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Intracellular distribution of the ΔNp73 protein isoform in medulloblastoma cells: a study with newly generated rabbit polyclonal antibodies

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Summary. The p73 protein is a member of the p53 family of transcription factors that has two N-terminal isoforms: the TAp73 isoform is reported to have a tumor suppressor function, whereas the ΔNp73 isoform likely has oncogenic potential. The expression of these isoforms and the differences in their intracellular distribution have been described in many cancer types; however, little is known about the p73 isoforms in brain tumors. Our study is focused on the intracellular localization of ΔNp73 in medulloblastoma cell lines. Due to a lack of suitable anti-ΔNp73 antibodies, we developed two new rabbit polyclonal antibodies, ΔNp73-26 and ΔNp73-27, with sufficient specificity, as demonstrated by immunodetection methods using transiently transfected cell lines. Both of these new antibodies were subsequently used for analysis of the ΔNp73 distribution in medulloblastoma cells using immunofluorescence, immunoblotting and immunogold labeling for transmission electron microscopy. We found a nuclear localization of the ΔNp73 isoform in all of the medulloblastoma cell lines included in this study. Furthermore, a non-random accumulation of the ΔNp73 isoform near the cell nuclei was observable in all of these cell lines. By double-labeling with ΔNp73 and golgin-97, we showed the co-localization of the ΔNp73 isoform with the Golgi apparatus. Nevertheless, further detailed analyses of possible interactions of ΔNp73 with the proteins accumulated in the Golgi apparatus should be performed to explain the dynamics of ΔNp73 outside the cell nucleus.

Key words: Medulloblastoma, DeltaNp73, Immunocytochemistry, Immunoblotting, Transmission electron microscopy

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Introduction

The p73 protein is a member of the p53 family of transcription factors, together with p53 and p63 (Kaghad et al., 1997). All three of these proteins show a high degree of homology, particularly in their functional domains, including the sequence-specific DNA-binding domain. The *TP73* gene is transcribed from two distinct promoters. This gene expression generates two main N-terminal isoforms, TAp73 and ΔNp73; however, alternative splicing within the 5' end of the primary transcript leads to the p73Δex2, p73Δex2/3, and ΔN'p73 isoforms. Alternative splicing within the 3' end of the primary transcript produces the C-terminal isoforms α-η (Kaghad et al., 1997; De Laurenzi et al., 1998, 1999; Ueda et al., 1999; Fillippovich et al., 2001; Ishimoto et al., 2002; Stiewe et al., 2002; Moll and Slade, 2004; Murray-Zmijewski et al., 2006).

The full-length TAp73 isoform contains a transactivation domain and thus is reported to have a tumor suppressor function. Truncated N-terminal isoforms without the transactivation domain show a dominant negative effect towards both the TAp73

isoform and wild-type p53; this observation may indicate an oncogenic potential of certain p73 N-terminal isoforms (Zaika et al., 2002; Melino et al., 2003; Moll and Slade, 2004; Oswald and Stiewe, 2008; Rufini et al., 2011).

In general, the activity of transcription factors is regulated in different ways, including their intracellular distribution. Nuclear export apparently blocks the transactivation activity of transcription factors; the same effect is also achieved by their retention in cytoplasmic organelles (Inoue et al., 2002; Dobbelstein et al., 2005; Di Vinci et al., 2009). During the last decade, the expression and intracellular localization of p73 isoforms were intensively studied in various human malignancies, including hepatocellular carcinoma (Müller et al., 2005; Tannapfel et al., 2008), breast carcinoma (Dominguez et al., 2006a; Bozzetti et al., 2007), colorectal carcinoma (Dominguez et al., 2006b), lung carcinoma (Uramoto et al., 2004; Di Vinci et al., 2009), and thyroid cancer (Frasca et al., 2003).

The p73 protein is known to be essential for the normal development and maintenance of neural tissue (Hernandez-Acosta et al., 2011; Killick et al., 2011). Nevertheless, little is known about p73 isoform expression in brain tumors, including any possible prognostic or predictive potential. Our first study on p73 isoforms in medulloblastoma tissue showed that the overexpression of p73α and ΔNp73, as detected by immunohistochemistry, is associated with a more aggressive clinical course of the disease (Zitterbart et al., 2007). In contrast, TAp73 expression was reported to be related to a better prognosis in children with medulloblastoma (Castellino et al., 2007).

In consideration of the contrasting results reported for various types of human cancer (see above), we decided to perform a detailed *in vitro* study on the intracellular distribution of the TAp73 and ΔNp73 isoforms in medulloblastoma cell lines (Nekulova et al., 2010). Nevertheless, the initially obtained results for the ΔNp73 isoform were not fully convincing, probably due to the specific but very weak signal of the anti-ΔNp73 antibody used for the experiments. To improve our previous results concerning the intracellular distribution of ΔNp73 in medulloblastoma cell lines, two new polyclonal antibodies with high specificity were generated, and these antibodies were used for ΔNp73 detection in the present study.

Materials and methods

Generation of new anti-ΔNp73 polyclonal antibodies

New anti-ΔNp73 rabbit polyclonal antibodies were produced by Moravian Biotechnology (Brno, Czech Republic). To obtain ΔNp73-specific polyclonal antibodies, New Zealand white rabbits were immunized with a peptide containing unique residues from the N-terminus of human ΔNp73 (MLYVGDPARHLAT) coupled to keyhole limpet hemocyanin. An over-expression screening system was used to test the polyclonal sera during the production process, and the new anti-ΔNp73 polyclonal antibodies were evaluated for specificity, both by SDS PAGE and immuno-cytochemical staining.

Cell culture and transient transfection for antibody characterization

MCF7 breast cancer cells with ectopic expression of p53 and various isoforms of p63 and p73 were used to test the cross-reactivity of the new antibodies with other p53 family members. The MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 2 mM L-glutamine (all from Invitrogen, Paisley, UK) in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The MCF7 cells were transfected with expression vectors encoding p53 and N-terminal and C-terminal isoforms of p63 and p73 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Vectors encoding the p63 isoforms were a generous gift from Dr. Karin Nylander (Yang et al. 1998); vectors encoding the p73 isoforms were kindly provided by Dr. Gerry Melino (De Laurenzi et al. 1998, Grob et al. 2001). pcDNA3 construct encoding p53 was generated according to standard protocols.

