interestingly, in the

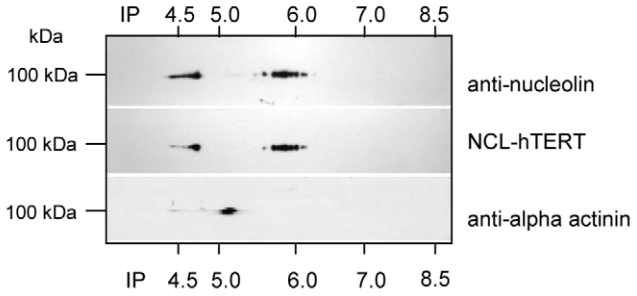

Fig. 6. Identification of nucleolin and alpha actinin in NB4-LR1 cells by two dimensional electrophoresis and immunoblotting. NB4-LR1 proteins separated by isoelectric focusing in a pH 3-10 Bio-Rad IPG gel in the first dimension and by electrophoresis in an SDS 10% polyacrylamide gel in the second dimension. The proteins were blotted onto PVDF membranes and revealed using the indicated antibodies. Positions of the molecular weight marker are indicated on the left. IP, isoelectric point.

same experiment, the two-dimensional blot analysed with anti-alpha-actinin antibody shows a clearly different pattern. Although alpha-actinin migrates at the same molecular weight as nucleolin, its pI is clearly different (4.96). Altogether, these findings demonstrate that NCL-hTERT does not recognize telomerase but nucleolin on western blots.

Immunofluorescence experiments show that RCK-hTERT and NCL-hTERT recognize distinct structures.

As the above experiments relied only on western blot analyses, immunofluorescence experiments were performed in order to support the above results. These experiments were performed on both NB4-LR1 cells, expressing a high level of endogenous hTERT, and on GM847 cells used as a negative control for hTERT expression.

In NB4-LR1 cells, endogenous hTERT detected by RCK-hTERT is clearly observed throughout the cells, indicating that it exists in both the nucleus and cytoplasm (Fig. 7A,B). It is clear from confocal images that RCK-hTERT and NCL-hTERT do not recognize the same protein even though some co-localization in nucleolar structures exists (Fig. 7A,f and B,f). This observation is not surprising, as it was previously demonstrated that hTERT is present in the nucleolus (Yang et al., 2002). The distribution of the protein recognized by NCL-hTERT is similar to that of nucleolin (Fig. 7A,l).

In the GM847 cell line, the detection of hTERT by RCK-hTERT was rather faint (Fig. 7C,a) supporting the results obtained in western blot experiments. By contrast, NCL-hTERT labelled the same structures as in NB4-LR1 cells, with the same intensity (Fig. 7C,b). Therefore, using a different experimental approach, these results corroborate the findings previously achieved.

