The deubiquitinaning enzyme UBPy/USP8 interacts with TrkA and inhibits neuronal differentiation in PC12 cells.

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Abstract

The tropomyosin-related kinase (Trk) family of receptor tyrosine kinases control synaptic function, plasticity and sustain differentiation, morphology, and neuronal cell survival. Understanding Trk receptors down-regulation and recycling is a crucial step to point out sympathetic and sensory neurons function and survival. PC12 cells derived from pheochromocytoma of the rat adrenal medulla have been widely used as a model system for studies of neuronal differentiation as they respond to nerve growth factor (NGF) with a dramatic change in phenotype and acquire a number of properties characteristic of sympathetic neurons. In this study we demonstrated that in PC12 cells the TrkA receptor interacts with the deubiquitinating enzyme USP8/UBPy in a NGF-dependent manner, and that it is deubiquitinated *in vivo* and *in vitro* by USP8. USP8 overexpression blocked NGF-induced neurites outgrowth while the overexpression of the catalytically inactive mutant USP8/UBPy^{C748A} caused a marked increase of cell differentiation. Localization and biochemical experiments have point out that USP8 and TrkA partially co-localize in endosomes after NGF stimulation. Finally we have studied the role played by USP8 on TrkA turnover; using specific siRNA for USP8 we found that USP8 knockdown increase TrkA half-life, suggesting that the deubiquitinating activity of USP promotes TrkA degradation.

Introduction

Neurons are highly polarized cells that pose a formidable challenge for growth factor signaling (Ascano et al. 2012); owing to their unique architecture, neurotrophins that act locally on the axonal terminals must convey their signals across their entire axon for subsequent regulation of gene transcription in the cell nucleus (Chowdary et al. 2012). Different lines of evidence have supported the "signaling endosome" model in which neurotrophins and their Trk receptor, as well as an array of downstream signaling molecules, are internalized as ligand –receptor complexes and retrogradely transported along microtubules to cell bodies (Ascano et al. 2012; Chowdary 2012). NGF-TrkA complexes are internalizes through at least two endocytotic pathways: clathrin-mediated endocytosis and Pincher-mediated macro pinocytosis (Shao et al. 2002; Valdez et al. 2005). In particular, in clathrin-mediated TrkA-NGF endocytosis some vesicles become specialized endosomes that serve as platforms for sustained signaling and are transported to the cell body using a dynein- and microtubule-dependent transport mechanism (Ginty and Segal 2002). The vesicles containing NGF-TrkA complexes include activated signaling proteins of the Ras-MAPK pathway and Rap1 GTPase (Howe et al. 2001; Wu et al. 2007). The macro pinocytosis mechanism of endocytosis involves Pincher, a membrane trafficking protein, that mediates the internalization of activated TrkA at plasma membrane ruffles; the resulting macroendosomes are processed into multivesicular bodies (MVB) distinctively refractory to signal termination by lysosomal processing, resulting in sustained signaling and neuronal gene expression (Philippidou et al. 2011). Endocytosis of receptor tyrosine kinases in non-neuronal cells follows a common scheme that consists of an internalization reaction and a delivery step, during which cargos are transferred to an endosomal station to be either directed to the lysosome for degradation or recycled back to the cell surface (Goh and Sorkin 2013). At each stage short motifs within protein cargos and/or post-translational modifications such as ubiquitination act as signals for the internalization and sorting of plasma membrane proteins (Acconcia et al. 2009). At least three mechanisms of TrkA ubiquitination have been reported: the ligand-dependent K63-polyubiquitination mediated by the Tumor necrosis factor Receptor-Associated Factor 6 (TRAF6) (Geetha and Wooten 2008) the monoubiquitination of this receptor at multiple sites mediated by Nedd4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) (Arevalo et al. 2006; Georgieva et al. 2011) and the c-Cbl mediated ligand-induced ubiquitination of TrkA receptor (Takahashi et al. 2011). TrkA ubiquitination and degradation required direct interactions between c-Cbl and phosphorylated TrkA. c-Cbl and ubiquitinated TrkA are found in a complex after NGF stimulation and are degraded in lysosomes (Takahashi et al. 2011). Removal of ubiquitin is accomplished by the action of deubiquitinating enzymes (DUBs), proteases specific for the isopeptide bond that links ubiquitin chains and ubiquitin to substrate proteins (Nijman et al. 2005). Recently Telebian et al. (2013) has demonstrated that the guanine nucleotide exchange factor, RasGRF1, binds NGF-activated TrkA and facilitates neurotrophin-induced neurite outgrowth in PC12 cells. RasGRF1is ubiquitinated and interacts with a deubiquitinating enzyme UBPy/USP8 that deubiquitinates and stabilizes RasGRF1 (Gnesutta et al. 2001). Ub-specific protease Y (UBPy) also designated as Ub-specific protease 8 (USP8), is a ubiquitin isopeptidase that belongs to the USP family of cysteine proteases (Naviglio et al. 1998; Gnesutta et al. 2001) and could

deubiquitinate monoubiquitinated receptor tyrosine kinases, as well as process Lys-48- and Lys-63-linked polyubiquitin to lower denomination forms in vitro (Row et al. 2006). In cultured cell lines, USP8 has been shown to interact with the SH3 domain of the signal-transducing adaptor molecule (STAM) that, on its own, associates with the hepatocyte growth factor-regulated substrate (Hrs), giving rise to the ESCRT-0 complex of the endosomal sorting machinery (Kato et al. 2000; Niendorf et al. 2007). Besides, by pull down assays and immunofluorescence experiments it has been demonstrated that USP8 interacts and co-localize with STAM2, a component of ESCRT-0 (endosomal-sorting complex required for transport-0), also known as Hbp in differentiating spermatids (Berruti et al. 2010). When overexpressed, USP8 moves to endosomes upon cell growth stimulation and plays a key role in the down-regulation of tyrosine kinase membrane receptors as EGFR and c-MET (Mizuno et al. 2005; Row et al. 2006; Alwan and Van Leeuwen 2007; Oh et al. 2014); consequently, USP8 is described to regulate cargo sorting and membrane traffic at early endosomes (Mizuno et al. 2007). Furthermore, analysis of a conditional USP8 mouse knock-out has revealed a drastic loss of growth factor receptors, including the EGFR, its family member, Erb-B3, and c-Met (Niendorf et al. 2007). As TrkA interact with RasGRF1 and as RasGRF1 interacts with USP8 it is possible that USP8 could interacts also with TrkA receptor In this work we investigated the role of USP8 in TrkA trafficking in PC12 cell and we have found that USP8 interacts with TrkA and its overexpression inhibits NGF induced differentiation, likely through a stimulation of TrkA degradation.

