A Combined Immunoprecipitation and Mass Spectrometric Approach to Determine ΔNp63-Interacting Partners

Kombinace přístupů imunoprecipitace a hmotnostní spektrometrie v analýze interakčních partnerů ΔNp63

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Summary
Expression of p63 is essential for the formation of epidermis and other stratifying epithelia. Moreover p63 is highly expressed in several epithelial cancers and is involved in tumourigenesis and controlling chemo-sensitivity. The identification of p63 interacting partners is essential for understanding the complex network of gene regulation managing epithelial development and could also help to reveal signalling pathways participating in UV-damage response in human skin. We used a proteomic approach to identify proteins that interact with ΔNp63. Proteins were isolated by immunoprecipitation with ΔNp63 specific antibody and analysed by mass spectrometry. We identified 23 proteins as potential ΔNp63 binding partners that were not present in negative control samples. These results will be evaluated using other methods.

Key words
antibodies – p63 – immunoprecipitation – proteomics – mass spectrometry – protein-protein interactions

Souhrn
Exprese p63 je nezbytná pro tvorbu epidermis a dalších vrstev epitelu. Je přítomen ve vysokých koncentracích v různých kožních nádorech, podílí se na rakovinném bujení a kontroluje chemosenzitivitu. Identifikace interakčních partnerů p63 je nezbytná pro pochopení celého systému genové regulace řídící vývoj epitelu. Tyto znalosti mohou také pomoci objasnit signální dráhy podílející se na odpovědi lidské kůže poškozené UV zářením. K identifikaci proteinů tvořících komplexy s ΔNp63 byly použity proteomické přístupy. Proteiny byly izolovány imunoprecipitací ΔNp63-specifickou protilátkou a následně analyzovány hmotnostní spektrometrií. Identifikovali jsme 23 proteinů, potenciálních interakčních partnerů ΔNp63, které nebyly nalezeny v kontrolních vzorcích. Získané výsledky budou ověřeny dalšími metodami.

Klíčová slova
protilátky – p63 – imunoprecipitace – proteomika – hmotnostní spektrometrie – protein-proteinové interakce

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Introduction

The transcription factor p63, a member of the p53 family, is required mainly for the development of limbs and epidermal differentiation [1] and is believed to be a robust biomarker for epithelial progenitor or stem cells [2,3]. The *TP63* gene expresses at least 6 different transcripts by utilising two distinct promoters (TA and ΔN) and alternative splicing within the 3′ end of the mRNA that generates α, β and γ isoforms [4]. It is supposed that p63 isoforms possess different transactivating and transcriptional repressing properties and regulate a wide range of target genes, nevertheless their functions are diverse. TAp63 isoforms show clear pro-apoptotic activity, while ΔNp63 isoforms protect from apoptosis by directly competing for TAp63 (or p53 and p73) target promoters or sequestering these proteins, forming inactive tetramers. It is emerging that p63 is involved in tumourigenesis and in controlling chemo-sensitivity. It is highly expressed in several epithelial cancers, and regulates apoptosis and sensitivity to drug treatments at least *in vitro*. However, a large body of evidence indicates that the main role of p63 lies in the regulation of epithelial development and in the formation of epidermis [5]. The level of isoforms fluctuates during epidermal development. The TA- and ΔN-isotype specific reagents revealed that ΔNp63 expression is confined to the basal layer of stratified squamous epithelium, whereas TAp63 variants predominate in the suprabasal layers [6]. Identification of targets is crucial in order to understand the developmental strategy sustained by p63. Many studies focused on down-stream genes of p63 at mRNA level, but the inaccessibility of reliable and high affinity p63 isoform-specific antibodies complicates identification of p63 interacting partners.