SDS-PAGE and immunoblotting for antibody characterization

Cells were grown to 80% confluence and lysed in a buffer consisting of 1% NP-40, 150 mM NaCl, and 20 mM Tris, pH 7.4, in the presence of a complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA). 20 μg of total protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blotted membranes were blocked in 5% non-fat dry milk for 1 hour at room temperature and probed overnight with our ΔNp73-26 and ΔNp73-27 rabbit polyclonal sera (dilution 1:1000), 4A4 mouse monoclonal anti-p63 antibody (dilution 1:250; Santa Cruz Biotechnology, Santa Cruz, USA), H-79 rabbit polyclonal anti-TAp73 antibody (dilution 1:100; Santa Cruz Biotechnology), IHC-00197 rabbit polyclonal antibody (dilution 1:200; Bethyl Laboratories, USA), 5B429 mouse monoclonal antibody (dilution 1:200; Santa Cruz Biotechnology) or IMG-313A mouse monoclonal antibody (dilution 1:100; Imgenex, San Diego, USA). Peroxidase-conjugated swine anti-rabbit (cat. No. P0217, dilution 1:1000; Dako, Glostrup, Denmark) and rabbit anti-mouse (cat. No. P0161, dilution 1:1000; Dako) immunoglobulin antisera were used as the secondary antibodies. To visualize the peroxidase activity, ECL reagents (Amersham Pharmacia Biotech, Little Chalfont, UK) were used according to the

manufacturer's protocol.

Immunocytochemistry for antibody characterization

MCF7 cells transiently transfected with plasmids encoding p53 and N-terminal isoforms of p63 and p73 were grown on slides for 24 hours and fixed in 10% ice-cold methanol-aceton (1:1) for 10 minutes. The endogenous peroxidase activity was blocked with 10% hydrogen peroxide in PBS for 5 minutes. The primary polyclonal antibodies ΔNp73-26 and ΔNp73-27 were diluted 1:20000, 4A4 monoclonal antibody was diluted 1 :10000 and H-79 polyclonal antibody was diluted 1:2000 in antibody diluent (Dako) and applied overnight at 4°C. The reactive sites were identified with biotinylated anti-rabbit secondary antibody and peroxidase ABC reagents (cat. No. PK-6101, PK-6102 Vectastain Elite ABC Kit, Vector Laboratories, USA) according to the manufacturer's protocol, and the peroxidase activity was visualized with DAB+ reagents (Dako).

Medulloblastoma cell lines and cell culture

Two reference and four in-house medulloblastoma cell lines were used in this study. The Daoy (HTB-186™) and D238Med (HTB-185™) reference cell lines were purchased from ATCC (LGC Standards, Teddington, UK). The MBL-02, MBL-06, MBL-12, and MBL-13 in-house cell lines were derived from primary tumors in our laboratory according to a protocol described previously (Veselska et al., 2006). Samples of tumor tissue were taken from patients surgically treated for medulloblastoma; a brief clinical description of these patients is given in Table 1. The samples were coded and processed in the laboratory in an anonymous manner. The Research Ethics Committee of the University Hospital Brno approved the study protocol, and a written statement of informed consent was obtained from the legal guardian of each child before participation in this study. All four in-house medulloblastoma cell lines were cultured in DMEM supplemented with 20% FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from PAA Laboratories, Linz, Austria) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The Daoy cells were maintained under the same conditions in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 1% non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from PAA). The D238Med cells were grown in Ham's F-12/DMEM medium mixture (1:1) supplemented with 20% FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from PAA). Experiments using in-house medulloblastoma cell lines were performed between passages 6-11 for MBL-06, MBL-12 and MBL-13 cell lines, and between passages 20-24 for MBL-02 cell line.

Table 1. Basic clinical description of patients from whom the in-house medulloblastoma cell lines were established.

Cell line	Age	Gender	Stage	Histology
MBL-02	7	F	Localized	Desmoplastic
MBL-06	5	M	Localized	Classic
MBL-12	1	M	Metastatic	Desmoplastic/extensive nodularity
MBL-13	2	M	Metastatic	Classic

M, male; F, female. The age at the time of diagnosis is given in years.

SDS PAGE and immunoblotting for medulloblastoma cell line analysis

For the total protein analysis, trypsinized cells (grown to 80% confluence) were washed with PBS and incubated in RIPA buffer containing 50 mM Tris-HCl at pH 8.0, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100 (all from Sigma), and protease inhibitors (Roche, Basel, Switzerland) on ice for 10 minutes. The lysate was then centrifuged at 14,000 RPM for 10 minutes at 4°C. To obtain nuclear and cytoplasmic fractions, a Ne-Per Kit (Pierce, Rockford, IL, USA) was used according to the manufacturer's instructions. Twenty-five micrograms of protein extract was loaded onto an 8% or 10% polyacrylamide gel (depending on the expected protein molecular weight) and separated by electrophoresis. Next, the proteins were transferred to PVDF membranes (BioRad) and blocked in 5% non-fat dry milk in 1% PBS-Tween for 60 minutes at room temperature. ΔNp73-27 polyclonal anti-ΔNp73 (dilution 1:500; Moravian Biotechnology; for details, see above), DO-1 mouse monoclonal anti-p53 antibody (dilution 1:5000; Sigma), 4A4 mouse monoclonal anti-p63 antibody (dilution 1:1000; Sigma), H-79 rabbit polyclonal anti-TAp73α antibody (dilution 1:200; Santa Cruz Biotechnology), TU-01 monoclonal anti-α-tubulin (dilution 1:1000; Exbio, Prague, Czech Republic), and EM-11 monoclonal anti-lamin C (dilution 1:1000; Exbio) were applied overnight at 4°C. Anti-α-tubulin and anti-lamin C served as controls for the purity of the cytoplasmic and nuclear cell fractions, respectively. After being washed with 1% PBS-Tween, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies anti-mouse IgG HRP (cat. No. A8786, Sigma) and anti-rabbit IgG HRP (cat. No. A2074, Sigma), both of which were diluted 1:5000, for 60 minutes at room temperature. Signal detection was achieved using the ECL Plus chemiluminescence detection system (Amersham) according to the manufacturer's instructions.

Immunofluorescence for medulloblastoma cell line analysis

For indirect immunofluorescence, cells were cultivated on coverslips in Petri dishes for 1–3 days (grown to 80% confluence), depending on the cell proliferation rate. The cells were then rinsed with PBS, fixed with 3% paraformaldehyde (Sigma) for 20 minutes at room temperature, and permeabilized in 0.2% Triton

X-100 (Sigma) for 1 minute. After being washed with PBS, nonspecific binding was blocked by 3% bovine serum albumin (BSA; PAA) in PBS for 10 minutes. For the simple indirect immunofluorescence, cells were then incubated with primary antibody in a humidified chamber at 4°C overnight, rinsed with PBS, and incubated with the corresponding secondary antibody at 37°C for 45 minutes. ΔNp73-26 and ΔNp73-27 rabbit polyclonal anti-ΔNp73 (dilution 1:400; Moravian Biotechnology; for details, see above), CDF4 mouse monoclonal anti-golgin-97 (dilution 1:100; Molecular Probes), and TU-01 mouse monoclonal anti-α-tubulin (dilution 1:200; Exbio) serving as a control were used as primary antibodies. Anti-mouse FITC-conjugated IgG (cat. No. F8521, dilution 1:100; Sigma) and anti-rabbit TRITC-conjugated IgG (cat. No. T6778, dilution 1:100; Sigma) were used as secondary antibodies. After a final rinse with PBS, the coverslips were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). An Olympus BX-61 fluorescence microscope was used for cell evaluation, and the micrographs were collected using a Vosskühler 1300D CCD camera and analyzed using a Lucia 1.5.9 imaging system (Laboratory Imaging, Prague, Czech Republic).

