Ku86 is important for TrkA overexpression-induced breast cancer cell invasion

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**Purpose:** We have recently shown that breast tumors express high levels of TrkA compared with normal breast tissues, with TrkA overexpression enhancing breast cancer cell invasion in vitro and metastasis in animal models. In this study, we tried to identify molecules involved in TrkA overexpression-mediated biological effects in breast cancer cells.

**Experimental design:** We used a proteomic-based approach to identify proteins involved in TrkA overexpression-stimulated invasion of MDA-MB-231 breast cancer cells. Proteins from control and TrkA overexpressing cells were separated using a cup-loading two-dimensional electrophoresis system before MALDI and LC-MS/MS mass spectrometry analysis.

**Results:** Among several putative regulated proteins, Ku86 was found increased in TrkA overexpressing cells. Moreover, Ku86 was co-immunoprecipitated with TrkA, suggesting the interaction of these two proteins in TrkA overexpressing cells. Interestingly, inhibition with small-interfering RNA and neutralizing antibodies showed that Ku86 was required for TrkA-stimulated cell invasion.

**Conclusions and clinical relevance:** These data allowed the identification of Ku86 as a new player involved in metastasis in breast cancer cells. Our findings suggest that TrkA and its downstream signaling pathways should be regarded as potential new targets for the development of future breast cancer therapy.

**Keywords:**
Breast cancer / Cell invasion / Ku proteins / TrkA tyrosine kinase receptor

1 Introduction

Several sets of growth factors and their cognate receptors are known to be involved in the regulation of cancer development [1–4]. Nerve growth factor (NGF) is the prototypic member of the neurotrophin family of proteins, well known for promoting survival and differentiation of neuronal cells during nervous system development. However, accumulating data indicate that NGF is also involved in cancer development [5–7]. NGF exerts its effects through two membrane receptors: the tyrosine kinase receptor TrkA and the receptor p75\(^{NTR}\), a common receptor for all neurotrophins and pro-neurotropins. NGF binding to TrkA induces TrkA receptor dimerization and autophosphorylation of cytoplasmic tyrosines, leading to the activation of various signaling pathways, including the Ras/MAPK pathway, the PLC\(_7\) pathway, and the P13K/Akt pathway [7]. The biological consequences of TrkA activation...
vary according to cell types. Hence, TrkA activation induces differentiation of neuronal precursors and neuroblastoma cells [8], whereas it induces proliferation in breast adenocarcinoma cells [9, 10] and apoptosis in medulloblastoma cells [11]. Recently, we have shown that NGF and its tyrosine kinase receptor TrkA are overexpressed in breast cancers compared with normal breast tissues [12, 13]. Inhibition of NGF with neutralizing antibodies or small interfering RNA strongly reduces tumor growth and metastasis of breast cancer cells xenografted in immunodeficient mice [12]. Moreover, TrkA overexpression in breast cancer cells leads to a constitutive activation of its tyrosine kinase, resulting in an increased cell growth and tumorigenicity [13]. Together, these findings point out the importance of the NGF/TrkA axis in breast cancer development. In addition, upregulation of TrkA has also been shown in other cancers including thyroid [14], lung [15], pancreatic [16, 17], prostatic [18, 19] and ovarian carcinomas [20, 21]. Thus, identification of molecules involved in the enhancement of aggressiveness of TrkA overexpressing cancer cells would be important for both better understanding of oncogenesis and research of new molecular targets. Here, we have used a functional proteomic approach to identify molecules involved in TrkA-mediated biological effects in breast cancer cells. We first separated proteins of total lysate using a cup-loading 2-DE system and then identified a series of putative modified proteins by MALDI and LC-MS/MS analysis. We found that Ku86, initially described to form a heterodimer with Ku70 to regulate DNA-dependent protein kinase (DNA-PK) that is crucial to DNA repair, was upregulated in TrkA overexpressing cells. Moreover, Ku86 was required for TrkA-stimulated invasion of breast cancer cells.