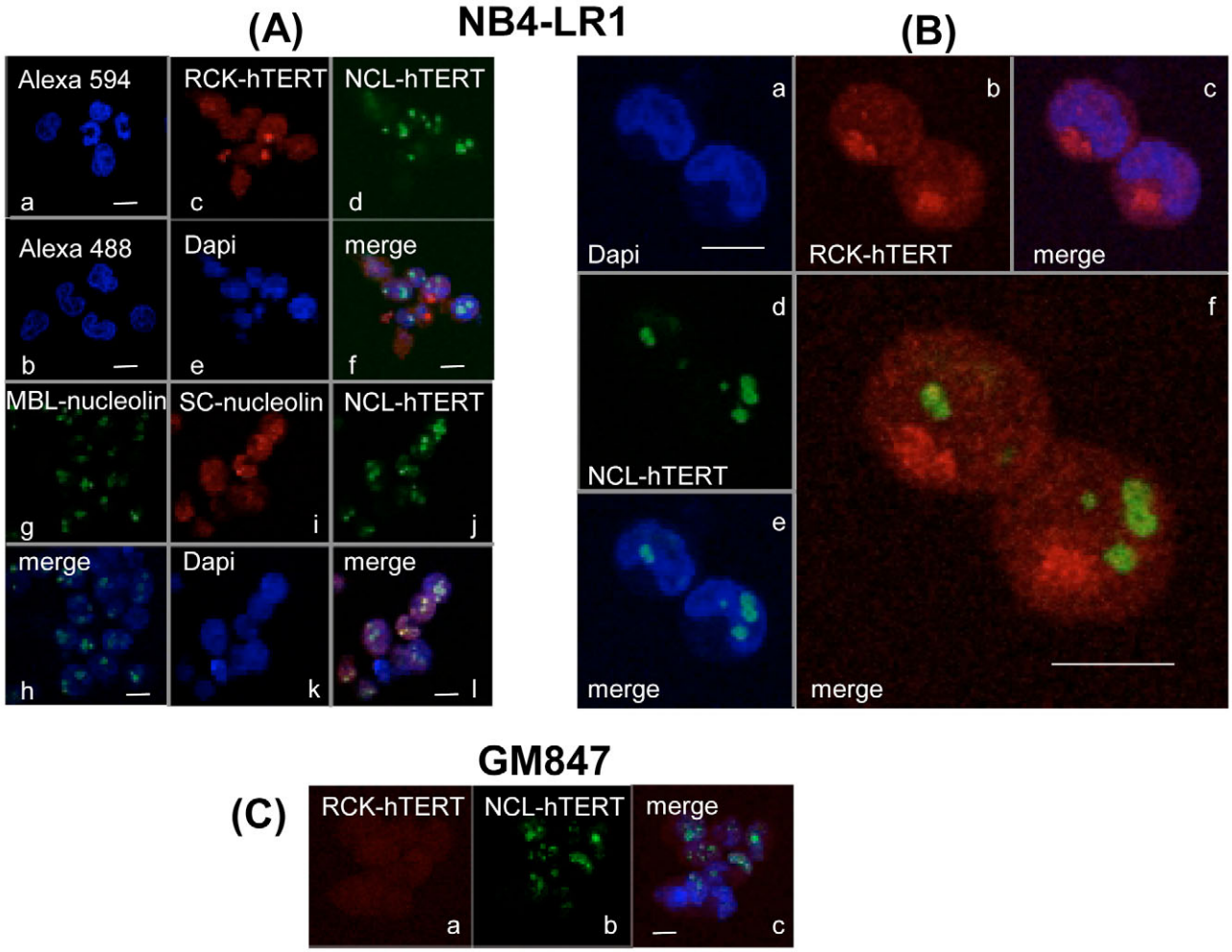

Fig. 7. Immunofluorescence and confocal microscopy. (A) hTERT was detected in NB4-LR1 cells using RCK-hTERT (red; Alexa Fluor 594-conjugated anti-rabbit antibody). Nucleolin was detected either by MBL-nucleolin (green; Alexa Fluor 488-conjugated anti-mouse antibody) or by SC-nucleolin (red; Alexa Fluor 594-conjugated anti-rabbit antibody). NCL-hTERT antibody detected a protein (green; Alexa Fluor 488-conjugated anti-mouse antibody) that colocalized with nucleolin. Nuclei are stained in blue with DAPI. Panels f and l show the superimposition of RCK-hTERT, NCL-hTERT, DAPI signals and SC-nucleolin, NCL-hTERT and DAPI signals, respectively. (B) Higher magnification of two NB4-LR1 cells stained using either RCK-hTERT (b) or NCL-hTERT (d). Panels c, e and f are superimposed images of DAPI and RCK-hTERT or NCL-hTERT signals and RCK-hTERT and NCL-hTERT signals, respectively. (C) Staining of GM847 cells using either RCK-hTERT (red; Alexa Fluor 594-conjugated anti-rabbit antibody) or NCL-hTERT (green; Alexa Fluor 488-conjugated anti-mouse antibody) antibodies. Nuclei are stained blue with DAPI. Bars, 10 μm.