Immunogold labeling and transmission electron microscopy for medulloblastoma cell line analysis

For the immunodetection of ΔNp73 and golgin-97 in ultrathin sections, cells grown on coverslips were washed with PBS and fixed in 2% paraformaldehyde in PBS for 60 minutes at room temperature. After a PBS rinse and dehydration, the cells were embedded in LR White medium (Polysciences Inc., London, UK). The labeling of the ultrathin sections was performed on grids. ΔNp73 was detected using ΔNp73-27 polyclonal anti-ΔNp73(dilution 1:400; Moravian Biotechnology; for details, see above) primary antibody and anti-rabbit 10-nm gold particle-conjugated IgG (cat. No. G7402, dilution 1:40; Sigma) secondary antibody. Golgin-97 was detected using a CDF4 monoclonal anti-golgin-97 (dilution 1:50; Molecular Probes) primary antibody and anti-mouse 20-nm gold particle-conjugated IgG (cat. No. ab27242, dilution 1:40; Abcam, Cambridge, UK) secondary antibody. Ultrathin sections incubated without primary antibodies or with the TU-01 primary monoclonal antibody against α-tubulin (dilution 1:200; Exbio) were used as controls. After immunodetection, the specimens were contrasted with 2.5% uranyl acetate (Lachema-Pliva, Brno, Czech Republic) for 20 minutes and with Reynolds' solution (Sigma) for 8 minutes at room temperature. The specimens were then observed using a Morgagni 268 (D) transmission electron microscope (FEI Company, Hillsboro, OR, USA). The images were captured by a MegaView III CCD camera (Soft Imaging System) and analyzed using AnalySIS software (Soft Imaging System).

Results

In this study, we performed a detailed analysis of the intracellular distribution of the ΔNp73 protein isoform in six medulloblastoma cell lines. To give precision to our pilot study concerning the localization of N-terminal isoforms of the p73 protein in medulloblastoma cells (Nekulova et al., 2010), we used two newly generated rabbit polyclonal antibodies against the ΔNp73 protein isoform. Therefore, the presented study is the first immunodetection of the ΔNp73 isoform using the antibodies ΔNp73-26 and ΔNp73-27. First, we confirmed the high specificity of these antibodies by immunoblotting and immunocytochemistry using MCF7 cells transiently transfected with p53 and different p63 and p73 N-terminal isoforms. We also performed immunoblotting of the p53 family members using a panel of four medulloblastoma cell lines chosen for morphological studies. Second, the intracellular distribution of the ΔNp73 isoform in six medulloblastoma cell lines was studied using the ΔNp73-26 and ΔNp73-27 antibodies by indirect immunofluorescence, immunogold labeling for transmission electron microscopy and immunoblotting of cell fractions.

Verification of the specificity of the new anti-ΔNp73 polyclonal antibodies

First, the specificity of the ΔNp73-26 and ΔNp73-27 antibodies was verified using MCF7 cells over-expressing p53 and various p63 and p73 N-terminal isoforms. Both the ΔNp73-26 and the ΔNp73-27 antibodies specifically detected the ΔNp73 isoform, in contrast to the 4A4 monoclonal antibody, which should have been p63-specific but recognized both p63 and p73 isoforms, and the H-79, 5B429 and IHC-00197 antibodies, which recognized the TAp73 isoform (Fig. 1). Furthermore, the new polyclonal antibodies diluted at 1:1000 gave a markedly strong and specific signal indicating their high affinity for the ΔNp73 isoform (Fig. 1).

To evaluate the specificity of the new ΔNp73-26 and ΔNp73-27 antibodies for immunocytochemical and immunohistochemical staining, immunocytochemistry was performed using MCF7 cells transiently transfected with p53 and with p63 and p73 N-terminal isoforms. Both of these antibodies, diluted at 1:20000, specifically detected only the cells transfected with the ΔNp73 isoform; 4A4 antibody recognized both p63 and p73, H-79 antibody was TAp73-specific (Fig. 2).

Immunoblotting of the p53 family proteins in medulloblastoma cell lines

Subsequently, the expression of the p53 family members, including the ΔNp73 isoform was also detected in four of the medulloblastoma cell lines used

in this study: the Daoy and D238Med reference cell lines, as well as in MBL-02 and MBL-13 in-house cell lines (Fig. 3). Two of the in-house cell lines (MBL-06 and MBL-12) were excluded from this analysis due to their low proliferating activity; we prioritize the completion of the morphological studies on these cell lines.

The p53 and p63 proteins were detected in all of the medulloblastoma cell lines; however, the expression pattern of the p63 bands was different among the cell lines: a set of bands ranging about 50 kDa was detected (Fig. 3). These multiple bands obviously represent various C-terminal isoforms of p63 or could be a result of post-translational modifications or protein degradation as previously reported (Ying et al. 2005, Petitjean et al. 2008, Conforti et al. 2012).

The TAp73 isoform was also detected in all of the examined medulloblastoma cell lines; two bands corresponding to the TAp73α and TAp73ß isoforms were detected (Fig. 3).

The ΔNp73 isoform was also successfully identified in all four medulloblastoma cell lines as a set of several bands ranging from 60 to 75 kDa; however, the pattern of the bands differs among the cell lines (Fig. 3). The same multiple bands were already reported to be C-terminal isoforms also in medulloblastoma cells (Castellino et al., 2007).