Mass spectrometry (MS) and data analysis techniques have been used to identify co-precipitated proteins from immunoprecipitated samples [7]. A major advantage of immunoprecipitated samples is their reduced complexity and therefore MS analysis is undemanding, enables faster scan speeds, improved mass accuracy and allows identification of proteins/peptides at low concentrations. Two different mass spectrometry approaches can be applied for this type of protein identification: (1) peptide fingerprinting (PMF) and (2) shotgun proteomics where PMF is connected with electrophoretic protein separation [8]. The obtained gel is Coomassie blue stained and bands unique to the test samples are excised, enzymatically digested and analysed by mass spectrometry. The effective mass (m/z) of these peptides is determined and matched to a peptide database to identify the corresponding protein. Advantages of this approach are compatibility with elution conditions mainly with composition of buffers and estimation of the molecular weight of the protein. Disadvantages appear only when unique bands are cut out and background bands are not identified or less abundant proteins my fall below the limits of detection by staining. Alternatively, the second approach, shotgun proteomics, is based on direct enzymatic digestion of the eluted protein complex. The resulting peptides are chromatographically separated and analysed by tandem mass spectrometry (MS/MS) [9] that comprises three steps. In the first step, the m/z of the peptide is measured while during second step the peptide is fragmented. The third step measures the m/z of the fragment ions. Protein identification is usually achieved by comparing experimental tandem mass spectra with theoretically generated spectra and selecting the most likely sequence match via search engines such as Mascot [10]. The identification is then filtered according to quality score and a false-discovery rate. An advantage of this approach is the ability to analyse complex protein mixtures *en block* but on the other hand elution conditions of protein complexes has to be compatible with mass spectrometry analysis. For example, the presence of surfactant sodium dodecyl sulfate (SDS) which can solubilise proteins is problematic for their trypsin digestion [11].

In view of our previous data indicating a role for p63 in regulating DNA damage response [12], we are interested in clarifying the role of ΔNp63 in UV-damage-response mechanisms in epithelial stem cells. The identification of ΔNp63 interacting partners in model cell line could help to reveal signalling pathways participating in UV-damage response in human skin. We obtained unique ΔNp63-specific antibody which we tested for immunoprecipitation of ΔNp63 and identification of its interacting partners in immortal human keratinocytes (HaCaT cells). Moreover for future studies we would like to find an optimal and compatible protocol combining immunoprecipitation approach and mass spectrometry analysis suitable for analysis of interacting partners of ΔNp63.

Materials and Methods

Transient Transfection and Western Blotting

H1299 cells (that do not express p53, p63 or p73) were transiently transfected with vectors encoding p53 and various isoforms of p63 and p73 using Lipofectamine 2000 (Invitrogen, USA). Cells were harvested into lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris-Cl, pH 8.0, 50 mM NaF, 5 mM EDTA, pH 8.0, protease inhibitor cocktail) and 20 μg of protein was loaded on polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk for 1 h and then incubated overnight at 4 °C with ΔNp63(44) rabbit polyclonal antibody specific for the N-terminus of ΔNp63 diluted 1 : 1,000; 4A4 mouse monoclonal antibody that recognizes all p63 isoforms (Santa Cruz Biotechnology, USA) diluted 1 μg/ml; or anti-actin AC-40 antibody (Sigma-Aldrich, MO, USA). After washing in PBS with 0.1% Tween, membranes were incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) diluted 1 : 1,000 in 5% milk. Detection was performed using ECL reagents (Amersham Pharmacia Biotech, UK).

Cell Lysates

HaCaT cell line was maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum, 300 μg/ml L-glutamine, 105 IU/ml penicillin and 100 μg/ml streptomycin. Cell lysates were prepared by deta-

ching cells with scraper, washing three times with ice-cold PBS and resuspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40) containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, MO, USA). The concentrations of proteins were measured by the Bradford colorimetric protein assay and 1.15 mg of total protein was used for immunoprecipitation.