2 Materials and methods

2.1 Materials

Cell culture reagents were purchased from Bio-Whittaker (Emerainville, France). Recombinant NGF was from R&D Systems (Lille, France). Nucleofection reagents were from Amaxa Biosystems (Lonza, Cologne, Germany). Dynabeads protein A and protein G were obtained from Dynal Biotech (Invitrogen, Cergy Pontoise, France). Electrophoresis reagents, bicinecinonic acid reagents, protease inhibitor cocktail and rabbit polyclonal anti-actin antibody were from Sigma (Saint-Quentin Fallavier, France). The monoclonal anti-Ku86 for immunofluorescence (S5C11) and neutralizing antibodies anti-Ku70 (clone N3H10) and anti-Ku86 (clone 111 or S10B1) were purchased from Thermo Scientific (Courtaboeuf, France). For Western blotting, the polyclonal anti-Ku70 (AHP316) and the monoclonal anti-Ku86 were purchased from AbD Serotec (Düsseldorf, Germany), GeneTex (Euromedex, Mundolsheim, France) and Sigma, respectively. The rabbit polyclonal anti-TrkA IgG, as well as irrelevant IgG, were from Upstate-Millipore. Peroxidase-conjugated donkey anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (Suffolk, UK). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Thermo Scientific). Lab-Tek chamber slides and Boyden chamber (8 mm) were obtained from Nalge Nunc International (Roskilde, Denmark). Alexa Fluor dyes-conjugated secondary antibodies were from Invitrogen. siRNA was purchased from Eurogentec (Angers, France).

2.2 Cell culture

The MDA-MB-231 human breast cancer cell line was from the American Type Culture Collection. MDA-MB-231 TrkA overexpressing cells were stably transfected and characterized in our laboratory [13]. Cells were routinely maintained in EMEM supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% FCS, 40 U/mL penicillin-streptomycin, 40 μg/mL gentamycin. All cells were cultured at 37°C in a humidified atmosphere of 5% CO2.

2.3 Protein extraction, separation and identification

2.3.1 Sample preparation and 2-DE

Protein extraction was performed as previously described [22]. IEF was carried out using 18 cm Immobiline DryStrips pH 3–10 (GE Healthcare Bio-Sciences). IPG strips were reswollen overnight in 345 μL DeStreak (hydroxyethyl disulfide, GE Healthcare Bio-Sciences) rehydration solution and 0.2% v/v carrier ampholytes 3–10 (Bio-LyteBio-Rad), under 2 mL mineral oil. Prior to IEF, protein samples (150 μg) were first reduced (1 h) by adding tributylphosphine to a final concentration of 5 mM and secondly alkylated in the dark with 15 mM iodoacetamide for 90 min at room temperature. The samples were then cup-loaded near the anode of the IPG strips and focused in a Protein IEF cell (Bio-Rad) at a temperature of 20°C. The IPG strips were initially conditioned for 30 min at 250 V (rapid voltage ramping), linearly ramped to 1000 V (1 h) and maintained at 1000 V for 1 h more. Then the electric voltage was slowly increased to reach 10 000 V in 1 h and focused at this voltage to give a total of 60 kVh. After focusing, the strips were equilibrated for 2 × 15 min in 6 M Urea, 30% w/v glycerol, 2% w/v SDS, 0.125 M Tris, 0.1 M HCl, containing either 50 mM DTT (first equilibration step) or 150 mM iodoacetamide (second equilibration step) [23]. SDS-PAGE was performed as previously reported [24]. The gel patterns were visualized by silver nitrate staining [25] for analytical purposes or by colloidal CBB G-250 [26] in the case of micropreparative separations.
2.3.2 2-D gel evaluation

Digitized images of 2-D gels were acquired by scanning with a GS-800 calibrated densitometer under control of PDQuest Advanced software version 8.0 (Bio-Rad), which was also used for image analysis and construction of a local 2-D database. Image alignment, spot detection, background removal and expression analysis were performed using PDQuest Advance software (Bio-Rad). Fold changes and all statistical analysis were calculated based on normalized spot volumes where the global spots volume was used to perform normalization. A total of three gels per protein extraction and three extractions from independent experiments were made (nine gels in total per condition) for the study.