Intracellular distribution of ΔNp73 in medulloblastoma cells

With the employment of both of the new anti-ΔNp73 polyclonal antibodies, we found a primarily nuclear localization of the ΔNp73 isoform in the cells of all six

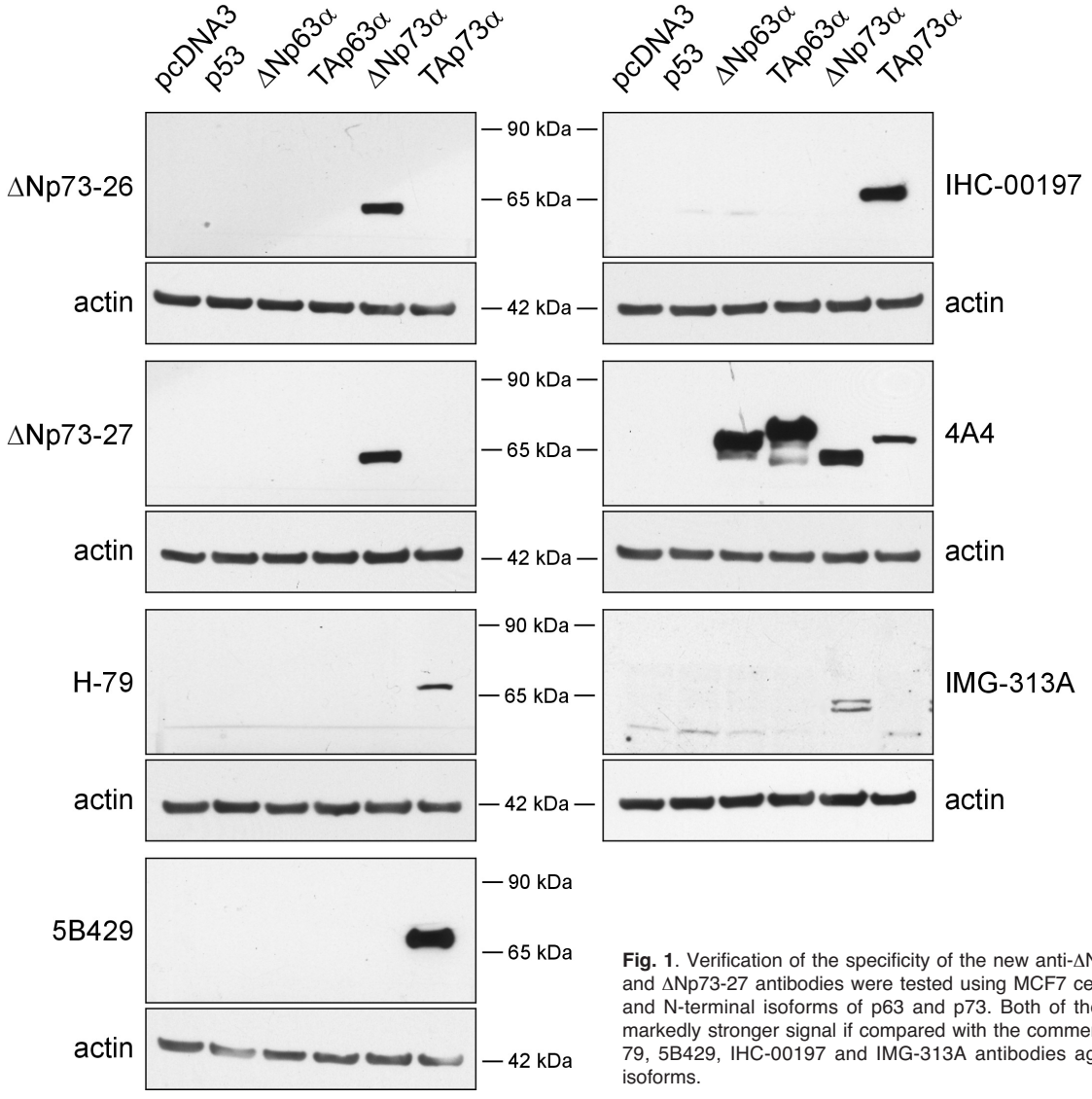

Fig. 1. Verification of the specificity of the new anti-ΔNp73 antibodies. ΔNp73 and ΔNp73-27 antibodies were tested using MCF7 cells over-expressing p53 and N-terminal isoforms of p63 and p73. Both of these antibodies showed markedly stronger signal if compared with the commercially available 4A4, H-79, 5B429, IHC-00197 and IMG-313A antibodies against the different p73 isoforms.

medulloblastoma cell lines included in this study; these findings are in accordance with our pilot experiments using a commercially available antibody (Nekulova et al., 2010).

Using indirect immunofluorescence, a strong nuclear signal for the ΔNp73 isoform was detected in most cells of all six medulloblastoma cell lines (Fig. 4). A semiquantitative analysis by cell fractionation and immunoblotting of both nuclear and cytoplasmic fractions also confirmed a predominantly nuclear localization of the ΔNp73 isoform, especially in the Daoy, MBL-02 and MBL-13 cell lines (Fig. 5).

Surprisingly, a localized cytoplasmic accumulation of the ΔNp73 isoform near the cell nuclei was also observable in all six examined cell lines (Fig. 4). The area of the cytoplasmic accumulation of the ΔNp73 isoform partly varied in both size and shape; however, its position suggests a possible co-localization with the Golgi apparatus.