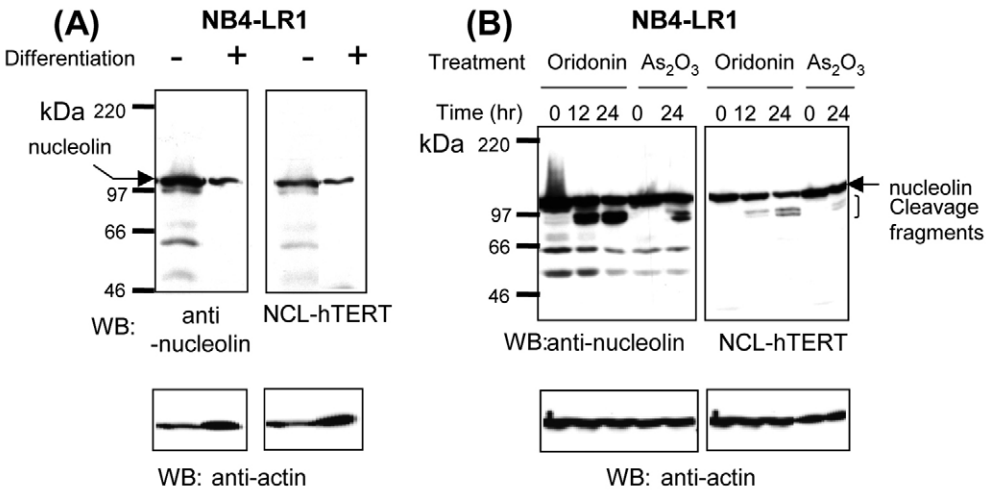

Fig. 8. Nucleolin protein in NB4-LR1 cells after differentiation and apoptosis. (A) Differentiation (+) of NB4-LR1 cells was obtained by a 72-hour treatment with a combination of ATRA (1 μM) and 8-CPT-cAMP (200 μM). (B) Apoptosis was induced either by a 24-hour treatment with oridonin (4 μg/ml) or a 24-hour treatment with As_2O_3 (5 μM). Proteolytic fragments are indicated.

Nucleolin is downregulated during differentiation and cleaved during apoptosis of NB4-LR1 cells

We have shown (Fig. 1) that the level of the protein detected by NCL-hTERT antibody decreased during differentiation of NB4-LR1 cells concomitantly with the decrease of hTERT mRNA. To further address the functional relevance of the above results, we investigated whether nucleolin was similarly regulated during differentiation. Fig. 8A clearly shows that, like the protein detected by NCL-hTERT antibody, nucleolin protein is reduced in differentiated cells.

The proteolysis of nucleolin during apoptosis has been reported earlier (Ikezoe et al., 2003; Liu et al., 2005). It generates a discrete cleavage product of ~90 kDa (Brockstedt et al., 1998; Mi et al., 2003; Pasternack et al., 1991; Sengupta et al., 2004). Therefore, if NCL-hTERT recognizes nucleolin, it would be expected to recognize these cleavage products. NB4-LR1 cells were treated with Oridonin or Arsenic, two apoptosis inducers. Fig. 8B shows that the protein detected by NCL-hTERT is rapidly degradated with the same cleavage pattern and kinetic as for nucleolin, generating proteolytic fragments of 88-90 kDa.

Collectively, these results demonstrate that the protein detected by NCL-hTERT is not hTERT but a protein endowed with the biochemical and biological features of nucleolin.

Discussion

The results obtained using mass peptide spectrometry analysis and confirmed by co-immunoprecipitation experiments and two-dimensional gel electrophoresis force us to conclude that among the commercially anti-hTERT antibodies used in this study, only RCK-hTERT antibody (Rockland) is suitable for hTERT protein detection.

Besides this, our work has confirmed physiologically interesting protein interaction in the hTERT enzymatic complex (hTERT, nucleolin, Hsp90β) and revealed a new one between nucleolin and alpha-actinin.

Importantly, we demonstrate here that the NCL-hTERT antibody clearly does not recognize hTERT, but nucleolin or a nucleolin-related protein. This can be concluded for the following reasons. (1) On SDS-PAGE and 2-DGE immunoblotting, NCL-hTERT recognizes a protein that shares a number of properties with nucleolin (similar molecular weight and isoelectric point, degradation during apoptosis into similar fragments, downregulation during differentiation); (2) this protein is detected in western blot and in immunofluorescence experiments independently of the expression of hTERT; (c) this protein band was then identified as nucleolin by classical microsequencing.

Although the predicted molecular weight of nucleolin according to its coding cDNA is 77 kDa (Srivastava et al., 1989), when run on a SDS-PAGE gel, it demonstrates an apparent weight of 100 kDa (Srivastava et al., 1989). This discrepancy has been attributed to the highly acidic N-terminal domain of this protein, which contains a high number of negatively charged amino acids (Ginisty et al., 1999). Nucleolin has four RNA binding domains in the central portion of the molecule and a carboxy-terminal RGG domain that binds RNA, as well as some proteins (reviewed in Bouvet et al., 1998; Creancier et al., 1993). Of note, this protein has been identified to interact with telomerase (Khurts et al., 2004) through its RNA binding domain 4 and its carboxy-terminal RGG domain. This binding also involves the telomerase RNA subunit hTR. This interaction between nucleolin and hTERT would be critical for the nucleolar localization of hTERT. Nucleolin has also been found to bind to the human telomeric DNA sequence d(TTAGGG)n (Ishikawa et al., 1993; Pollice et al., 2000).