2.3.3 Immunoprecipitation and SDS-PAGE

Cells were harvested by scraping in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 1 mM sodium orthovanadate and protease inhibitor cocktail) as previously described [27]. Lysate (5 mg of proteins) was first precleared using an isotype rabbit IgG (R&D system). TrkA and interacting proteins were then co-immunoprecipitated using 25 μg of anti-TrkA antibody (Upstate) and Dynabeads protein A according to manufacturer’s instructions. Immunoprecipitated proteins were eluted in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.002% bromophenol blue) and boiled 5 min before analysis on 10% polyacrylamide gels. For staining, SDS-polyacrylamide gels were fixed overnight in solution containing 50% ethanol and 1.4% orthophosphoric acid. After three washes for 30 min in MilliQ water, gels were incubated in impregnation solution (1.3 M ammonium sulfate, 34% methanol, 1.4% orthophosphoric acid) for 1 h and placed in staining solution (1.3 M ammonium sulfate, 34% methanol, 1.4% orthophosphoric acid, 0.07% CBB G250) for 24 h. Finally, gels were destained with several washes of MilliQ water until the background was clear.

2.3.4 In-gel trypsin digestion and protein identification by MS

Coomassie blue-stained protein bands were excised from SDS-PAGE gel and processed for trypsin digestion as previously described [27]. Protein identification was realized using both MALDI-TOF (Voyager DE STR instrument, Applied Biosystems) and NanoLC-NanoESI-MS/MS (LCQ Deca XP+, Thermo-electron, San Jose, CA) as previously described [27]. Database searching was done with SwissProt 56.8 (410 518 sequences; 148 080 998 residues). Carbamidomethylation of cysteine was set as fixed modification, oxidation of methionine was set as a variable modification for all MASCOT searches. To ascertain unambiguous identification, searches were performed in parallel with Phenyx software using the same parameters.

2.4 Immunoassays

2.4.1 Western blot

After 2-DE or SDS-PAGE separation, proteins were electro-transferred onto nitrocellulose membrane using a semi-dry transfer system (Trans-Blot SD cell, BioRad). Non specific protein binding sites were saturated for 1 h 30 at room temperature in TBS-0.1% Tween-20 reagent (TBST) containing either 5% BSA for Ku70, Ku86 and actin immunodetection. Membranes were then incubated overnight at 4°C with 1:5000 anti-Ku70, 1:500 anti-Ku86, 1:500 anti-TrkA or 1:5000 anti-actin antibodies. After washes in TBST, peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted in saturated solution was added for 1 h at room temperature and the membranes were washed several times in TBST before detection of peroxidase activity using chemiluminescent system.

2.4.2 Flow cytometry

Cells detached by trypsin-EDTA solution were incubated for 1 h at 4°C with 20 μg/mL of the indicated antibodies or matched control isotypes at similar concentrations. After washing with PBS containing 0.5% BSA, cells were incubated for 30 min at 4°C with secondary fluorescein-labelled IgG. Cells were then analyzed in Coulter Epics XL/XL-MCl cytometer (Beckman Coulter, Villepinte, France).

2.4.3 Immunocytochemistry and confocal microscopy

MDA-MB-231 cells were seeded on Lab-Tek chamber slides pre-coated with type I collagen. Cells were washed in PBS pH 7.5, fixed in 4% paraformaldehyde for 20 min. Non specific protein binding sites were then blocked in PBS pH 7.5 containing 2% BSA and cells were incubated in blocking solution containing 10 μg/mL rabbit anti-TrkA and 10 μg/mL mouse anti-Ku70 or anti-Ku86 antibodies overnight at 4°C. After washes in PBS pH 7.5, 10 μg/mL Alexa Fluor 546 goat anti-rabbit IgG and 10 μg/mL Alexa Fluor 488 donkey anti-mouse IgG were added for 1 h at 37°C. Cells were washed in PBS pH 7.5 and mounted. Scanning fluorescence images were acquired using a Zeiss Axiohot microscope.