To verify this hypothesis, we performed a double

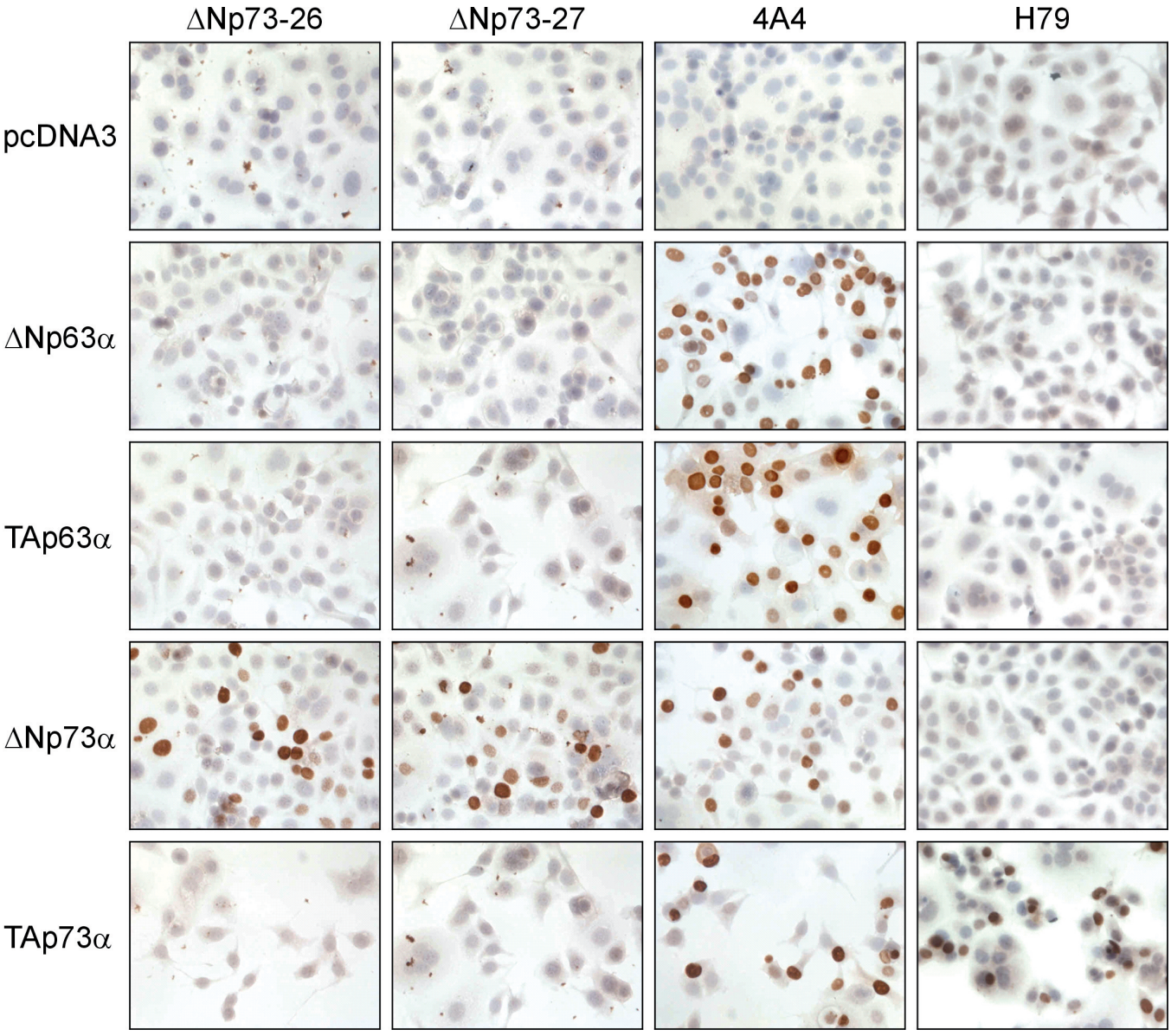

Fig. 2. Evaluation of the specificity of the new antibodies using immunocytochemistry. MCF7 cells were transiently transfected with p53, and p63 and p73 N-terminal isoforms, grown on slides, fixed and incubated with ΔNp73-26 and ΔNp73-27 antibodies diluted at 1:20000. Subsequently, staining was developed using immunoperoxidase detection system. The ΔNp73-26 and ΔNp73-27 antibodies specifically detected only the cells transfected with the ΔNp73α isoform, H-79 antibody recognized only TAp73α, 4A4 antibody detected both isoforms of p63 and p73. x 400

labeling of the ΔNp73 isoform and of human golgin-97 as a marker of the Golgi apparatus (Lock et al. 2005). Indirect immunofluorescence clearly confirmed the co-localization of the ΔNp73 cytoplasmic signal and the signal for golgin-97 in both the reference and the in-house medulloblastoma cell lines (Fig. 6). To investigate this finding on the ultrastructural level, the same primary antibodies were used for the labeling of ultrathin sections for transmission electron microscopy. The results from the double immunogold labeling showed the presence of membranous vesicles positive for both the ΔNp73 isoform and golgin-97 near the cell nuclei (Fig. 7). Therefore, we conclude that the ΔNp73 isoform - in addition to its typical nuclear localization - is also stored in vesicles of the Golgi apparatus.

Discussion

The availability of antibodies with high specificity is a crucial aspect of every experiment based on immunodetection methods. We were aware of this fact in our previous studies aimed at the detection of endogenous p73 N-terminal isoforms using commercially available antibodies: the ΔNp73 isoform was detected using the mouse monoclonal anti-ΔNp73 IMG-313A (or OP181) antibody (clone 38C674.2; Imgenex), which was developed against amino acid residues 2 - 13 of human ΔNp73 (Zitterbart et al., 2007; Nekulova et al., 2010). This antibody is the most commonly used commercially available antibody against the ΔNp73-specific epitope; however, its specificity was only demonstrated on transiently transfected cell lines and not on systems with endogenous expression of the ΔNp73 isoform (Sayan et al., 2005; Rosenbluth et al., 2009).

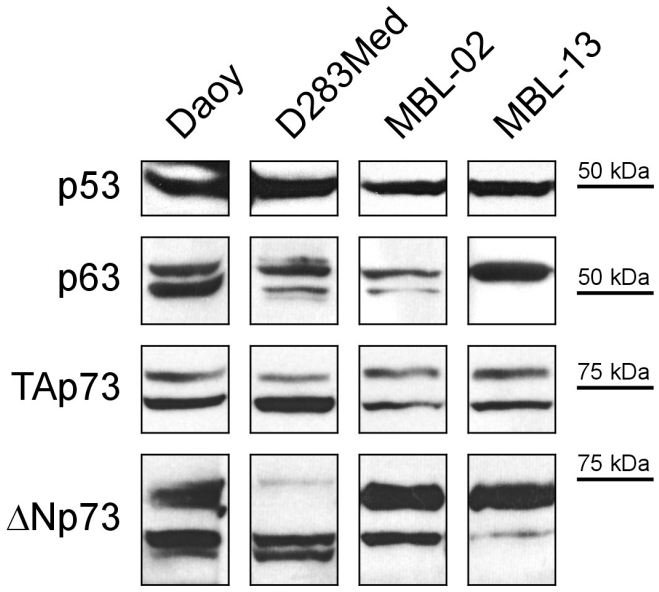

Fig. 3. Immunoblot analysis of the expression of the p53 family proteins in medulloblastoma cell lines. DO-1 mouse monoclonal anti-p53 antibody, 4A4 mouse monoclonal anti-p63 antibody, H-79 rabbit polyclonal anti-TAp73α antibody, and ΔNp73-27 rabbit polyclonal anti-ΔNp73 antibody were used for the experiment. The expression of all detected isoforms was demonstrated in all of the examined medulloblastoma cell lines.

Our previous results obtained with the IMG-313A antibody on medulloblastoma cells were questionable. Although a predominantly cytoplasmic positivity for the endogenous ΔNp73 isoform was shown in medulloblastoma FFPE tissues using immunohistochemistry (Zitterbart et al., 2007), we then observed a mostly nuclear positivity for this isoform and only a weak fluorescent signal in the cytoplasm in medulloblastoma cell lines when immunofluorescence was employed (Nekulova et al., 2010).

To clarify this issue, we decided to generate new polyclonal anti-ΔNp73 antibodies in rabbits. Both of these antibodies gave satisfying results in experiments on their specificity using transiently transfected MCF7 cell line (Figs. 1, 2), and they also showed high specificity in the immunoblotting detection of endogenous ΔNp73 isoform in the medulloblastoma cell lines used in this study (Fig. 3). Strong expression was confirmed in both in-house medulloblastoma cell lines examined (MBL-02 and MBL-13) and moderate expression in the Daoy reference cell line. In accordance with the immunofluorescence detection, which will be discussed below, very low expression of the ΔNp73 isoform was detected in the D238Med reference cell line (Fig. 3). The lack of observable cross-reactivity with either the TAp73 isoform or other members of the p53 family should also be highlighted. Thus, both the ΔNp73-26 and ΔNp73-27 antibodies represent efficient tools for immunodetection studies of the endogenous expression of the ΔNp73 isoform.