Materials and Methods

Plasmids, reagents and antibodies

HA_6-ubiquitin vector and pCDNA3-hUBPy plasmid were obtained by G. Draetta (Naviglio et al. 1998). pEGFP-C1-hUBPy was kindly provided by S. Urbè, University of Liverpool (Row et al. 2006). pME18S-FLAG-mUBPy^{C748A} was a generous gift of J.E.M. Leeuwen, Radbound University Nijmegen, Netherlands (Alwan et al. 2007). pRC/CMV-TrkA was a gift by Pierotti C. (Greco et al. 1993), Department of Experimental Oncology and Molecular Medicine, Istituto Nazionale dei Tumori, Milan, Italy; pEGFP-C1 vector was from Clontech while pCDNA3 plasmid was from Life technologies. NGF was purchased by Promega and N-Ethylmaleimide (NEM) was from Sigma. pRc/CMV- HA-TrkA was a generous gift by Yves-Alain Bardè, Max-Planck-Institute of Neurobiology, department of Neurobiochemistry, Biozentrum of the University of Basel (Bibel et al. 1999). Anti-UBPy rabbit antibodies have been produced as described in Gnesutta et al 2001 (Gnesutta et al. 2001). Anti-TrkA rabbit antibodies (C-14) were from Santa Cruz Biotechnology and were used for western blot analyses. For immunofluorescence assays has been used rabbit polyclonal anti-TrkA antibodies bought by Novus Biologicals or mouse monoclonal anti- TrkA antibodies bought by Abcam. Purified mouse anti-EEA1 antibody was from BD Transduction Laboratories. α-GFP antibody was from Clontech. Anti-HA (12CA5) antibodies were from Roche.

Cell culture and transfection

PC12 cells were grown at 37°C in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 5% heat inactivated Foetal Bovine Serum (EuroClone) and 10% Horse Serum (Sigma). PC12-TrkA cells, obtained by M.V. Chao, N.Y. University School of Medicine (Hempsted et al. 1992) were cultured in Dulbecco's modified Eagle's medium supplemented with heat inactivated 10% Horse Serum, 5% Foetal Bovine Serum, and 200 μg/ml G418 (Sigma) at 37°C. Transient transfections were performed using LipofectAMINE 2000 (Gibco-BRL). HEK 293 Phoenix cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (EuroClone).

RNA interference

For siRNA experiments, PC12 cells were plated at a density of 5x 10^6 cells /ml in 100 mm dishes in complete medium. The day after cells were transfected with either Control Stealth siRNA duplex (Nonspecific) (scr=scramble) or USP8-specific Stealth siRNA duplex (siUSP8) (sense CAAUGCUCCACAUUUGGCUGAUUAU, antisense 5′ AUAAUCAGCCAAAUGUGGAGCAUUG; Invitrogen) at 50 nM concentration using Lipofectamine RNAiMAX (Invitrogen). 24h, 48h and 72h after transfection cells were harvested and lysed by Lysis Buffer (Tris HCL pH 7.4, NaCl 100 mM, NP40 0.5% w/v, NaF 50 mM, Na_3VO_4 1mM, PMSF 1mM, Aprotinin 2μg/ml, Leupeptin 1μg/ml, Pepstatin 1μg/ml). 30 ug of total protein extracts were loaded and separated on 8% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were cut into two pieces and immunodecorated with anti-USP8 and

anti-Actin antibodies. Signals were detected using peroxidase-conjugated donkey anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, Inc) and revealed by ECL detection systems (Genespin).

Western Blot

Cell lines protein extracts were prepared using RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, sodium deoxycholate 0.5% w/v, SDS 0.1% w/v, Triton X-100 1%) supplemented with Complete ™EDTA Free (Roche) and 30 ug of total protein extracts were loaded and separated on 8% polyacrylamide gels. Western blotting was done according to standard procedures using nitrocellulose membranes (Protran). Blots were probed with anti-TrkA (C-14) antibodies Santa Cruz Biotechnologies or anti-UBPy primary antibodies (Gnesutta et al. 2001). Signals were detected using peroxidase-conjugated donkey anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, Inc) and revealed by ECL detection systems (Genespin).

TrkA-UBPy co-immunoprecipitation

PC12 cells were plated at a density of 5x 10^6 cells /ml in 60mm dishes in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 5% heat inactivated Foetal Bovine Serum (EuroClone) and 10% Horse Serum (Sigma). The day after cells were serum starved for 18h and then stimulated with 50 ng/ml of NGF 2.5S (Promega) for 0, 15, 30 and 60 minutes. Cells were then lysed mechanically in Lysis Buffer (Tris HCl pH 7.4, NaCl 100 mM, NP40 0.5% w/v, NaF 50 mM, Na_3VO_4 1mM, PMSF 1mM, Aprotinin 2μg/ml, Leupeptin 1μg/ml, Pepstatin 1μg/ml) and total protein extracts were clarified by centrifugation (13000 rpm 10 min). Equal amounts (1050 μg) of total protein extracts for each sample were incubated with 4 μl of anti-TrkA (C14) antibodies on a wheel at 4°C over-night. Immunocomplexes were then recovered with protein A-Sepharose beads (Sigma) and analysed by Western blot with anti-UBPy antibodies and then revealed with anti-rabbit HRP secondary antibodies (Jackson Immunoresearch Laboratories, Inc) and ECL system (Genespin).

TrkA deubiquitination "in vivo"

PC12 cells were plated at a density of 5x 10^6 cell/ml in 100mm dishes and grown in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 5% heat inactivated Foetal Bovine Serum (EuroClone) and 10% Horse Serum (Sigma). Cells were then transiently transfected or with Control Stealth siRNA duplex (50nM) (Scr) and HA_6-ubiquitin (3μg) or with a siRNA specific for USP8 (50nM) (siUSP8) and HA_6-ubiquitin (3μg) using Lipofectamine RNAiMAX (Invitrogen). 24h after transfection cells were serum starved for 18h. Prior to lysis in Lysis Buffer (Tris HCl pH 7.4, NaCl 100 mM, NP40 0.5% w/v, NaF 50 mM, Na_3VO_4 1mM, PMSF 1mM, Aprotinin 2μg/ml, Leupeptin 1μg/ml, Pepstatin 1μg/ml) cells were stimulated with 50ng/ml of NGF for 30 min. An equal amount (815 μg) of clarified total protein extracts for each sample was immunoprecipitated with 2.5μg of anti-HA antibodies (12CA5) (Roche) pre-coupled with protein A-Sepharose beads (Sigma). Samples were loaded onto 8% polyacrylamide gels, blotted and analysed with

anti-TrkA (C-14) antibodies and then revealed with anti-rabbit HRP secondary antibodies (Jackson Immunoresearch Laboratories, Inc) and ECL system (Genespin).

TrkA deubiquitination "*in vitro*"

HEK 293 cells were plated at a density of 5x 10^6 cell/ml in 100 mm dishes. pCDNA3, hUBPy and UBPy^{C748A} plasmid were each separately transfected in two 100 mm plate by means of Lipofectamine 2000. Cells were harvested 24h after transfection and lysed in Lysis Buffer (Tris HCl pH 7.4, NaCl 100mM, NP40 0.5% w/v, NaF 50mM, Na_3VO_4 1mM, PMSF 1mM, Aprotinin 2µg/ml, Leupeptin 1µg/ml, Pepstatin 1µg/ml). Total protein extracts were immunoprecipitated with anti-UBPy antibodies (4µl of antibodies for each sample) coupled to protein A beads over-night at 4°C on a wheel. Immunocomplexes , after several washes, were split into two fractions; one was treated with 20mM of N-Ethylmaleimide (NEM), a specific inhibitor for cysteine protease, and the other was treated with the same amount of ethanol (vehicle). Four 100mm dishes were transfected with TrkA and Ubiquitin-HA$_6$ constructs. These cells were then starved for 18h and then stimulated with 50ng/ml of NGF for 30 minutes to induce ubiquitination of TrkA receptor; cells were then lysed and immunoprecipitated with anti-TrkA antibodies coupled to protein A beads 1.5 h at 4°C on a wheel. Beads were then washed extensively and split into two fractions. TrkA beads were then reconstituted in DUB buffer (10mM Tris-HCl, pH 8.0, 1mM dithiothreitol, 1mM EDTA). TrkA immunocomplexes were combined with UBPy immunocomplexes in 100µl of DUB buffer and incubated at 37°C for 90 min with regular shaking. Beads and 30 µg of total protein extracts (ET) were then analysed by Western blot. Filters were probed with anti-HA (12CA5) (Roche) primary antibodies and then revealed with anti-mouse HRP secondary antibodies and ECL system.