2.5 Real-time PCR

RNA extraction and real-time PCR (RT-PCR) amplifications were performed as previously described [27]. The primers used...
were as following: 5'-CCCAATGCAGCATATT-3' and 5'-CTCTCAGCCGACTGAGAC-3' for Ku86, 5'-AAAGAGAC
TGGGCCTCTGGT-3' and 5'-TGTGGCTCTCAAGCTC
CTCT-3' for Ku70, 5'-GATTCACCGCCAAAGGAGA-3' for RPLP0 (human acidic ribosomal phosphoprotein P0), which
was used as a reference gene. The amplification was
performed during 40 cycles (95°C for 20 s, at 55°C for 30 s,
and at 72°C for 30 s). Data were analyzed using the MX4000
PCR system software (Stratagene, Amsterdam, The Neth-
erlands) with the SYBRGreen option (with dissociation
curves).

2.6 siRNA transfection

MDA-MB-231 cells (2 x 10^6) were transiently transfected with
3 μg of siKu70 and/or siKu86 using the nuclease transfection
(Anmaxa Biosystems) according to manufacturer’s
instructions. Sequences of 5'-GAUGCCCUUAUCU-
GAAAAA-3' and 5'-UUUUUCAGUAAACGGCUGAU-3' were
used as sense and anti-sense for siKu70, 5'-CCGGUUUC-
CAACAGCGCC-3' and 5'-CACCCUGUGAAGACCUCCG-3'
were used as sense and anti-sense for siKu86, siRNA
against GFP (sense 5'-GUAGACCCGUGAAUAUC-3',
anti-sense 5'-GAUGAAACUAGGGCUG-3') were used as

![Image](m/z_1031.24.png)

**Figure 1.** Detection of Ku86 up-regulation in TrkA overexpressing
cells. (A), a representative 2-D gel image after silver staining. The
arrow indicates spots corresponding to Ku86. (B), zoomed regions
of Ku86 spots. (C) MALDI-TOF spectrum of tryptic digest for Ku86
identification. (D) A tandem mass spectrum of the doubly-
charged ion at m/z 1031.24. The bold letters indicate the detected
b and y ions matching the predicted ion mass in the database.
(E) Peptides sequenced by LC-MS-MS (underlined). The detected
fragments (bold letters) by MALDI-TOF are also indicated in the
sequence of full-length of Ku86.
control. Twenty-four hours after transfection, cells were seeded for the evaluation of apoptosis induction or invasion.

2.7 Invasion assay

BD Falcon inserts with a PET membrane/6.5 μm pores (BD Biosciences) were used for invasion assay. The inserts were pre-coated with GFR Matrigel (1:10 dilution, BD Biosciences). Cells (5 × 10⁴) were seeded on polycarbonate membrane insert and maintained in EMEM containing 0.1% FCS. For antibody neutralization, cells were pre-treated with 20 μg/mL of neutralizing antibodies against Ku70 and Ku86 during 30 min before seeding. After 16 h of culture, the insert was washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that invaded the Matrigel and migrated to the bottom surface of the insert were fixed with methanol, stained by Hoechst 33258 and then counted on 10 random fields at 200 magnification under a Nikon Eclipse Ti-U fluorescent microscope.

2.8 Apoptosis analysis

Cells were treated with 5 ng/mL TNF-related apoptosis inducing ligand (TRAIL) for 6 h. Apoptosis was determined by morphological analysis after fixation with methanol (10 min, –20°C) and staining with 1 μg/mL Hoechst 33258 (10 min, room temperature, in the dark). A minimum of 500–1000 cells was examined for each case under fluorescent microscope and the results represented the number of apoptotic cells over the total number of counted cells.

2.9 Statistical analysis

Statistical significances were determined with two-tailed Student’s t tests. All p-values were two-sided. \( p < 0.01 \) was considered as statistically significant.