Immunoprecipitation

Magnetic Dynabeads Protein G (Invitrogen, Dynal AS, Norway) were coated with ΔNp63-specific rabbit polyclonal antibody ΔNp63(44) and with normal rabbit sera as negative control using protocol „Preparing Protein a bead-antibody affinity columns – direct coupling" [13]. The immunoprecipitation was performed according to product manual. Target protein and its binding proteins were eluted with different procedures: (1) 200 mM glycine, pH 2.8, at RT/1 h (according to standard Dynal Dynabeads Protein G protocol, Invitrogen, Dynal AS, Norway), (2) 6 M urea, Tris-Cl, pH 7.5, at RT/1 h, (3) 30 mM TCEP 85 °C/5 min (Sigma-Aldrich, MO, USA), (4) 4% SDS, 125 mM Tris-Cl, pH 6.0, at 85 °C/5 min and (5) sample buffer (20% SDS, glycerol, 2% bromphenol blue, 1 M Tris-Cl, pH 6.8, 5% mercaptoethanol) at 85 °C/5 min; according to standard Dynal Dynabeads Protein G protocol, Invitrogen, Dynal AS, Norway. All eluted samples (5 µl) were separated by 1-D electrophoresis on 10% SDS-PAGE gels and stained with NOVEX Colloidal Blue Staining Kit (Invitrogen, Dynal AS, Norway) to check efficiency of particular elution procedures.

Acetone Precipitation

Cold acetone (HPLC grade, Sigma-Aldrich, MO, USA) was added to the protein sample (in ratio 5 : 1, acetone : sample, v/v) and the mixture was vortexed thoroughly. The mixture was then incubated overnight at –20 °C and then the precipitate was spun down at 4 °C for 15 min at 12,000× g. The supernatant was decanted and the pellet was air dried for 10 min and immediately used for digestion.

Filter Assisted Sample Preparation

The filter assisted sample preparation (FASP) method was based on procedures described by Wisniewski et al [14,15]. The protein sample from immunoprecipitation was diluted by 8 M urea (8UA) in 100 mM Tris-Cl, pH 7.4 and applied on the 10 kDa cut-off filter (Vivacon 500, Sartorius Stedim, Germany) together with polyethyleneglycol (25 µl of 1% solution in water). After centrifugation (14,000× g, 30 min, 25 °C) the proteins were washed three-times with 400 µl of 8 UA and subsequently three--times with 50 mM ammonium bicarbonate (ABC). After the last centrifugation step, 50 µl of ABC containing 100 ng of trypsin (Promega, WI, USA) was added. After on-filter protein digestion in thermomixer (14 h, 37 °C; Eppendorf, Germany) the resulting peptides were spin down (14,000× g, 30 min, 25 °C) and the filter unit was washed two times by 50 µl of ABC. Peptide solution was concentrated under vacuum (Savant Speed Vac system, Thermo Fisher Scientific, MA, USA) and peptides were then extracted with acetonitrile (ACN) : 5% formic acid (FA), 1 : 1; v/v, into LC-MS vial prior to LC-MS/MS analysis.

In Solution Digestion

The acetone-precipitated pellet was resuspended with 100 mM triethyl ammonium bicarbonate (Sigma-Aldrich, MO, USA) and 2.5 µg of trypsin (Promega, WI, USA) per 100 µg of protein was directly added. The sample was digested overnight at 37 °C. The digestion was stopped by addition of mixture of ACN/5% FA, 1 : 1, v/v. The peptide solution was dried in a vacuum centrifuge and dissolved in 25 µl 50% ACN and 2.5% FA.

Mass Spectrometric Analysis and Protein Identification

LC-MS/MS analyses of peptide mixture were done using RSLCnano system connected to Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific, MA, USA). Prior to LC separation, tryptic digests were concentrated online and desalted using trapping column (100 µm × 30 mm) filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters, MA, USA). After washing the trapping column with 0.1% FA, the peptides were eluted (flow 300 nl/min) from the trapping column onto an Acclaim Pepmap100 C18 column (2 µm particles, 75 µm × 250 mm; Thermo Fisher Scientific, MA, USA) by the following gradient program (mobile phase A: 0.1% FA in water; mobile phase B: ACN : methanol : 2,2,2-trifluoroethanol (6 : 3 : 1; v/v/v) containing 0.1% FA): the gradient elution started at 2% of mobile phase B and increased from 2% to 45% during the first 90 min (11% in the 30th, 25% in the 60th and 45% in 90th min), then increased linearly to 95% of mobile phase B in the next 5 min and remained at this state for the next 15 min. Equilibration of the trapping column and the column was done prior to sample injection to sample loop. The analytical column outlet was directly connected to the Nanospray Flex Ion Source (Thermo Fisher Scientific, MA, USA).