There are a number of reasons why the results obtained with NCL-hTERT (Novocastra) antibody were not questioned earlier. First, like telomerase, nucleolin is associated with many of the processes that are dysfunctional in neoplastic cells. Nucleolin expression is highly proliferation dependent in human tumour cells and nucleolin was used in studies of different cancer cell lines as a useful marker for cell proliferation (Derenzini et al., 1995). Furthermore, high levels of silver-staining nucleolar proteins (of which nucleolin is a major component) predict a poor prognosis in many types of cancer (Derenzini, 2000). In nondividing cells, nucleolin autocatalyzes its own degradation, and is present at a very low level (Sirri et al., 1995). Nucleolin is also regulated both at transcriptional and post-translational levels during development of somatic tissue (Bicknell et al., 2005).

Second, as for telomerase, we show that differentiation of APL cells was accompanied by a downregulation of nucleolin

in agreement with recent results obtained in other cell models (Murakami et al., 1991; Otake et al., 2005).

Third, telomerase and nucleolin share the same intracellular distribution. Indeed like telomerase, nucleolin is not only found in the nucleus but also in the cytoplasm, depending on the cell type. Furthermore, in the nucleus both telomerase and nucleolin can be mobilized between nucleoli and the nucleoplasm depending on the state of the cell (Daniely and Borowiec, 2000; Daniely et al., 2002; Khurts et al., 2004; Wong et al., 2002). Given these observations, it is not surprising, therefore, that the pattern of expression for nucleolin and telomerase were found to be almost identical.

NCL-hTERT was claimed to be made against a fragment of the protein corresponding to a part of the N-terminal domain of hTERT (amino acids: 173-320). However, we were not able to identify any amino-acid sequence similarity between the two proteins that could have the immunological properties of NCL-hTERT, and the question of this misrecognition remains non-elucidated. Nevertheless, the identification of nucleolin as the protein recognized by NCL-hTERT antibody should lead to an urgent re-evaluation of the results obtained with this antibody and that are already published (Brustmann, 2005; Dalerba et al., 2005; Domont et al., 2005; Dutu et al., 2005; Elkak et al., 2005; Falchetti et al., 2003; Fullen et al., 2005; Gulmann et al., 2005; Kraemer et al., 2003; Lantuejoul et al., 2005; Lantuejoul et al., 2004; Luzar et al., 2005a; Luzar et al., 2005b; Maes et al., 2005; Sabah et al., 2004; Sato et al., 2004; Smith et al., 2004; Uziel et al., 2005; Yan et al., 2004).

Far from being detrimental to published work, our finding should renew interest after reconsideration of the conclusions previously drawn. Because part of the studies that used NCL-hTERT antibody were designed to validate hTERT expression as a diagnostic and prognostic marker of cancer, our present findings confirm that the expression pattern of nucleolin could also be associated with clinicopathological characteristics or prognosis of tumours. Undoubtedly, future pathological and biomolecular investigations should now consider these new features.

Materials and Methods

Reagents

All-trans retinoic acid (ATRA; dissolved in ethanol as stock solution at 10^{-3} M), 8-(4-chlorophenylthio)adenosine cyclic 3′,5′-monophosphate (8-CPT-cAMP; dissolved in RPMI 1640 at 10^{-2} M), and As_2O_3 were purchased from Sigma (St Louis, MO). Oridonin, a gift from Dr Q. J. Tang (Shanghai Academy of Agricultural Sciences) was dissolved in DMSO as stock solution at 10 mg/ml.