3 Results

3.1 Ku86 is upregulated in breast cancer cells overexpressing TrkA

Proteins of mock and TrkA overexpressing MDA-MB-231 cells were separated by 2-DE before analysis of protein spots with PDQuest software. A representative example of proteins separated on 2-D gel is shown in Fig. 1A. Nearly 1500 spots were obtained in the ranges of MW

Table 1. Ku86 identification with MALDI-TOF

| Position | Corresponding peptide sequences | Observed precursor m/z | Theoretical precursor neutral mass | Delta mass (Da) |
|----------|---------------------------------|------------------------|------------------------------------|----------------|
| 36–44    | KVITMFVQR (Ox)                  | 1136.64                | 1136.64                            | 0              |
| 37–44    | VITMFVQR                        | 992.59                 | 992.55                             | 0.04           |
| 131–141  | HIEIFTDLSR                      | 1316.69                | 1316.67                            | 0.02           |
| 172–184  | EDGSGDRGDPFRR                   | 1363.61                | 1363.58                            | 0.03           |
| 185–195  | LGGHGSFPLK                      | 1108.63                | 1108.6                             | 0.03           |
| 196–209  | GITEQIQKGEIIVK                  | 1570.87                | 1570.86                            | 0.01           |
| 243–250  | HSIHWPCR (Carb)                 | 1091.59                | 1091.51                            | 0.08           |
| 251–260  | LTIGSNLISR                      | 1072.66                | 1072.62                            | 0.04           |
| 275–282  | TWTVVDAK                        | 918.49                 | 918.48                             | 0.01           |
| 287–307  | EDIQKETVVCLNDDETEVLK            | 2498.92                | 2498.14                            | 0.06           |
| 316–325  | YGSDIVPSFK                      | 1111.57                | 1111.56                            | 0.01           |
| 316–332  | YGSDIVPSFRVDEEQMK (Ox)          | 1986.94                | 1986.92                            | 0.02           |
| 354–363  | RFFMGQNLK (Ox)                  | 1254.7                 | 1254.65                            | 0.05           |
| 355–363  | FFMGQVLK (Ox)                   | 1098.57                | 1098.55                            | 0.02           |
| 403–413  | ANPGVGAVPHIK                    | 1376.79                | 1376.76                            | 0.03           |
| 423–439  | QYMFSSLK (Ox)                   | 1018.52                | 1018.48                            | 0.04           |
| 444–465  | YAPTEAQNAVDALIDSMLAK (Ox)       | 2336.09                | 2336.16                            | −0.07          |
| 470–481  | TDTLEDLFTPTK                    | 1379.73                | 1379.68                            | 0.05           |
| 487–497  | FROLFOCLLRH (Carb)              | 1516.76                | 1516.81                            | −0.05          |
| 490–497  | LFQCLLRH (Carb)                 | 1085.61                | 1085.58                            | 0.03           |
| 533–543  | IKTLFPLIEAK                     | 1271.78                | 1271.79                            | −0.01          |
| 535–543  | TLFPLIEAK                       | 1030.6                 | 1030.61                            | −0.01          |
| 546–565  | DVQTAQIEFQDNHDGPTAK             | 2242.04                | 2242.01                            | 0.03           |
| 641–648  | AFRQEAIF                        | 962.54                 | 962.52                             | 0.02           |
| 709–732  | DKPSGDTAAVEFEEGGDVDDLDMI (Ox)    | 2524.04                | 2524.12                            | −0.08          |

Spots indicated in Fig. 1 were cut out of the gel and digested with trypsin before MALDI-TOF analysis. MASCOT search program was used to identify proteins. The table lists all the 25 peptides identified by MALDI-TOF. The underlined peptides are also identified by nanoLC-nanoESI-MS/MS.