The difference between the results obtained with the previously used IMG-313A antibody and those obtained with these newly developed antibodies was also apparent in the immunofluorescence experiments. Our previous experiments concerning detection with the IMG-313A antibody showed a predominantly nuclear positivity for the ΔNp73 isoform in all of the examined medulloblastoma cell lines, and the cytoplasmic positivity was very weak (Nekulova et al., 2010). Both of these new antibodies confirmed nuclear positivity, on the one hand, but also substantiated non-random accumulation of the ΔNp73 isoform in the cytoplasm near the cell nucleus, on the other (Fig. 4).

The nuclear localization of the ΔNp73 isoform was observed in all of the medulloblastoma cell lines examined in this study. These results are in accordance with our previous findings using the IMG-313A antibody (Nekulova et al., 2010). Using our new ΔNp73-26 and ΔNp73-27 antibodies for immunofluorescence detection, the intensity of the nuclear staining for ΔNp73 was high in all four in-house medulloblastoma cell lines (Fig. 4C-F) and in the Daoy reference cell line (Fig. 4A),

while being weak only in the D238Med reference cell line (Fig. 4B). These observations were unmistakably confirmed by immunoblotting of both nuclear and cytoplasmic cell fractions (Fig. 5); the ΔNp73 isoform clearly prevailed in the nuclear fraction of the Daoy, MBL-02 and MBL-13 cell lines, whereas only the weaker band was detected in the nuclear fraction of the D238Med cell lines. Already-published studies on the

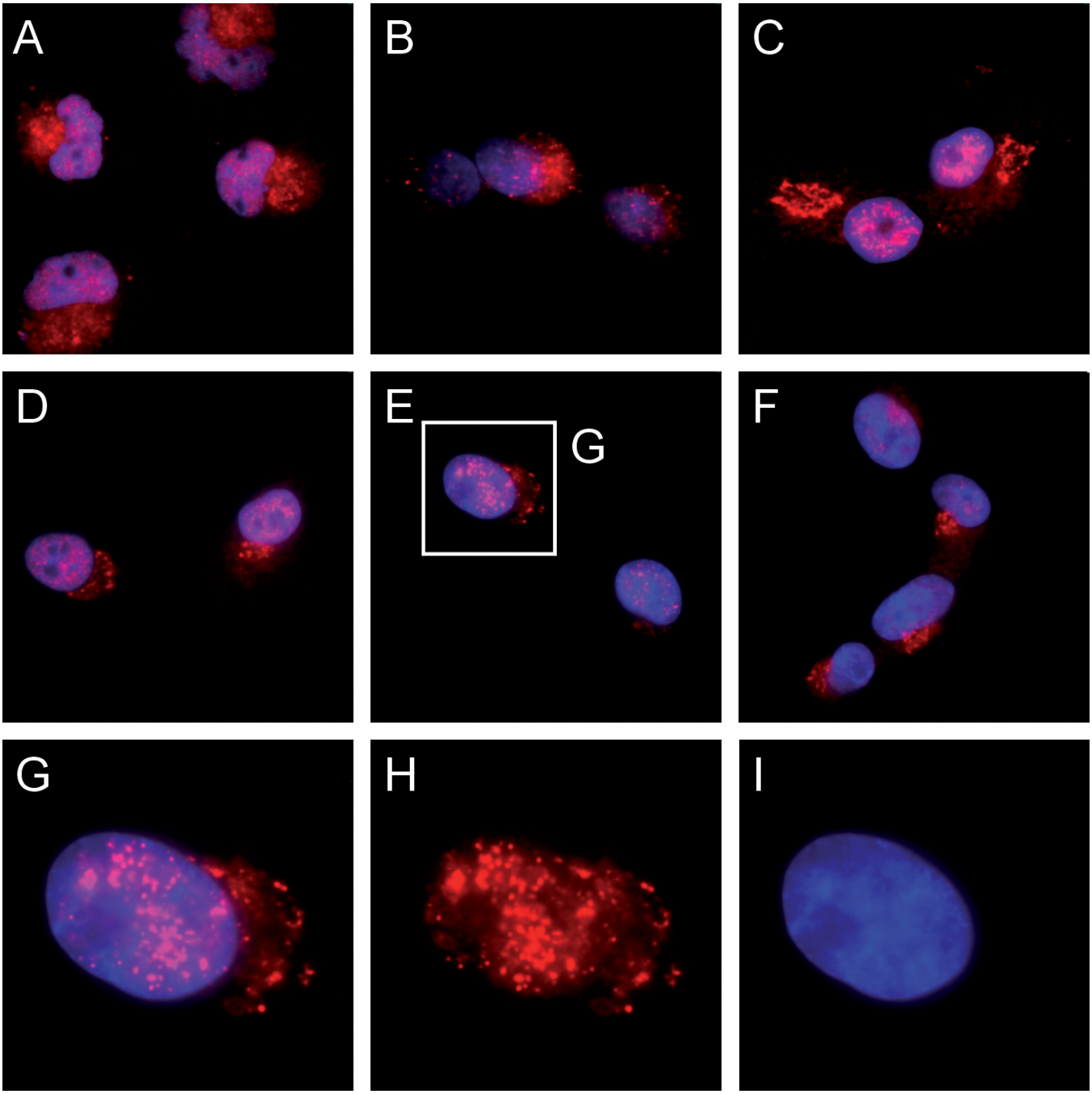

Fig. 4. Immunofluorescence analysis of the intracellular localization of the ΔNp73 isoform in medulloblastoma cell lines. ΔNp73-26 and ΔNp73-27 rabbit polyclonal anti-ΔNp73 antibodies stained by indirect immunofluorescence using TRITC-labeled secondary antibody (**A-H**, red) were used for the experiments; counterstaing with DAPI (**A-G, I,** blue). Daoy **(A)**, D283Med **(B)**, MBL-02 **(C)**, MBL-06 **(D)**, MBL-12 **(E, G-I)**, and MBL-13 **(F)** cells are shown. Predominantly nuclear localization **(A-F)** and the localized cytoplasmic accumulation of the ΔNp73 isoform near the cell nuclei were demonstrated in all six examined cell lines **(A-F)**. x 400

intracellular localization of the ΔNp73 isoform in cells of various tumor types yielded partially contrary results. ΔNp73 was described solely in the nuclei of hepatocellular carcinoma (Müller et al., 2005; Tannapfel et al., 2008), breast carcinoma (Dominguez et al., 2006a) and colorectal carcinoma (Dominguez et al., 2006b). Predominantly nuclear and only weak cytoplasmic localization of ΔNp73 was detected in cells of thyroid