Cell lines, generation of stable clones and cell culture

The retrovirally infected acute promyelocytic leukemia (APL) NB4-LR1/hTERT-GFP cells (Pendino et al., 2001) expressing both hTERT protein and the green fluorescent protein (GFP) reporter from the same transcript and the NB4-LR1/GFP subline expressing only the GFP were cultured as described previously (Lanotte et al., 1991; Pendino et al., 2003; Pendino et al., 2002) in RPMI1640 medium supplemented with 10% fetal bovine serum (PAA Laboratories, Les Mureaux, France), penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and sodium bicarbonate (70 mg/l), and incubated at 37°C in a 5% CO_2 atmosphere (Binder Incubator).

The GM847 (SV40 immortalized human skin fibroblasts) cell line, a gift from Dr Olivia Pereira-Smith (Baylor College of Medicine, Waco, TX) and HeLa cells were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories), L-glutamine (2 mM) and penicillin (50 IU/ml), streptomycin (50 μg/ml), and incubated at 37°C in a 5% CO_2 atmosphere (Binder Incubator). pBabe-puro, pBABE-puro-hTERT and pBABE-puro-$hTERT_{HA}$, provided by Dr Weinberg (MIT, Cambridge, MA), were transfected in GM847 cells (GM847-pBabe, GM847-pBabe-hTERT) and in HeLa cells (HeLa-pBabe, HeLa-pBabe-hTERT and HeLa-pBabe-$hTERT_{HA}$) at about 80% confluency using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Forty eight hours after transfection, cells were selected continuously in puromycin (1 μg/ml). Stable clones were isolated.

Analysis of hTERT expression by Real-time PCR

Full-length hTERT mRNA expression was quantified by fluorescence real-time RT-PCR using the LightCycler® technology and the LightCycler Telo*TAGGG* hTERT Kit from Roche Diagnostics (Meylan, France) according to the manufacturer's instructions. The hTERT level was normalized to the expression of the housekeeping gene porphobilinogen deaminase (PBGD).

Western blotting

Whole cell pellets (2×10^6 cells) were washed twice with ice-cold PBS, resuspended in PBS (v/v), lysed by addition of an equal volume of 2× SDS buffer [1× = 50 mM Tris-base (pH 6.8), 2% SDS, 100 mM dithiothreitol and 10% glycerol], and boiled for 5 minutes, then put on ice for 5 minutes and boiled for another 5 minutes. Insoluble material was removed by centrifugation at 14,000 ***g*** for 5 minutes. The proteins (10 μg) were separated by SDS-PAGE using a 7% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were then stained with Ponceau S to ensure transfer and equal loading. When necessary, the membranes were cut into segments to allow immunoblotting with different antibodies. After blocking in Tris-buffered saline containing 3% nonfat-milk and 0.1% Tween 20 (TBS-T) for 2 hours at room temperature, the blots were incubated overnight at 4°C in fresh blocking solution with an appropriate dilution of primary antibody.

The sources, properties and dilutions of anti-hTERT antibodies are described in Table 1 and in Fig. S1 in supplementary material. Anti-actin (rabbit polyclonal; Sigma, MO; dilution 1:5000), anti-HA (mouse monoclonal, clone F7; Santa Cruz Biotechnology, sc-7392; dilution 1:2000), anti-Hsp90β (rabbit polyclonal; Chemicon, AB3468; dilution 1:1000), anti-nucleolin (mouse monoclonal, clone 4E-2; MBL, MO19-3, dilution 1:1000 for western blotting and rabbit polyclonal, H-250; Santa Cruz Biotechnology, sc-13057, for immunoprecipitation) and anti-alpha-actinin (mouse monoclonal; Chemicon; dilution 1:150) antibodies were used. Blots were washed three times for 5 minutes each in TBS-T and then incubated with a 1:5000 dilution of appropriate horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. Blots were again washed three times for 5 minutes each in TBS-T and then developed by enhanced chemiluminescence (ECL, Perkin Elmer Life Sciences).

Immunoprecipitation

NB4-LR1/hTERT cells (2×10^7) were lysed with 1.0 ml of RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100 and 1 mM EDTA) with protease inhibitors (Sigma, MO) for 30 minutes on ice and then centrifuged at 14,000 ***g*** for 10 minutes. The soluble part was subjected to a preclearing step in which it was incubated with 50 μl of protein G-Sepharose (4 Fast Flow, Amersham Biosciences, Piscataway, NJ) for 1 hour at 4°C. The precleared cell extracts were then incubated with the adequate first antibody overnight at 4°C. Protein G-Sepharose beads (50 μl of 50% slurry) were then added for 1 hour at 4°C with gentle shaking and centrifuged. The immunoprecipitates were washed three times in cold RIPA buffer containing protease inhibitors. They were then resuspended in 60 μl 1× SDS buffer, boiled for 5 minutes and centrifuged for 1 minute at 100 ***g***. The supernatants were saved as immunoprecipitate samples, fractionated by SDS-PAGE as described above. Immunoprecipitation reactions to control for nonspecific binding were performed using normal IgG.