Immunofluorescence

PC12 cells were plated at a density of 1x 10^5 cell/ml on polylisine pre-treated coverslips. Cells were then starved in RPMI supplemented with 0.5 % of Horse Serum (Euroclone). After 18 h, cells were stimulated with 50 ng/ml of NGF for 15 minutes. Cells were then fixed for 10 min with 3.7% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized for 4 min with 0.1% Triton X-100 in PBS, and stained with different antibodies. In particular for UBPy/USP8 and TrkA co-localization anti-mouse monoclonal (6B2) anti-TrkA antibodies (1:500, Abcam) and rabbit anti –UBPy (1:1000, Gnesutta et al. 2001) primary antibodies were used; secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (1:1000, Life technologies). For EEA1 and USP8 co-localization were used mouse monoclonal anti-EEA1 (1:500, BD Transduction Laboratories) and rabbit anti-UBPy (1:1000, Gnesutta et al. 2001) primary antibodies; secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (1:1000, Gnesutta et al. 2001). EEA1 and TrkA co-localization was performed with mouse monoclonal anti-EEA1 (1:500, BD Transduction Laboratories) and rabbit polyclonal anti-TrkA (1:400, Novus Biologicals, Inc) primary antibodies and Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (1:1000, Life technologies). Fluorescence images were captured with a Leica

DMIRE2 inverted microscope and TCSSP2 confocal microscope (images were analysed with the MBF Image J software https://www.macbiophotonics.ca/). Colocalization analysis was done using the ImageJ plugin *Image correlation analysis* calculating the PDM (Product of the Differences from the Mean, i.e. for each pixel: PDM = (red intensity- mean red intensity)×(green intensity – mean green intensity) values (Li et al. 2004).

Endosome isolation

PC12 cells were plated into 100mm dishes at a density of 5x 10^6 cell/ml and growth in complete medium. Cells were then starved in RPMI supplemented with 0.5% of HS for 18h and then let un-stimulated or stimulated with 50ng/ml of NGF for 15 minutes. Cells were washed with PBS and suspended in 10mM HEPES, pH 7.2, 100mM KCl, 1mM EDTA, 25mM sucrose. The cell suspension was passed through a 22-gauge syringe needle 10 times and then centrifuged at 3000 x g for 10 min at 4°C. Equal amounts of total protein extracts for each sample were incubated on a wheel at 4°C over-night with mouse anti-EEA1 (BD Transduction Laboratories) antibodies (2.5 μg, 1 h, 4°C). Immunocomplexes were then recovered with protein A-Sepharose beads (Sigma) and analysed by Western blot with anti-TrkA (C-14) (Santa Cruz) antibodies and then revealed with anti-rabbit HRP secondary antibodies (Jackson Immunoresearch Laboratories, Inc) and ECL system (Genespin). Filters were stripped (stripping buffer: SDS 10%, Tris-HCl 0.5M pH 6.7 and β-mercaptoethanol 100mM) and stained again with anti-UBPy and anti-EEA1 (BD Transduction Laboratories) antibodies.

Neurite outgrowth

PC12 and PC12-TrkA cells were plated in 60 mm dishes and transfected with UBPy-GFP or UBPy^{C748A} and GFP. 24h after transfection cells were maintained for 24, 48 or 72 h in basal medium with 0.5% Horse Serum with or without NGF (50 ng/ml). After different period of stimulation cells were observed with a Nikon Eclipse C600 fluorescence microscope and photographed with a Leica DG350F CCD camera. About 200 cells were counted at each experiment. Cells were counted as positive for neurite outgrowth if one or more neurites exceeded two cell body diameters in length.

TrkA turnover assay

PC12 cells were plated in2 set (each composed of 6 dishes) of 60 mm plates. After reaching of sub-confluence, each set of plates was transiently transfected with either Control Stealth siRNA duplex (Nonspecific) (scr) or USP8-specific Stealth siRNA duplex (siUSP8). As transfecting agent was used RNAiMAX (Invitrogen) as recommended by manufacturer's instructions. Cells were then starved in RPMI supplemented with 0.5 % of HS (Euroclone); after 18 h cells were stimulate with 50ng/ml of NGF for the 15, 30, 60, 90, 120 minutes. One sample of each set was left un-stimulated as control. Cells were then washed 2 times with phosphate-buffered saline (PBS) and lysed in Lysis Buffer (Tris HCl pH 7.4, NaCl 100mM, NP40 0.5% w/v, NaF 50mM, Na_3VO_4 1mM, PMSF 1mM, Aprotinin 2μg/ml, Leupeptin 1μg/ml, Pepstatin 1μg/ml).

Total protein extracts were quantified by Biorad protein assay (Biorad) and 30 µg of total protein extracts (ET) were then analysed by Western blot. Filters were probed with anti-TrkA (C14) (Santa Cruz Biotechnology) and then revealed with anti-mouse HRP secondary antibodies and ECL system. Anti-actin antibodies produced in rabbit (Sigma) were used to normalize sample.

Results

USP8 is expressed in PC12 cell line and interacts, in an NGF-dependent manner, with TrkA receptor.