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12–120 kDa and pI 3–10. In TrkA overexpressing cells, more than 20 spots were found to be down- or upregulated (at least two-fold changes with p<0.05) after analysis with PDQuest software. Proteins were identified by MALDI-TOF and LC-MS/MS. Among a dozen of differentially expressed proteins which remain to be validated, a significant increase of Ku86 protein was observed in TrkA overexpressing cells. Interestingly, Ku86 was found as a set of four close spots in empty vector transfected cells (mock) and TrkA overexpressing cells (Fig. 1B). MALDI-TOF spectrum showed 25 experimental tryptic peptides that matched to theoretical masses, leading to 32.5% sequence coverage with an average error mass of 0.034 Da (Fig. 1C, Table 1). This identification was consolidated by independent MS/MS analysis of corresponding spot from 2-D gel, as sequencing of five peptides revealed 8% sequence coverage with an average error mass of 0.295 Da (Fig. 1D and E, Table 2). Importantly, Western blotting analysis also showed a similar increase of Ku86 in TrkA overexpressing cells (Fig. 2A and B), thus validating the 2-DE and MS analysis. However, RT-PCR showed no modification of Ku86 mRNA level (Fig. 2C), indicating that Ku86 upregulation is post-translationally controlled.

Table 2. Ku86 identification with NanoLC-nanoESI-MS/MS

| Position | Corresponding peptide sequences | Observed precursor m/z | Theoretical mass | Delta mass (Da) | Score |
|----------|--------------------------------|------------------------|-----------------|----------------|-------|
| 316–325  | YGSDIVPFSK                     | 1111.96                | 1111.56         | 0.40           | 45    |
| 355–363  | FFMGNQVLK                      | 1082.83                | 1082.56         | 0.27           | 42    |
| 470–481  | TDTLEDLFPPTK                   | 1380.05                | 1379.68         | 0.37           | 90    |
| 535–543  | TLFPLEAK                       | 1031.25                | 1030.61         | 0.64           | 45    |
| 546–565  | DQVTQEIEFQDNHEDGPTAK           | 2242.49                | 2242.01         | 0.48           | 21    |

Spots indicated in Fig. 1 were cut out of the gel and digested with trypsin before nanoLC-nanoESI-MS/MS. MASCOT search program was used to identify proteins. The table lists the five identified peptides.

Figure 2. Analysis of Ku86 expression by Western blot and RT-PCR. (A) Western blotting. Proteins (200 μg) were separated in a 2-D gel and Western blots were performed using an anti-Ku86 antibody. Actin was used to confirm the loading and transfer of equal amounts of protein. (B) Relative levels of Ku86. Quantification was performed by densitometry of spots revealed after Western blot. Values were normalized with actin. Results are the mean of three independent experiments. (C) RT-PCR detection of Ku86 mRNA. RPLP0 (human acidic ribosomal phosphoprotein P0) was used as a reference gene.
3.2 Ku86 is co-immunoprecipitated with TrkA

Ku86 and Ku70 can be associated to form a heterodimeric regulatory subunit of the DNA-PK that is crucial to DNA repair [28]. Ku proteins are also reported to be involved in cell proliferation, migration and invasion [29]. More recently, we have shown that Ku70 interacts with TrkA in MCF-7 breast cancer cells to stimulate cell survival in TRAIL-induced apoptosis [27]. To determine if TrkA interacts with Ku proteins in MDA-MB-231 TrkA overexpressing cells, we identified TrkA co-immunoprecipitated proteins by LC-MS/MS. Both Ku86 and Ku70 were found to be immunoprecipitated with TrkA. Identification of Ku86 was made based on the sequencing of three peptides (556.88; 689.45; 690.88) (Fig. 3A and B), with a MASCOT score of 208 and 4% coverage (blanket). On the other hand, two peptides corresponding to Ku70 were also sequenced (586.71 and 568.63), with a Mascot score of 48 and 3% of coverage (Fig. 3C). Moreover, Western blot confirmed the presence of both Ku86 and Ku70 in TrkA co-immunoprecipitated proteins but not in isotype IgG co-immunoprecipitated proteins (Fig. 3D), suggesting the specific association of Ku proteins to TrkA.

3.3 Membrane Ku86 and Ku70 are increased in TrkA overexpressing cells

Increased levels of Ku86 in TrkA overexpressing cells as well as the association of Ku proteins with TrkA prompted us to determine the levels of membrane Ku proteins by flow cytometry analysis. As shown in Fig. 4A and B, an increase of membrane Ku86 and Ku70 was observed in TrkA overexpressing cells compared to empty vector transfected cells. We then analysed subcellular distribution of these proteins in TrkA overexpressing cells by confocal microscopy after immunohistochemical staining (Fig. 4C). TrkA (blue fluorescence) and Ku proteins (green fluorescence) seemed to be mainly located in the nucleus and cytoplasmic membrane, though a faint and punctuate staining was also observed in the cytoplasm.