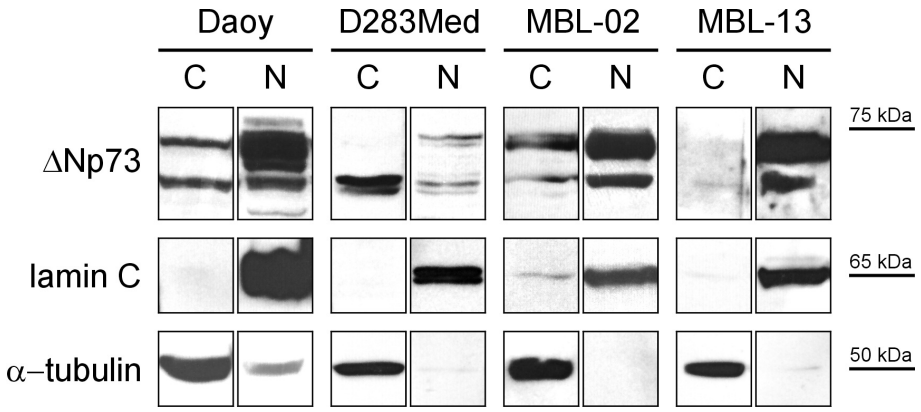

Fig. 5. Immunoblot analysis of the intracellular distribution of the ΔNp73 isoform in medulloblastoma cells. Nuclear/cytoplasmic fractionation followed by immunoblotting was performed using Daoy, D283Med, MBL-02, and MBL-13 cell lines. α-tubulin and lamin C were used to confirm a purity of nuclear fractions. Predominantly nuclear localization of the ΔNp73 isoform was found in Daoy, MBL-02, and MBL-13 cells.

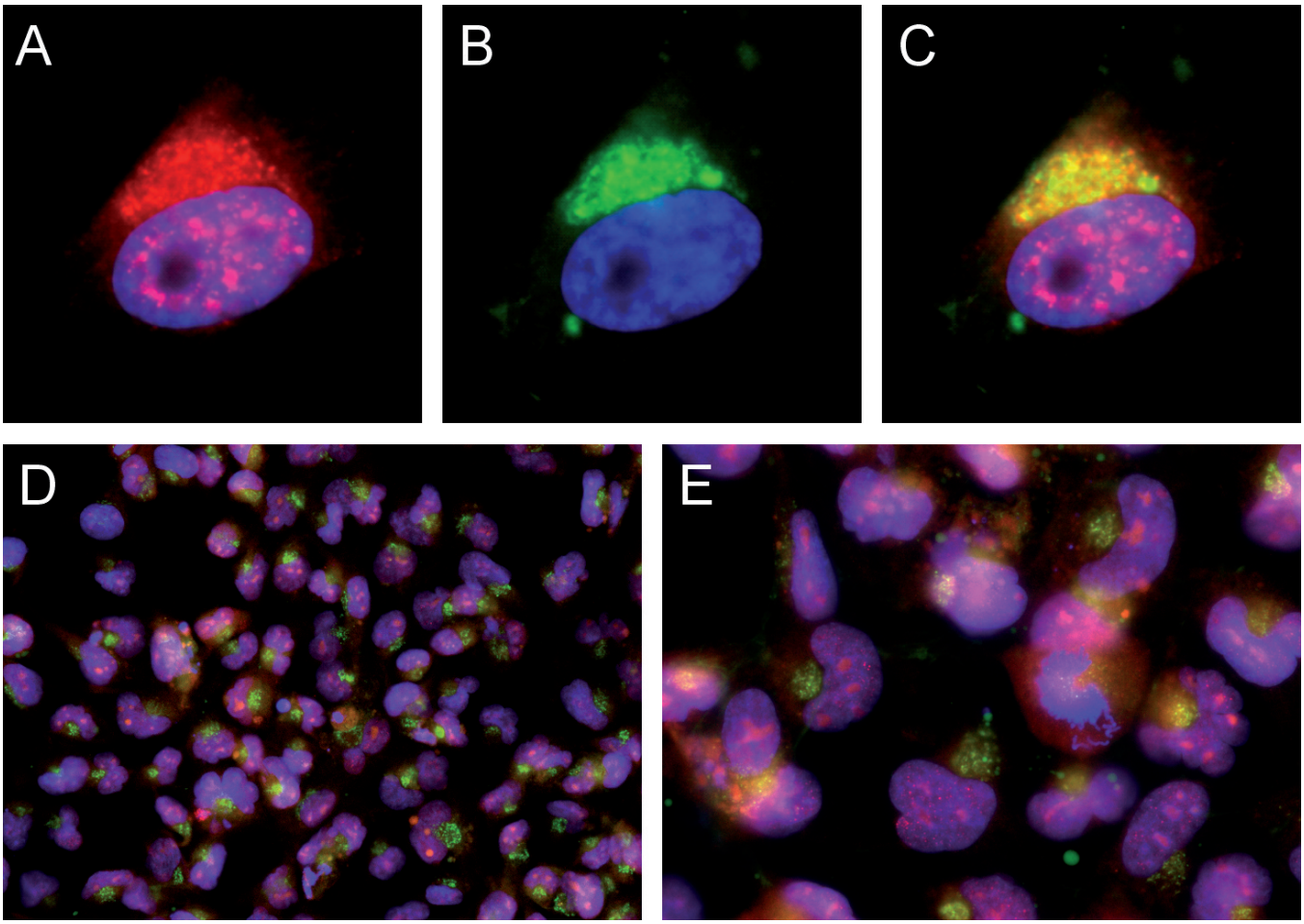

Fig. 6. Immunofluorescence analysis of the co-localization of the ΔNp73 isoform and Golgi apparatus. Representative double labeling for ΔNp73 and golgin-97 in MBL-06 **(A-C)** and Daoy **(D-E)** cell lines. ΔNp73 (**A, C, D, E**, red), golgin-97 (**B, E,** green) and counterstaining with DAPI **(A-E)** were performed. Merged micrographs **(C-E)** clearly confirmed the co-localization of the ΔNp73 isoform with Golgi apparatus. A-C, x 400; D, x 100; E, x 200

cancer (Frasca et al., 2003), non-small cell lung carcinoma (Di Vinci et al., 2009) and medulloblastoma (Nekulova et al., 2010). In contrast, predominantly or entirely cytoplasmic localization was reported in cells of lung carcinoma (Uramoto et al., 2004), breast carcinoma (Bozzetti et al., 2007), and medulloblastoma (Zitterbart et al., 2007). Surprisingly, the intracellular distribution of p73 isoforms in breast carcinoma cells was shown to be cell-density-dependent (Tophkhane et al., 2009). As mentioned above, these discrepancies are probably caused by the use of certain anti-ΔNp73 antibodies that show variable specificity among p53 family members (Sayan et al., 2005; Rosenbluth et al., 2009).