It has been demonstrated that the deubiquitinating enzyme USP8/UBPy interacts and deubiquitinates some receptor tyrosine kinases (RTKs): EGFR, c- Met, promoting their degradation (Mizuno et al. 2005; Row et al. 2006; Alwan and Van Leeuwen 2007; Oh et al. 2014). TrkA is a tyrosine kinase receptor expressed in neuronal cells that is essential for sensory and sympathetic neurons survival. TrkA is poly- and mono-ubiquitinated, a modification that might function as a sorting signal for trafficking in neuronal cells, and interacts with the Ras guanine exchange factor RasGRF1 (Telebian et al. 2013; Robinson et al. 2005); furthermore RasGRF1interacts with the deubiquitinating enzyme USP8 that deubiquitinates and stabilizes it (Telebian et al. 2013). As USP8 transcript and protein has a widespread distribution in the mouse brain (Bruzzone et al. 2008) we have investigated the hypothesis that USP8 activity might interact and deubiquitinate also TrkA receptor. First of all we analyzed the expression of USP8 in some cell lines; in particular we focused our attention on PC12 cells which is a model cell line for neuronal differentiation and expresses the TrkA receptor. The cDNA for USP8 of *Rattus norvegicus* has not been cloned yet but an analysis on rat genome database (www.ensembl.org, Acc: ENSRNOG00000010729; http://www.ncbi.nlm.nih.gov, Acc: NM_001106502) revealed that the USP8 gene is localized on chromosome 3, is composed of 19 exons and codes for a putative protein of 1092 residues. Alignment analyses performed with CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)reveals that mouse, human and rat sequences are homolog and have an identity that exceed 83% (Supplementary data S1). As shown in Fig.1A our anti-USP8 antibodies recognized a specific band in the different cell lines suggesting that this deubiquitinating enzyme was expressed also in the rat PC12 cells. In contrast with the sequence derived from the rat genome database rat USP8 protein seems to be slightly smaller than human and mouse protein; this is probably due to an alternative splicing. To demonstrate that the band observed in PC12 cells corresponds to the rat USP8 protein we treated PC12 cells with a specific Stealth siRNA (siUSP8) designed for the sequence of the putative rat USP8 mRNA. As shown in Fig. 1B the specific siRNA is able to knockdown the band corresponding to rat USP8, a partial depletion is evident just 24 hours after transfection while a strong inhibition occurred after 72 hrs. After that we performed a co-immunoprecipitation assay to determine if USP8 and TrkA interact in PC12 cells and if this interaction depends upon NGF stimulation. As shown in Fig.2 USP8 is co-immunoprecipitated by anti-TrkA antibodies and the amount of USP8 clearly increased after stimulation of PC12 cells with NGF. This demonstrated that these two proteins interact physiologically in PC12 cells and that this interaction is NGF-dependent.
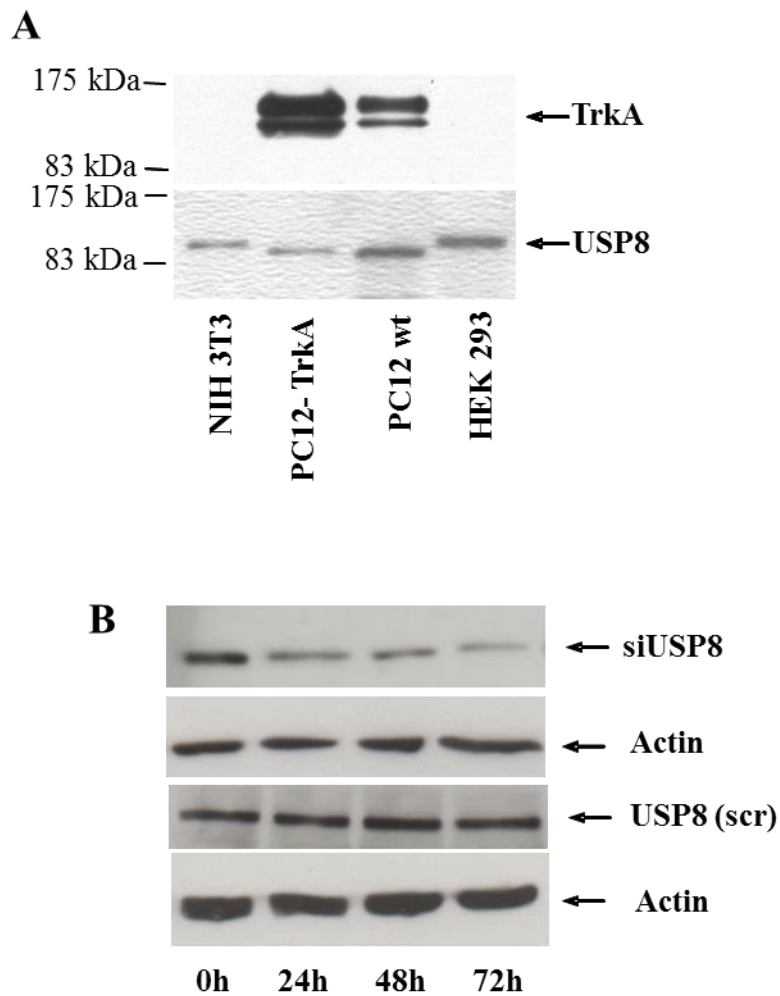
Figure 1. USP8 and TrkA expression. Panel A. *Expression of TrkA receptor and USP8 in different cell lines.* Total protein extracts (30 $\square$g) from different cell lines obtained in RIPA buffer were loaded onto SDS-polyacrylamide gels and blotted. Membranes were probed with anti-TrkA (C-14) and anti-USP8 antibodies. **Panel B.** *USP8 Depletion.* Whole cell lysates from PC12 cells transfected with control siRNA, USP8 (scr), or siRNA directed against human USP8 (siUSP8) were analyzed for endogenous proteins by Western blot, as indicated. Actin was use to normalize samples loading.

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R.n UBPy    MPAVASVPKELYLSSSLKDLNKKTEVKPEKTSTKNAKFYKFSSFLSYIHSAQKIFKAAEE 60
M.m UBPy    MPAVASVPKELYLSSSLKDLNKKTEVKPEKTSTKN-----------YIHSAQKIFKTAEE 49
H.s UBPy    MPAVASVPKELYLSSSLKDLNKKTEVKPEKISTKS-----------YVHSALKIFKTAEE 49
            ***************************** ***.           *:*** ****:***

R.n UBPy    CRLDRDEERAYVLYMKYVAVYNLIKKRPDFKQQQDYYLSILGPANIKKAIEEAERLSESL 120
M.m UBPy    CRLDRDEERAYVLYMKYVAVYNLIKKRPDFKQQQDYYLSILGPANIKKAIEEAERLSESL 109
H.s UBPy    CRLDRDEERAYVLYMKYVTVYNLIKKRPDFKQQQDYFHSILGPGNIKKAVEEAERLSESL 109
            ****************** :*************** : *****.*****:**********

R.n UBPy    KLRYEEAEVRKQLEEKDRREEEQLQQQKRQEMGREDSGAAAKRSVENLLDSKTKIQRVNG 180
M.m UBPy    KLRYEEAEVRKQLEEKDRREEEQLQQQKRQEMGREDSGAAAKRSVENLLDSKTKTQRING 169
H.s UBPy    KLRYEEAEVRKKLEEKDRQEEAQRLQQKRQETGREDGGTLAKGSLENVLDSKDKTQKSNG 169
            *********** :****** :** *  ****** ****.*: ** *:**:**** * *: **

R.n UBPy    EKSEGGAAAERGAVTAKELYTMMMDKNTSLIIMDARKIQDYQHSCILGSLSVPEEAISPG 240
M.m UBPy    EKSEGAAAAERGAITAKELYTMMMDKNTSLIIMDARKIQDYQHSCILDSLSVPEEAISPG 229
H.s UBPy    EKNEKCETKEKGAITAKELYTMMTDKNISLIIMDARRMQDYQDSCILHSLSVPEEAISPG 229
            **.*    : *:**:********* *** *******::****.****.**** ************

R.n UBPy    VTANWIEAKLSDDSKDTWKKRGSVDYVVLLDWFSSAKDLLLGTTLRSLKDALFKWESKAV 300
M.m UBPy    VTASWIEANLSDDSKDTWKKRGSVDYVVLLDWFSSAKDLLLGTTLRSLKDALFKWESKTV 289
H.s UBPy    VTASWIEAHLPDDSKDTWKKRGNVEYVVLLDWFSSAKDLQIGTTLRSLKDALFKWESKTV 289
            ***.****:*.**********.*:************** :*****************:*

R.n UBPy    LHHEPLVLEGGYENWLLCYPQFTTNAKVTPPPRSRPEEVSVSLDFTYPSLEEPVPSKLPA 360
M.m UBPy    LRHEPLVLEGGYENWLLCYPQFTTNAKVTPPPRSRAEEVSVSLDFTYPSLEEPVPSKLPT 349
H.s UBPy    LRNEPLVLEGGYENWLLCYPQYTTNAKVTPPPRRQNEEVSISLDFTYPSLEESIPSKPAA 349
            *:::***************** :*********** :  ****:***********.:*** .:

R.n UBPy    QMPPPPLEANEKAQLVTDQDGKPRPLVQSALAGPSVAPKAEASPIIQPVPATKNVPQVDR 420
M.m UBPy    QMPPPPIETNEKALLVTDQDEKLRLSTQPALAGPGAAPRAEASPIIQPAPATKSVPQVDR 409
H.s UBPy    QTPPASIEVDENIELISGQNERMGPLNISTPVEPVAASKSDVSPIIQPVPSIKNVPQIDR 409
            * **..:*.:*:   *::.*: :      .: . * .*.:::.******.*: *.***:**

R.n UBPy    TKKPAVKLPEDHRMKSESTD------QSGRVLSDRSTKPVFTSPATMLTDEEKARIHEET 474
M.m UBPy    TKKPSVKLPEDHRIKSENTD------QSGRVLSDRSTKPVFPSPTTMLTDEEKARIHQET 463
H.s UBPy    TKKPAVKLPEEHRIKSESTNHEQQSPQSGKVIPDRSTKPVVFSPTLMLTDEEKARIHAET 469
            ****:*****:**:***.*:      ***:*:.*******. **: *********** **

R.n UBPy    ALLMEKNRQEKELWERQQKEQKEKLRREEQERKAGKTQDAEERDFTENQHKAKDGQEKRD 534
M.m UBPy    ALLMEKNKQEKELWDKQQKEQKEKLRREEQERKAGKTQDADERDSTENQHKAKDGQEKKD 523
H.s UBPy    ALLMEKNKQEKELRERQQEEQKEKLRKEEQEQKAKKKQEAEENEITEKQQKAKEEMEKKE 529
            *******:***** ::**:*******:****:** *.*:*:*.: **:*:*** **::

R.n UBPy    SKQTKAEDREPPADGAQDATGTQRQSKSEHDASDAKVSVE--GKRCPMSEVQKRPADVSS 592
M.m UBPy    SKQTKTEDRELSADGAQEATGTQRQSKSEHEASDAKVPVE--GKRCPTSEAQKRPADVSP 581
H.s UBPy    SEQAKKEDKETSAKRGKEITGVKRQSKSEHETSDAKKSVEDRGKRCPTPEIQKKSTGDVP 589
            *:*:* **:* .*. .:: **.:*******::**** .** ***** .* **:.. .

R.n UBPy    ASSMSGELSAG---------------------------KAQREPLTRARSEEMGRIVP 623
M.m UBPy    AS-VSGELNAG---------------------------KAQREPLTRARSEEMGRIVP 611
H.s UBPy    HTSVTGDSGSGKPFKIKGQPESGILRTGTFREDTDDTERNKAQREPLTRARSEEMGRIVP 649
            : ::*: .:*                 ******************

R.n UBPy    GLPLGWAKFLDPITGTFRYYHSPTNTVHMYPPEMAPSSVPPSTPPTHKVKPQIPAERDRE 683
M.m UBPy    GLPLGWAKFLDPITGTFRYYHSPTNTVHMYPPEMAPSSAPPSTPPTHKVKPQVPAERDRE 671
H.s UBPy    GLPSGWAKFLDPITGTFRYYHSPTNTVHMYPPEMAPSSAPPSTPPTHKAKPQIPAERDRE 709
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R.n UBPy        PSKLKRSYSSPDITQALQEEEKRRPAVTPTVNRENKPPCYPKAEISRLSASQIRNLNPVF 743
M.m UBPy        PSKLKRSYSSPDITQALQEEEKRRPAVTPMVNRENKPPCYPKAEISRLSASQIRNLNPVF 731
H.s UBPy        PSKLKRSYSSPDITQAIQEEEKRKPTVTPTVNRENKPTCYPKAEISRLSASQIRNLNPVF 769
                ****************:******:*:***  ******.*********************

R.n UBPy        GGSGPALTGLRNLGNTCYMNSILQCLCNAPHLADYFNRNCYQDDINRSNLLGHKGEVAEE 803
M.m UBPy        GGSGPALTGLRNLGNTCYMNSILQCLCNAPHLADYFNRNCYQDDINRSNLLGHKGEVAEE 791
H.s UBPy        GGSGPALTGLRNLGNTCYMNSILQCLCNAPHLADYFNRNCYQDDINRSNLLGHKGEVAEE 829
                ***********************************************************

R.n UBPy        FGIIMKALWTGQYRYISPKDFKVTIGKINDQFAGSSQQDSQELLLFLMDGLHEDLNKADN 863
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H.s UBPy        FGIIMKALWTGQYRYISPKDFKITIGKINDQFAGYSQQDSQELLLFLMDGLHEDLNKADN 889
                **********************:*********** ************************

R.n UBPy        RKRHKEENNDHLDDFKAAEHAWQKHKQLNESIIVALFQGQFKSTVQCLTCHKKSRTFEAF 923
M.m UBPy        RKRHKEENNEHLDDLQAAEHAWQKHKQLNESIIVALFQGQFKSTVQCLTCRRRSRTFEAF 911
H.s UBPy        RKRYKEENNDHLDDFKAAEHAWQKHKQLNESIIVALFQGQFKSTVQCLTCHKKSRTFEAF 949
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M.m UBPy        MYLSLPLASTSKCTLQDCLRLFSKEEKLTDNNRFYCSHCRARRDSLKKIEIWKLPPVLLV 971
H.s UBPy        MYLSLPLASTSKCTLQDCLRLFSKEEKLTDNNRFYCSHCRARRDSLKKIEIWKLPPVLLV 1009
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R.n UBPy        HLKRFSYDGRWKQKLQTSVDFPLENLDLSQYVIGPKSSLKKYNLFSVSNHYGGLDGGHYT 1043
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H.s UBPy        HLKRFSYDGRWKQKLQTSVDFPLENLDLSQYVIGPKNNLKKYNLFSVSNHYGGLDGGHYT 1069
                ************************************..*********************

R.n UBPy        AYCKNAARQRWFKFDDHEVSDISVSSVRSSAAYILFYTSLGPRVTEAAT 1092
M.m UBPy        AYCKNAARQRWFKFDDHEVSDISVSSVRSSAAYILFYTSLGPRITDVAT 1080
H.s UBPy        AYCKNAARQRWFKFDDHEVSDISVSSVKSSAAYILFYTSLGPRVTDVAT 1118
                ***************************:****************:*:.**
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Figure S1.

Alignment of UBPy/USP8 sequence from *Mus musculus (M.m)*, *Rattus norvegicus (R.n)* and *Homo sapiens (H.s)*. The alignment was done with CLUSTALW2 tool (www.ebi.ac.uk/Tools/msa/**clustalw**2).