3.4 Ku86 is involved in TrkA-induced cell invasion

We have previously shown that TrkA overexpression leads to an increase in invasion and survival of breast cancer cells [13]. To determine whether Ku proteins were implicated in
TrkA-overexpression-induced biological effects, we inhibited Ku proteins by siRNA or neutralizing antibodies. As shown in Fig. 5A, specific siRNAs strongly decreased the expression of Ku86 and Ku70. Inhibition of Ku86 with both siRNA and the neutralizing antibody efficiently reduced invasion of TrkA overexpressing cells, whereas inhibition of Ku70 had no effect (Fig. 5B and C). Interestingly, simultaneous inhibition of both Ku86 and Ku70 reduced cell invasion at a similar degree to that observed upon Ku86 inhibition alone. We then evaluated if Ku proteins could regulate apoptosis induction in TrkA overexpressing cells. For this, we transfected cells with siKu and then treated them with TRAIL (TNF-related apoptosis inducing ligand), a cytokine well known for apoptosis induction in breast cancer cells. As shown in Fig. 5D, siKu86 had no effect on apoptosis of cells whatever TRAIL treatment. In contrast, siKu70 induced apoptosis even in the absence of TRAIL and could further increase apoptosis induction by TRAIL, confirming our previous finding in MCF-7 breast cancer cells [27]. When cells were co-transfected with siKu86 and siKu70, no significant difference was observed compared to siKu70 alone-transfected cells. All together, these results indicated that Ku proteins functioned independently of each other to mediate TrkA overexpression-induced biological effects: Ku86 was only involved in cell invasion whereas Ku70 was implicated in cell survival.

4 Discussion

We have previously shown that TrkA overexpression increases breast cancer cell growth, invasion, as well as survival. In order to understand the underlying mechanisms, we have used a functional proteomic approach to identify molecules involved in the TrkA-mediated biological effects. We first separated proteins using 2-DE system and then identified a series of putative modified proteins in TrkA overexpressing cells by MALDI and LC-MS/MS analysis. The cup-loading technology reduces the inter-experimental variations, allowing rapid identification of protein changes between samples on reproducible 2-D gels [30]. Among a dozen of differentially expressed proteins, which remain to be validated, we observed a significant increase of Ku86 in TrkA overexpressing cells, as revealed by direct quantification of corresponding spots in 2-D gels and Western blot analysis. This is of particular interest, as we have recently identified another member of the Ku family of proteins, namely Ku70, as a partner of TrkA signaling in breast cancer cells [27]. Indeed, we have shown that NGF treatment induces tyrosine phosphorylation of Ku70 upon its association to TrkA. Moreover, Ku70 is involved in TrkA-enhanced cell survival [27]. Here, we only found that Ku86 was up-regulated in TrkA overexpressing cells, although both Ku70 and Ku86 were co-immunoprecipitated with TrkA. Visualisation of Ku86 as four close spots in 2-D gels may be due to post-translational modifications, as Ku proteins have been described to be acetylated and phosphorylated [27, 31, 32]. In spite of the increase of protein levels, we were not able to observe any significant variation of Ku86 mRNA level by RT-PCR, suggesting a mechanism of regulation at post-transcriptional level. Reinforcing this hypothesis, it has been recently described that VEGF can activate AKT which in turn inhibits Ku70 proteolysis by phosphorylating Hdm2, the ubiquitin ligase of Ku70 [33]. Thus, further studies will
be needed to examine the exact underlying mechanism of Ku86 upregulation in our model. The fact that Ku86 was upregulated in TrkA overexpressing breast cancer cells is in line with previous demonstration that Ku86 is expressed in abundant levels in tissues with a high proliferative index or in cells stimulated to proliferate [34, 35]. Moreover, upregulation of Ku proteins has been associated with the progression of some types of tumors. For instance, the levels of Ku86 are positively correlated with that of anti-apoptotic Bcl-2 in B cell chronic lymphocytic leukemia [36]. Ku86 has also been reported to be upregulated in bladder, breast and primary hepatocellular carcinomas, compared to adjacent non-tumorous tissues [37–39].