The non-random accumulation of the ΔNp73 isoform in the cytoplasm near the cell nuclei in all of the examined cell lines (Fig. 4) was the most surprising

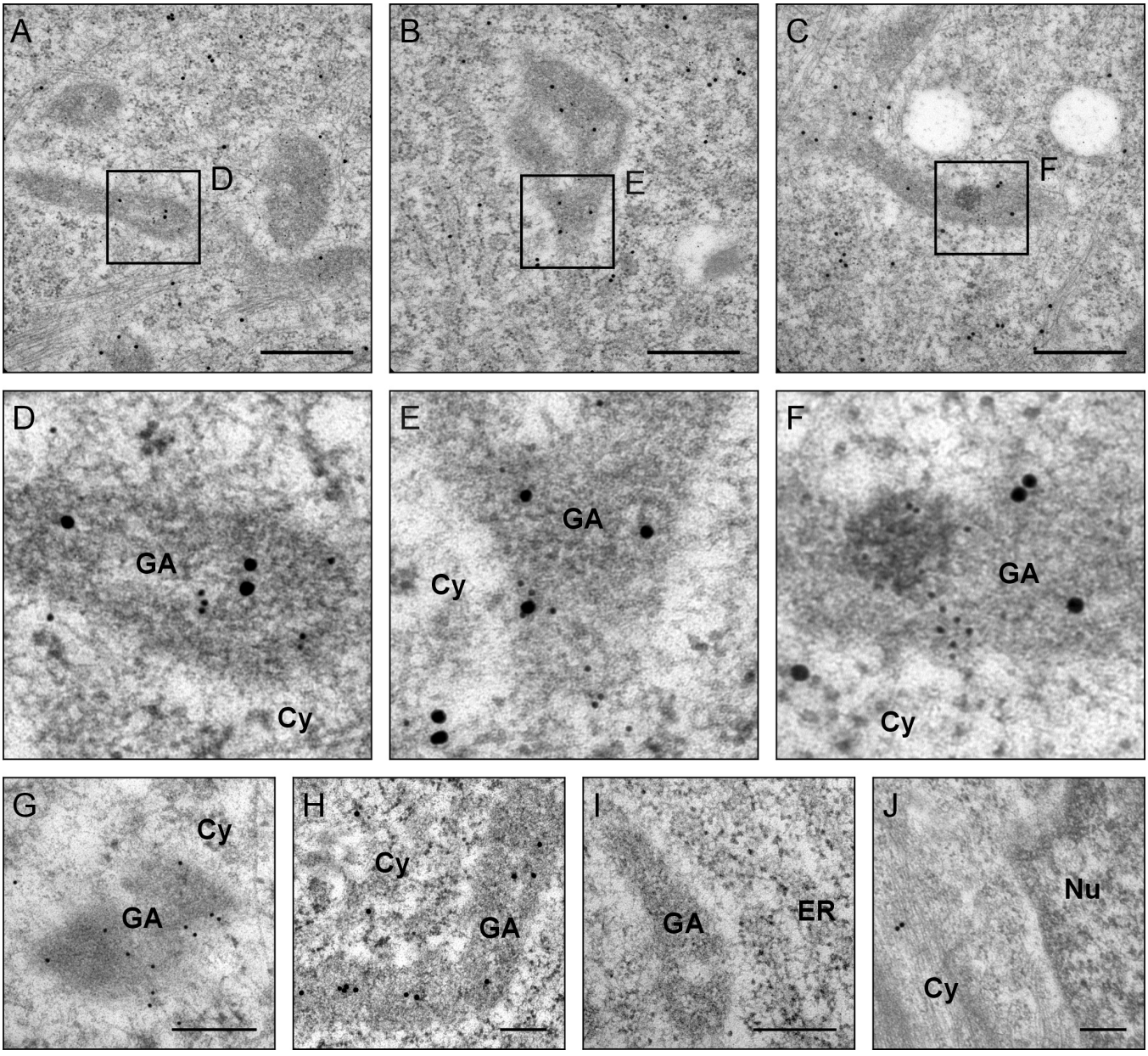

Fig. 7. Immunogold labeling of the ΔNp73 isoform and golgin-97 for transmission electron microscopy. Representative double labeling for ΔNp73 and golgin-97 in MBL-12 cells **(A-F)**: the ΔNp73 isoform was detected using 10 nm gold particles (small dots); golgin-97 was detected using 20 nm gold particles (large dots). A co-localization of the ΔNp73 and golgin-97 in the membranous vesicles **(D-F)** in the cytoplasm was demonstrated. Representative single labeling for ΔNp73 **(G)** and golgin-97 **(H)** in MBL-12 cells. Negative control stained with anti-rabbit IgG 10 nm gold conjugate antibody **(I)** and anti-mouse IgG 20 nm gold conjugate antibody **(J)**. Golgi apparatus (GA), cytoplasm (Cy), endoplasmic reticulum (ER), nucleus (Nu). Bars: A-C, 500 nm; G-J, 200 nm

result of this study. Based on the obvious similarity between this pattern of cytoplasmic signal for the ΔNp73 isoform and the pattern of Golgi apparatus staining with different markers, as reported in many papers (Kristiansen et al., 2008; Gill et al., 2010; Lee et al., 2011), we performed a double labeling of golgin-97 and theΔNp73 isoform by the employment of indirect immunofluorescence. The results of this experiment clearly proved the co-localization of the ΔNp73 isoform and the Golgi apparatus (Fig. 6); these observations were subsequently confirmed by double immunogold labeling of ultrathin sections for transmission electron microscopy (Fig. 7). The storage of the ΔNp73 isoform in the Golgi apparatus is a completely new finding that was not previously described.

Because p73 is not a membranous protein, which would be retained in the Golgi apparatus directly, we propose possible interactions between the ΔNp73 isoform and another protein localized in the Golgi apparatus. Such interaction may cause the retention of ΔNp73 in the Golgi apparatus. Although several proteins (PKCß, myosin VI or VRK1, for example) that may interact with various p53 family members, especially with p53, were shown to be linked to the Golgi apparatus (Brodie and Blumberg, 2003; Jung et al., 2006; Valbuena et al., 2007), there is no evidence regarding interactions of these proteins with p73. We hypothesize that the potential counterpart of ΔNp73 in such interaction may be the Wwox tumor suppressor, which is localized in the Golgi apparatus (Bednarek et al., 2001). Wwox triggers the translocation of p73 from the cell nucleus into the cytoplasm, and the interaction of these proteins in the cytoplasm has been reported (Aqeilan et al., 2004). Although a co-localization of Wwox and the ΔNp73 isoform was not proven in malignant salivary gland neoplasms (Gomes et al., 2011), the localization of Wwox in several transiently transfected cell lines resembles the pattern of cytoplasmic localization of golgin-97 and ΔNp73 obtained by immunofluorescence in our experiments (Aqeilan et al., 2004). Nevertheless, further detailed analyses of ΔNp73 localization in the Golgi apparatus and of its possible interactions with Wwox will be performed in our forthcoming study.

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