IP

IP: anti-TrkA; IB: anti-UBPy
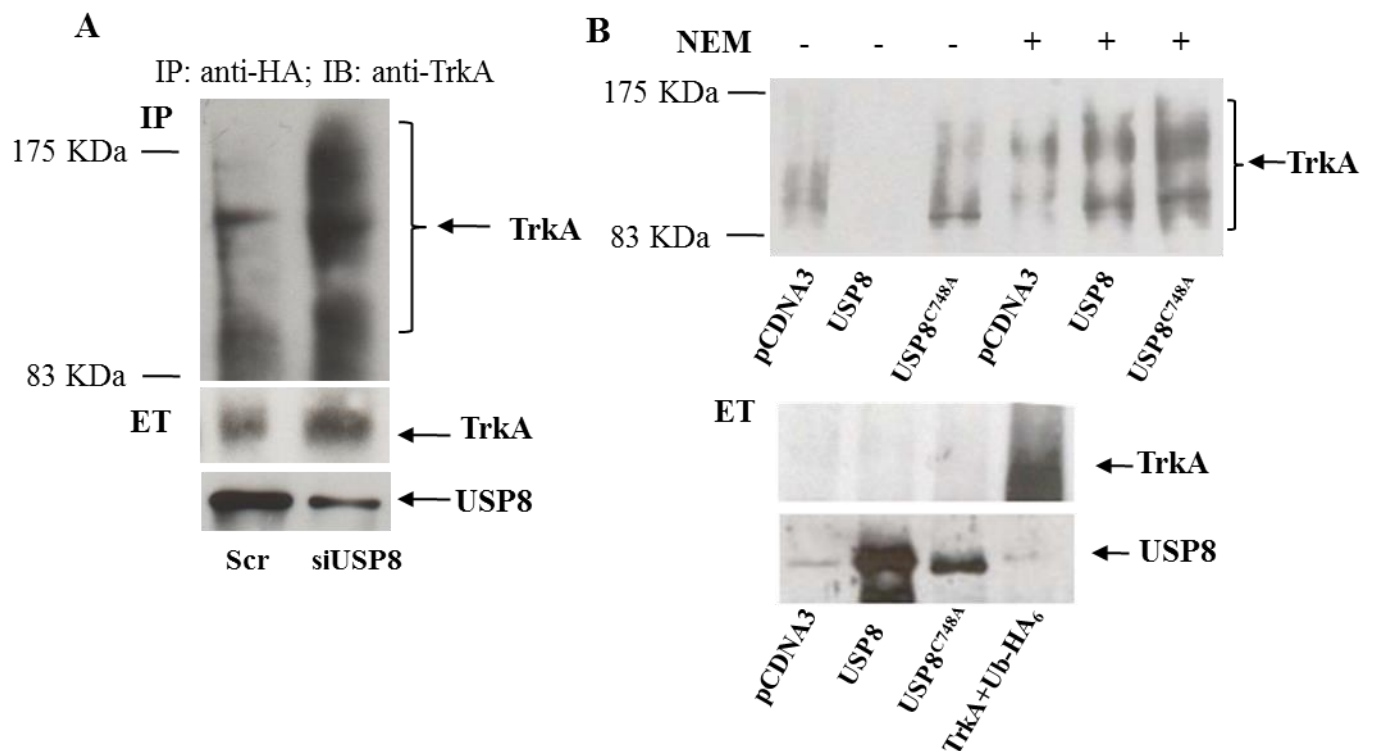
← USP8

ET

← USP8

← TrkA

NS   S10'   S30'   S60'
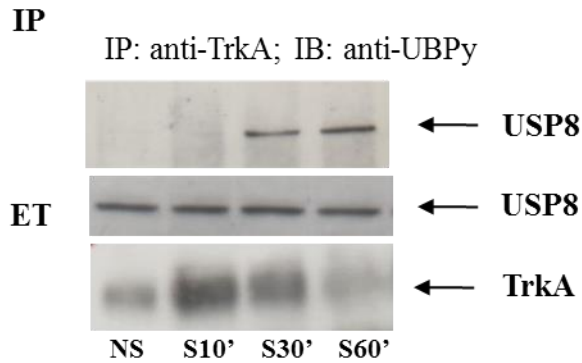
Figure 2

Figure 2. *TrkA receptor and USP8 interacts in an NGF-dependent manner in PC12 cells.* PC12 cells were starved overnight and then left unstimulated (NS) or stimulated (S) with 50 ng/ml NGF for different times (15, 30, 60 min). Subsequently, immunoprecipitation with anti- TrkA (C-14) antibodies was performed followed by immunoblotting with anti-USP8 antibody. *ET:* total extracts; *IP*: Immunoprecipitates; *IB*: immunoblottes.

TrkA receptor is a substrate for USP8 de-ubiquitinating activity "in vivo" and "in vitro".

To determine whether TrkA is a substrate for USP8 *in vivo,* PC12 cells were transiently transfected with a construct expressing ubiquitin tagged with HA epitope (Ubi-HA$_6$) and control stealth siRNA duplex (Scr) or USP8-specific Stealth siRNA duplex (siUSP8). After transfection cells were starved over-night and stimulated with 50 ng/ml of NGF for 30 minutes; an equal amount of total protein extract for each sample was immunoprecipitated with anti-HA antibodies. Control cells (scr) present a basal level of ubiquitinated TrkA receptor forms; ubiquitination levels of TrkA receptor in cells transfected with USP8-specific Stealth siRNA duplex (siUSP8) are enhanced (Fig.3A). These data indicate that the isopeptidase activity of USP8 is required for the de-ubiquitination of TrkA receptor *in vivo*. Afterwards, to confirm the de-ubiquitinating activity of USP8 on TrkA receptor, we performed an *in vitro* de-ubiquitination assay as described in "Materials and Methods". In particular it has been used an alkylating agent, N-Ethylmaleimide (NEM), that inhibits specifically the catalytic activity of de-ubiquitinating enzymes by covalent alkylation of their active cysteine (Otto and Schirmeister 1997). Human USP8 and the inactive USP8/UBPy^{C748A} were immunopurified from transfected HEK293 cells and tested for their ability to remove ubiquitin from immunoprecipitated TrkA receptors. As it could be seen in Figure 3B TrkA is expressed at high level in HEK 293 cells transfected with pRC/CMV-TrkA and Ubiquitin HA$_6$ (*Et TrkA*); USP8, that is present at endogenous level in HEK293 cells, is expressed at higher level in cells transfected with pCDNA3-hUBPy and pME18S-FLAG-mUBPy^{C748A} (a catalytically inactive, dominant negative version (Alwan and Van Leeuwen 2007).When assayed for its enzymatic activity USP8 was shown to be active, in fact,