MS data were acquired in a data-dependent strategy selecting up to top 20 precursors based on precursor abundance in the survey scan (350–1,700 m/z). The resolution of the survey scan was 120,000 (400 m/z) with a target value of 1×10^6 ions, one microscan and maximum injection time of 200 ms. Low resolution CID MS/MS spectra were acquired with a target value of 10,000 ions in rapid CID scan mode with m/z range adjusted according to actual precursor mass and charge. MS/MS acquisition in the linear ion trap was carried out in parallel to the survey scan in the Orbitrap analyser by using the preview mode. The maximum injection time for MS/MS was 50 ms. Dynamic exclusion was enabled for 45 s after one MS/MS spectra acquisition and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 m/z.

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (version 1.3) with in-house Mascot search engine utilisation. Mascot MS/MS ion searches were done against UniRef100 protein database (taxonomy *Homo sapiens*; downloaded from http://www.uniprot.

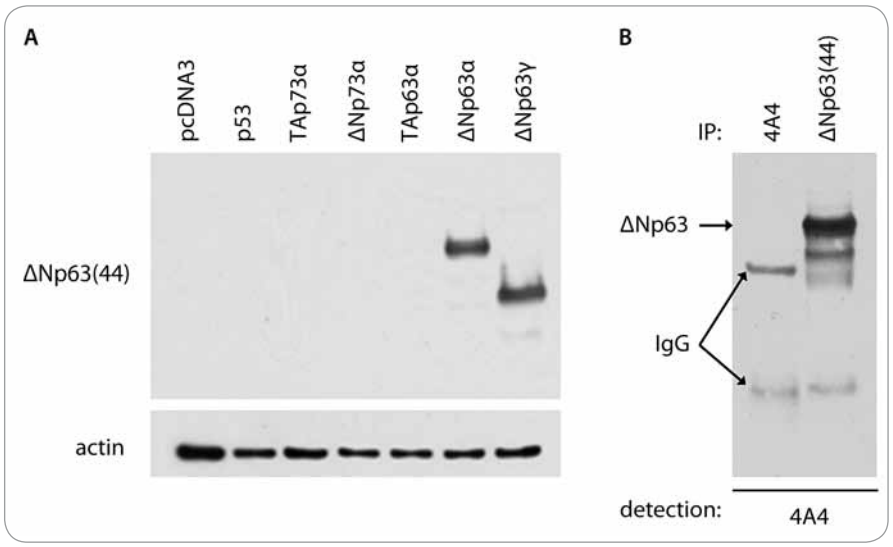

Fig. 1A) Evaluation of specificity of ΔNp63(44) polyclonal antibody. Specificity of ΔNp63(44) antibody was tested by Western blotting on lysates of H1299 cells transiently transfected by p53 and various isoforms of p63 and p73. ΔNp63(44) antibody was diluted 1 : 1,000.

Fig. 1B) Immunoprecipitation of endogenous p63 from HaCaT cells: We immunoprecipitated endogenous p63 from HaCaT cell lysate using 4A4 antibody and ΔNp63-specific rabbit polyclonal antibody ΔNp63(44). 4A4 antibody was used for detection by Western blotting (diluted 1 : 250).

org/downloads) [16]. Mass tolerance for peptides and MS/MS fragments were 5 ppm and 0.5 Da, respectively. Oxidation of methionine as optional modification and one enzyme miss cleavage were set for all searches. Percolator was used for post-processing of Mascot search results. Peptides with false discovery rate (FDR; q-value) < 1%, rank 1 and with at least 6 amino acids were considered.

Results and Discussion

Immunoprecipitation and Comparison of Elution Conditions

We tested ability of antibody to immunoprecipitate protein ΔNp63 from HaCaT cell lysates. Rabbit polyclonal antibody, which we used for immunoprecipitation, was obtained after immunisation of rabbit with ΔNp63-specific N-terminal peptide MLYLENNAQTQFSEC and tested for specificity and cross-reactivity (Fig. 1A). Immunoprecipitation of p63 was performed using mouse monoclonal PAN-specific p63 antibody 4A4 and ΔNp63-specific rabbit polyclonal antibody ΔNp63(44). However only polyclonal antibody ΔNp63(44) was able to immunoprecipitate p63 protein from HaCaT cell line lysates (Fig. 1B).