Ku86 and Ku70 are mainly localized in the nucleus, where they form heterodimers to recruit the catalytic subunit of DNA-PK, which is involved in multiple biological processes such as DNA double-strand break repair, telomere length maintenance, cell cycle progression and transcriptional regulation [32, 40]. However, cytosolic and membrane Ku proteins are increasingly reported to exert different functions independently of each other. Cytosolic Ku70 has been shown to bind to the pro-apoptotic protein Bax and inhibit Bax-mediated apoptosis by preventing its translocation to mitochondria. This anti-apoptotic function is mediated by a domain in the carboxy-terminal of Ku70 and does not require the cooperative effects of Ku86 [41]. Accordingly, here we showed that Ku70 but not Ku86 was involved in the increased survival of TrkA overexpressing breast cancer cells. On the other hand, membrane Ku proteins have been reported to be associated with cell adhesion and migration [42, 43]. It has been shown that hypoxia-stimulated invasion of neuroblastoma and breast carcinoma cells involves upregulation of membrane Ku86 [44]. Similarly, Ku proteins are found to interact with matrix metalloproteinase 9 at the membrane of highly invasive normal and tumoral hematopoietic cells [45]. Translocation of Ku proteins from the nucleus to the plasma membrane can enhance migration of monocytes [46]. In this study, we found an increase of membrane Ku86 and Ku70 in TrkA overexpressing cells. Moreover, inhibition of Ku86 with siRNA or neutralizing antibodies strongly reduced TrkA-stimulated invasion, indicating that Ku86, especially membrane Ku86, was involved in this process. Our previous work shows that activation of signalling pathways including PI3/AKT and MAP kinases is required for TrkA overexpression-enhanced cell invasion and survival [13]; here we showed that Ku86 was involved in TrkA-overexpression-stimulated cell invasion while Ku70 was only implicated in TrkA-overexpression-enhanced cell survival. Thus, it will be interesting to determine the link between classical signaling pathways and the specific implication of Ku proteins in these processes.

![Figure 5](https://www.clinical.proteomics-journal.com)}
Clinical Relevance

Increased receptor tyrosine kinase activity has been found in a number of different human cancers, motivating development of anti-cancer therapies designed to abrogate their functional contribution. Thus, Herceptin has been clinically used to target the tyrosine kinase receptor Erb-B2. However, less than 15% of patients with breast cancer are Erb-B2 positive, critically limiting the impact of Herceptin treatments. Better understanding of the involvement of other tyrosine kinase receptors in cancer development is therefore important for therapeutic improvements. The Trk family of neurotrophin receptors is emerging as an important player in the development of several types of cancers, including breast cancer. We have recently shown that breast tumors express high levels of TrkA compared to normal breast tissues, with TrkA overexpression enhancing breast cancer cell invasion in vitro and metastasis in animal models. In this study, we showed by functional proteomic exploration that Ku86 is upregulated in TrkA overexpressing breast cancer cells and is involved in TrkA-induced cell invasion. Therefore, upregulation of Ku86 in tumor cells overexpressing TrkA might be a mechanism participating to metastasis. Our findings suggest that TrkA and its downstream signaling pathways should be regarded as potential new targets for breast cancer therapy.

In conclusion, we showed by functional proteomic exploration that Ku86 is upregulated in TrkA overexpressing breast cancer cells and is involved in TrkA-induced cell invasion. Therefore, upregulation of Ku86 in tumor cells overexpressing TrkA might be a mechanism leading to an increase of metastasis. Although further in vitro and in vivo investigations will be required to test this hypothesis, these data reveal Ku86 as a new potential player in the intracellular signaling leading to breast cancer cell metastasis.

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