immunoprecipitated USP8 greatly reduced the amount of ubiquitinated TrkA receptors; conversely when NEM was added to the enzymatic assay mixture the immunoprecipitated USP8 failed to deubiquitinate TrkA.
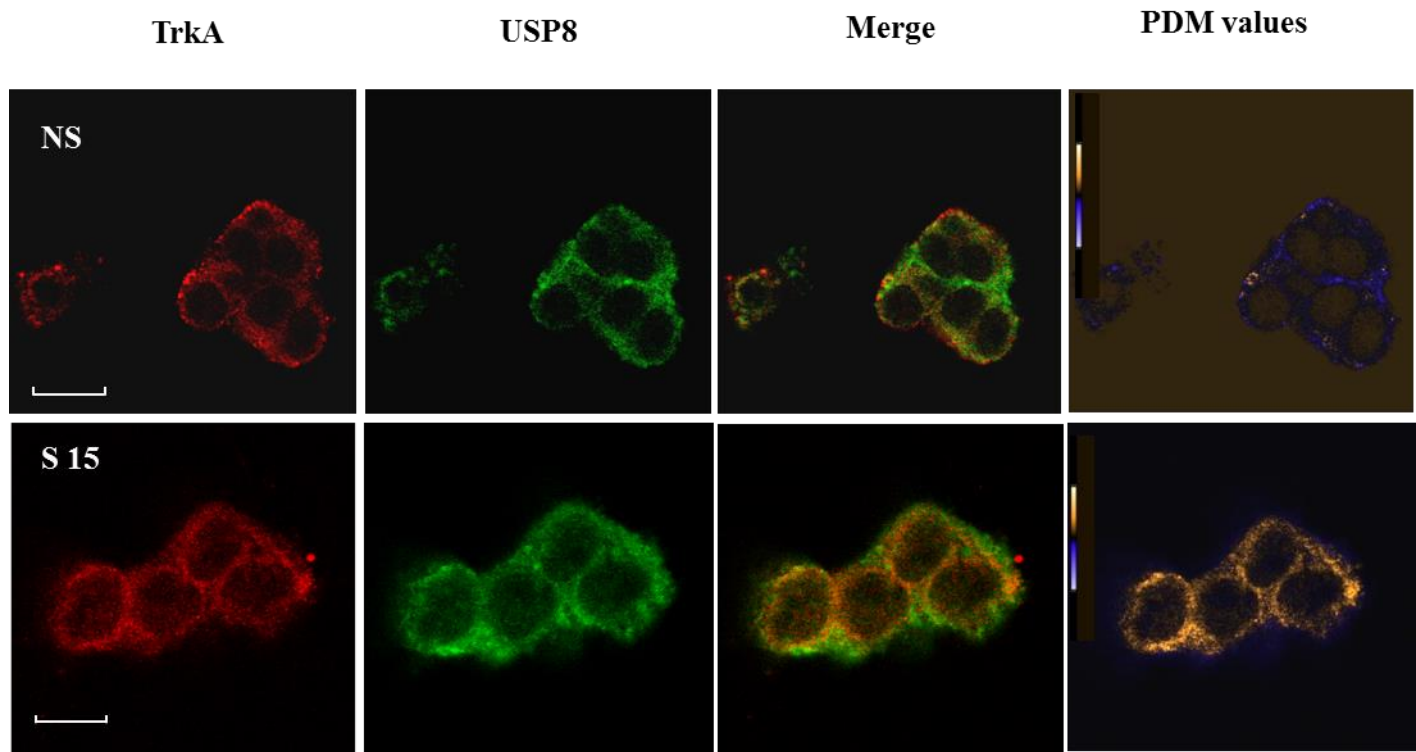

Figure 3

Figure 3. *USP8 deubiquitinates TrkA receptor "in vivo" and "in vitro".* **Panel A:** PC12 cells were transiently transfected or with Control Stealth siRNA duplex (*Scr*) and HA_6-ubiquitin or with a siRNA specific for USP8 (*siUSP8*) and HA_6-ubiquitin. Serum starved cells were stimulated with NGF for 30 minutes and lysed; an equal amount of total proteins (815μg) for each samples was incubated with 2.5 μg of anti-HA antibodies pre-coupled with protein A-Sepharose beads. Samples were loaded onto 8% polyacrylamide gels, blotted and analysed with anti-TrkA (C-14) antibodies. *ET:* total extracts; *IP*: Immunoprecipitates. **Panel B:** HEK 293 cells were transiently transfected with pCDNA3, pCDNA3-hUBPy or pME18S-FLAG-mUBPy^{C748A} mutant. Cells were harvested 24 h post-transfection. USP8 was immunoprecipitated with anti-USP8 antibody coupled to Protein-A Sepharose beads overnight at 4°C. Immunocomplexes were split into two fractions: one was treated with 20 mM N-Ethylmaleimide (NEM) and the other was treated with ethanol (vehicle). Four 100 mm dishes were transfected with pRC/CMV-TrkA and Ubiquitin-HA_6 vectors; cells were serum-starved overnight. To induce ubiquitination of TrkA receptors, cells were stimulated with 50 ng/ml NGF for 30 min. Cells were then lysed on ice, and immunoprecipitation of ubiquitinated TrkA was performed with anti-TrkA antibodies coupled to protein A beads at 4 °C for 1.5 h. Beads were then washed extensively in lysis buffer and split into two fractions. TrkA beads were then reconstituted in Dub buffer (10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA). TrkA immunoprecipitates were then combined with USP8 immunoprecipitates and the de-ubiquitination reaction

was performed at 37 °C for 90 min. Immunocomplexes and total extracts (ET) were then analysed by Western blot.

USP8 and TrkA partially co-localize in early endosomes in PC12 cells.

To investigate the extent of TrkA and USP8 co-localization, we performed immunofluorescence analysis carried out with a confocal microscope on PC12 cells. As shown in figure 4 in un-stimulated cells endogenous TrkA localized mainly at the cell periphery. After ligand binding, TrkA receptor is quickly removed from the cell surface. In the same cells endogenous USP8 is distributed in the cytosolic compartment both in un-stimulated and in stimulated cells, and after NGF addition seems to be more concentrated in spots. Co-localization analysis revealed that USP8 and TrkA marginally co-localize in un-stimulated cells, while co-localization was more evident after stimulation with NGF (Fig. 4).

Figure 4

Figure 4. *TrkA and USP8 colocalize after NGF stimulus in PC12 cells*

PC12 cells were plated on poly-lysine pretreated **coverslips**. Overnight starved cells were let unstimulated or stimulated for 15 minutes with 50 ng/ml NGF. Cells were fixed, permeabilized and stained with primary antibodies; in particular were used anti-mouse monoclonal (6B2) anti-TrkA antibodies (1:500, Abcam) and rabbit anti –UBPy (1:1000, Gnesutta et al. 2001). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (1:1000, Life technologies). The PDM image (PDM

(Product of the Differences from the Mean, i.e. for each pixel: PDM = (red intensity- mean red intensity)×(green intensity – mean green intensity) was calculated using the plugin Image Correlation Analysis of MBF ImageJ software. *Scale bar = 10 μm.*

To verify if TrkA and USP8 co-localize also in endosomes first we performed a confocal analyses with a specific marker for early endosomes (EEA1) (Stenmark et al.1996); USP8 partially co-localize with EEA1 in un-stimulated cells; however in NGF treated cells the co-localization between EEA1 and USP8 appear more evident suggesting that NGF stimulus promotes an increase of USP8 localization on early endosomes (Fig.5A). Also TrkA co-localizes with EEA1 and the co-localization extent is increased in NGF treated cells (Fig 5B). So, NGF stimulus promotes the internalization of the TrkA receptor by addressing the activated and ubiquitinated receptor to the early endosomes where also USP8 is recruited. The immunofluorescence data were confirmed using a biochemical approach. PC12 cells were starved overnight; the day after, cells were let un-stimulated or treated with NGF for 15 minutes and then lysed. Equal amounts of total protein extracts for each sample were incubated with anti-EEA1 antibodies. Immunoprecipitated samples were separated by SDS-PAGE and blotted on nitrocellulose filter; the blot was first immunodecorated with anti-TrkA antibodies and then stripped and stained with anti-USP8 antibodies. As it is shown in Fig. 5C while the USP8 immunoreactive band is present both in un-stimulated and in stimulated cells TrkA signal was detected only when cells are stimulated. TrkA receptor appears to be recruited to USP8-containing early endosomes upon stimulation. Hence the co-localization and biochemical experiments here described, together with the indication that USP8 deubiquitinates TrkA, suggest that USP8 could influence the trafficking of activated TrkA at early endosome level.