To identify binding partners of ΔNp63 we had to elute complexes from magnetic beads with elution buffer compatible with mass spectrometry measurement. We used four different buffers with specific elution conditions (as described in Materials and Methods) and sample buffer as control. The elution of proteins from magnetic beads was verified by Western blotting (Fig. 2). The best method for elution of protein complexes from magnetic beads as well as for mass spectrometry identification was 4% SDS, 125 mM Tris-Cl, pH 6.0, at 85 °C/5 min. The 1-D electrophoresis showed that elution buffer also released the antibody from beads (data not shown). The presence of antibody immunoglobulins could complicate measurement by mass spectrometry therefore we decided to covalently couple the antibody to magnetic beads (as described in Materials and methods). Magnetic beads with bound antibodies were subsequently used for final experiment (Fig. 3). To distinguish non-specific binding proteins we prepared magnetic beads coated with normal rabbit sera as negative control. Negative control samples were prepared in the same way as described previously (Fig. 3).

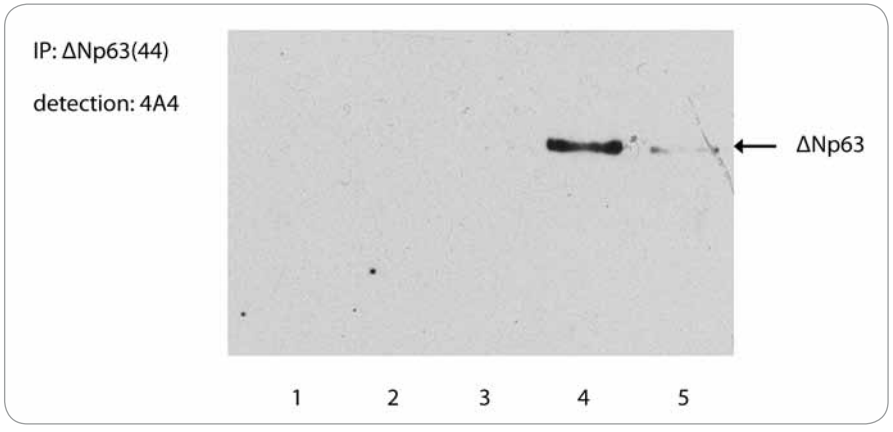

Fig. 2. Comparison of different elution conditions. The success of ΔNp63 elution from beads was evaluated by Western blotting. Different elution buffers were used: **Line 1.** 200 mM glycine, pH 2.8 **2.** 6 M urea, Tris-Cl, pH 7.5 **3.** 30 mM TCEP **4.** 4% SDS, 125 mM Tris-Cl, pH 6.0 **5.** sample buffer, 85°C/5 min. Detection was performed using 4A4 antibody (diluted 1 : 250).

Connection Between Immunoprecipitation and Mass Spectrometry

Elution of protein complexes is an important step in the shotgun proteomic approach and the composition of the elution buffer has to be appropriate for both immunoprecipitation and enzymatic digestion followed by mass spectrometric analysis. In our study we eluted ΔNp63 protein partners from magnetic beads using 4% SDS, 125 mM Tris-Cl, pH 6.0 buffer. Unfortunately, the concentration of SDS in this elution