Protein identification by peptide mass spectrometry

Immunoprecipitates prepared as described above were fractionated by SDS-PAGE in two 7% polyacrylamide gels run in the same conditions. One of the gels was used for immunoblotting with either RCK-hTERT or NCL-hTERT antibodies, whereas the other was stained with colloidal Coomassie Brilliant Blue G-250. In brief, the gel was fixed for 1 hour in 20% methanol containing 1.3% O-phosphoric acid. Then, Coomassie Brilliant Blue G-250 solution was added to a final concentration of 1%. Staining was carried out overnight with shaking at room temperature on an orbital rotator. The gel was washed with 1% acetic acid until all Coomassie particles are removed. Stained protein bands of interest were cut from the gel in the corresponding region of interest, deduced from immunoblotting analysis. The resulting peptides were extracted and digested with trypsin. Peptide mass spectrometry of the samples was performed by using matrix-assisted laser desorption-ionisation/time-of-flight mass spectrometry (MALDI-TOF/MS). The tryptic peptide masses obtained were searched in the National Center for Biotechnology Information nonredundant database using Mascot software (Matrix Science, London, UK).

Two-dimensional gel electrophoresis

NB4-LR1 cells (10^7) were collected by centrifugation at 300 ***g***, washed twice in ice-cold PBS and lysed in 1 ml of IEF buffer (7 M urea, 2 M thiourea, 4% Chaps, 0.24% Triton X-100, 0.48% Bio-Lyte 3/10 ampholyte and 2 mM tributylphosphine) containing 1% protease inhibitor cocktail (Sigma P8340), 1.25 mM NaF and 0.2 mM sodium orthovanadate. The lysate was incubated at room temperature for 20 minutes with gentle agitation and clarified by

centrifugation at 35,500 ***g*** for 1 hour. Protein concentration was determined by the Bradford assay. ReadyStrip Immobilized pH gradient (IPG) strips (17 cm, pH 3-10, Bio-Rad) were rehydrated with 100 μg protein extract diluted in 350 μl IEF buffer. The first dimension electrophoresis was performed with Multiphor II IEF system (Amersham Biosciences). Focusing was carried out at 20°C under a current limit of 50 μA/strip and achieved with a total of 55,000 V/hour. The second dimension electrophoresis was performed using a standard vertical slab gel electrophoresis (Protean II XL system, Bio-Rad). The strip previously incubated in 1% DTT then in 3% iodoacetamide in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol 2% SDS), for 15 minutes each, was loaded on the top of a vertical 10% polyacryamide gel (19×19 cm). SDS-PAGE was carried out at 15 mA/gel, 15°C, in classical SDS/Tris/glycine running buffer followed by immunoblotting.

Immunofluorescence and confocal microscopy

Cells (1×10^6) were collected by centrifugation, washed twice with PBS, fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature. Fixed cells were permeabilized by 0.1% Triton X-100 in PBS at room temperature for 8 minutes and blocked with 2% bovine serum albumin (BSA) in PBS overnight at 4°C. After three washes in blocking buffer (0.05% BSA in PBS), cells were incubated with primary antibody (NCL-hTERT, 1:50; RCK-hTERT, 1:150; MBL-nucleolin or SC-nucleolin, 1:200) in blocking buffer overnight, washed twice in PBS and incubated with secondary antibody (either Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 594-conjugated anti-rabbit both from Molecular Probes, Invitrogen) diluted 1:1000 in 2% BSA in PBS, for 1 hour at room temperature. Subsequently, cells were washed three times in PBS and dropped on glass slides which were mounted in Vectashield mounting medium with 4′,6-diaminidine-2-phenylindole (DAPI, Vector Laboratories) to counterstain nuclei. Immunofluorescent images were acquired with the LSM software on a Zeiss LSM510 Meta laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss Axiovert 200M (plan Apochromat 63× 1.40 NA oil immersion objective).

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