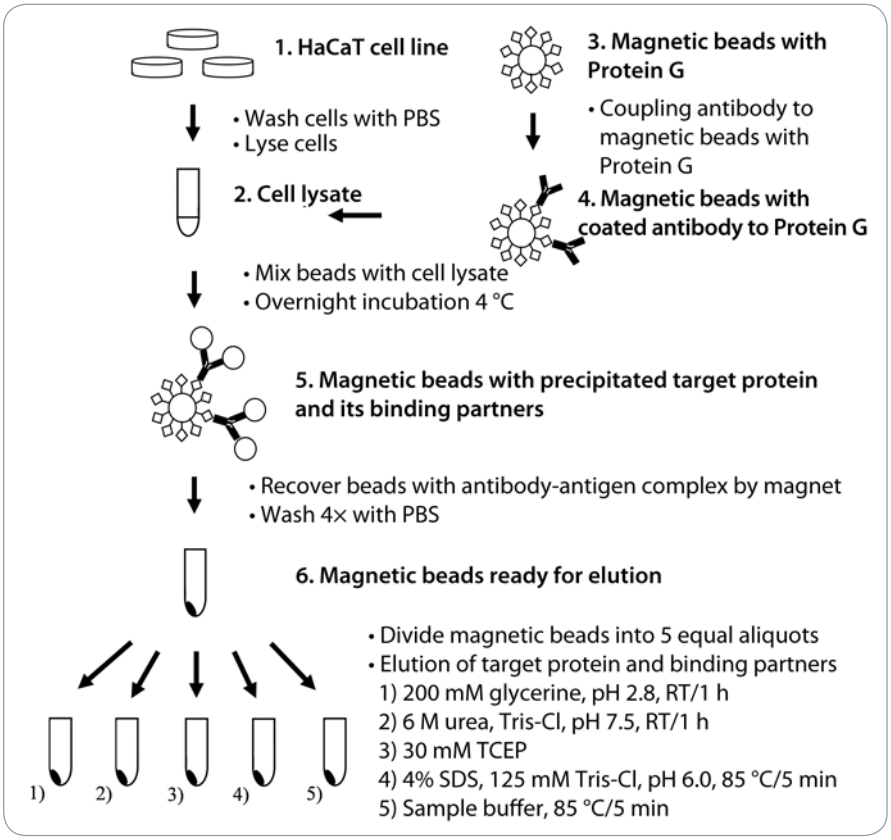

Fig. 3. Graphical overview of immunoprecipitation and elution conditions. The diagram highlights the key steps of the immunoprecipitation and elution process.

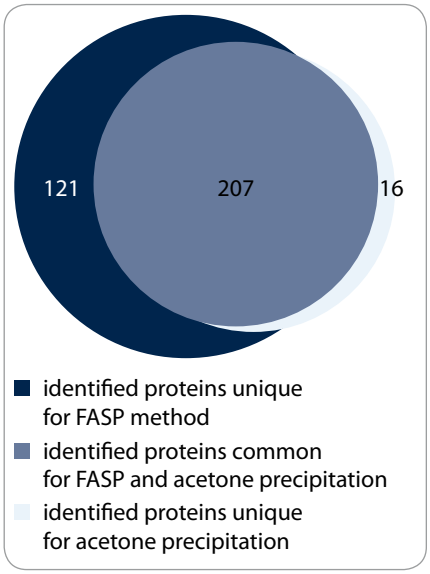

Fig. 5. Overview of numbers of identified proteins using different sample preparation procedures. Proteins uniquely identified in control and p63 samples processed by FASP method (dark blue, 121 proteins); proteins uniquely identified in control and p63 samples processed by acetone precipitation (light blue, 16 proteins), and proteins common for all analysed samples (medium blue, 207 proteins). All proteins were confidently identified by at least two peptides.

buffer was not compatible with trypsin digestion, therefore two different approaches for SDS elimination were applied: (1) Acetone precipitation and (2) FASP. The work-flow of whole procedure from detergent elimination to protein identification is shown in Fig. 4. Acetone precipitation is easy for operation, time-saving, low cost and suitable for treating protein samples of various volumes, particularly large volumes. On the other hand, recovery of proteins was not satisfactory and residual SDS was still present during mass spectrometry analysis. FASP analysis proved to be suitable for protein samples solubilised in buffers containing strong detergents. In the first step, SDS was exchanged by urea on a standard filtration device. Then proteins were directly digested on the filter and the resulting desalted peptides were eluted. Although this method was more laborious, the number of identified proteins was higher in comparison to acetone precipitation.

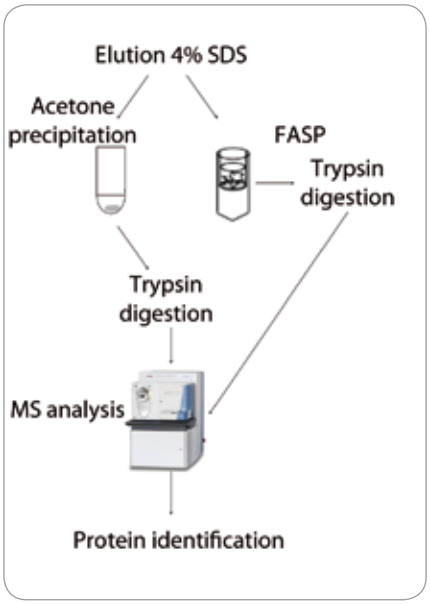

Fig. 4. Graphical overview of sample preparation for mass spectrometry analysis and protein identification. The diagram highlights key steps of sample detergents elimination (acetone precipitation and filter aided sample preparation), trypsin digestion, mass spectrometry analysis, and protein identification.

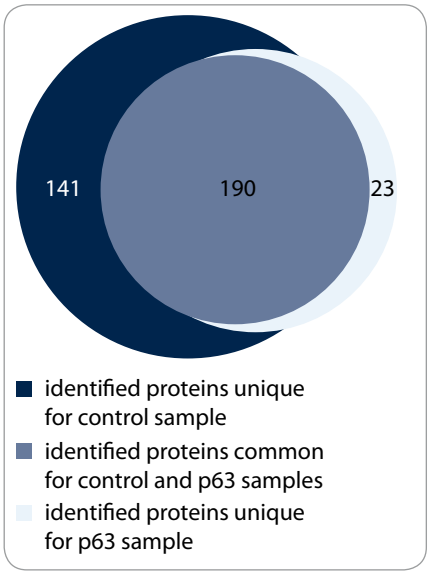

Fig. 6. Overview of numbers of identified proteins in control and p63 samples prepared with FASP method. Proteins uniquely identified in control sample (dark blue, 141 proteins); proteins uniquely identified in p63 sample (light blue, 23 proteins), and proteins common for all analysed samples (medium blue, 190 proteins). All proteins were confidently identified by at least two peptides.

Mass Spectrometry and Protein Identification

The protein digests of immunoprecipitates processed by two alternative sample preparation procedures were analysed by LC-MS/MS. To assess the efficiency of both procedures we compared the total number of identified proteins in control- and p63-immunoprecipitated samples. While 328 proteins were identified in p63 samples processed by FASP, only 223 proteins were found by acetone precipitation. In total, 207 identified proteins were identical in both FASP and acetone precipitated samples (Fig. 5). These results indicate that FASP is more favourable and robust for processing of immunoprecipitates. We performed another LC-MS/MS analysis using samples processed by FASP and finally identified 23 proteins which are potential ΔNp63 interacting partners (Fig. 6). These preliminary results should be verified preferably by another independent technique.

Conclusion

We compared identified proteins from our assay with results of Amoresano et al [17]. They analysed ΔNp63α interacting proteins by co-immunoprecipitation in mammalian cells and mass spectrometry. They used H1299 cell line transfected with vector containing myc-ΔNp63α. Cell extracts were incubated with anti-Myc 9E10 antibody and anti-IgG agarose beads, washed and eluted with Myc competitor peptide. Untransfected H1299 cells were used as negative control. They identified 49 potential ΔNp63α binding proteins.

A total of 33 proteins identified by Amoresano et al [17] were also found in our experiments, but with the exception of one protein (RNA-binding protein FUS) we also identified all of them in the negative control experiment (the comparison was made with proteins identified by at least one peptide in our MS/MS analysis). We suppose that these proteins are non-specifically bound to antibody immunoglobulins or agarose beads and the experimental design of Amoresano et al were insufficiently rigorous to uncover these contaminants, leading to false-positive results.

The large number of proteins that we identified in both p63 and negative control samples revealed that elimination of non-specific binding proteins, especially for DNA-binding proteins as ΔNp63α, is a crucial step for successful identification of binding partners. The subsequent careful optimisation of cell lysate preparation and washing steps after immunoprecipitation as well as verification by other methods are necessary.

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