A

| | NS | NS | NS |
| | EEA1 | USP8 | Merge |

B

| NS | NS | NS |
| EEA1 | TrkA | PDM values |

C

ET

EEA1→
USP8→
TrkA →

IP: anti-EEA1

Anti-TrkA →
Anti-USP8→
Anti-EEA1→

NS   S15'
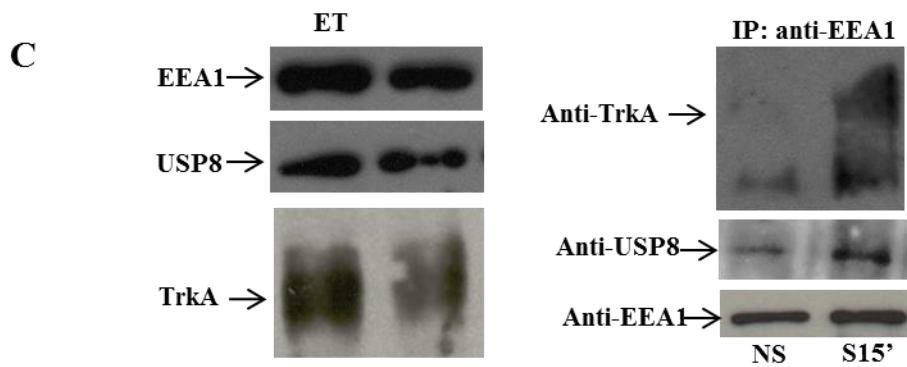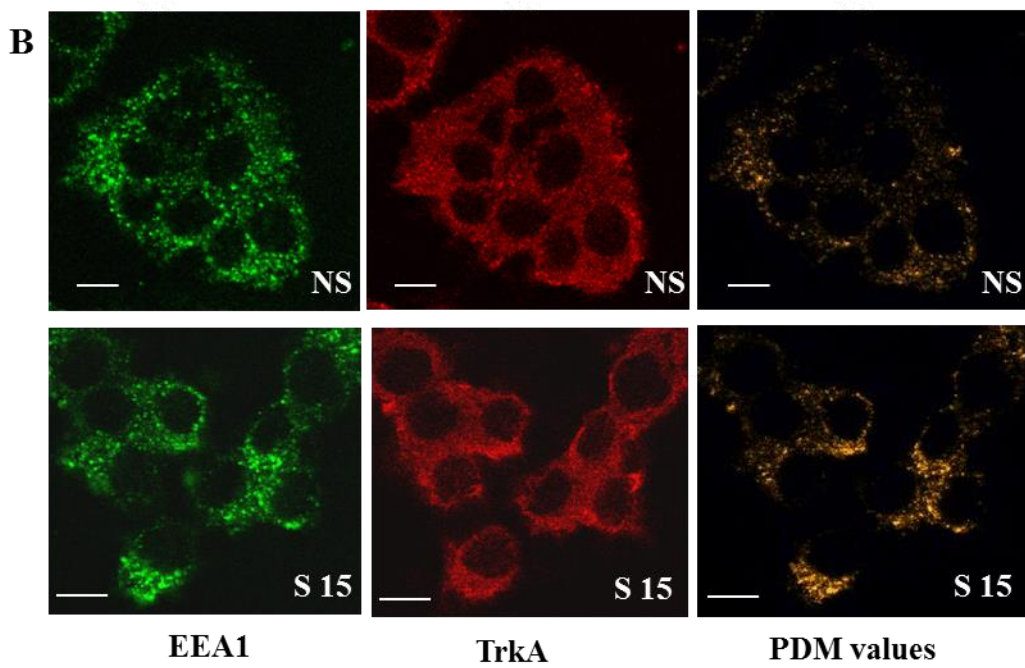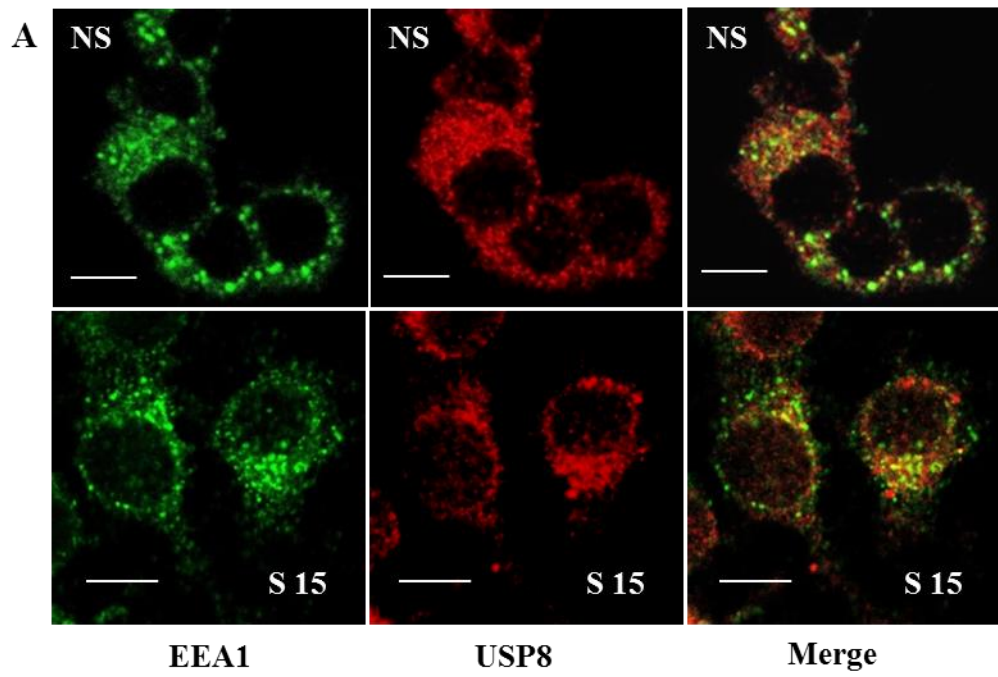
Figure 5

Figure 5. *USP8 co-localizes with TrkA receptor in endosomes.* **Panel A:** PC12 cells were plated on poly-lysine pretreated coverslips. Overnight starved cells were let un-stimulated or stimulated for 15 minutes with 50 ng/ml NGF. Cells were fixed, permeabilized and stained with anti-EEA 1 antibodies and anti-USP8 antibodies. *Scale bar = 10 μm.* **Panel B:** PC12 cells were plated on poly-lysine pre-treated **coverslips**. Overnight starved cells were let un-stimulated or stimulated for 15 minutes with 50 ng/ml NGF. Cells were fixed, permeabilized and stained with primary antibodies. In particular for EEA1 and USP8 co-localization were used mouse monoclonal anti-EEA1 (1:500, BD Transduction Laboratories) and rabbit anti-UBPy (1:1000, Gnesutta et al. 2001) primary antibodies; secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (1:1000, Gnesutta et al. 2001). EEA1 and TrkA co-localization was performed with mouse monoclonal anti-EEA1 (1:500, BD Transduction Laboratories) and rabbit polyclonal anti-TrkA (1:400, Novus Biologicals, Inc) primary antibodies and Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (1:1000, Life technologies). The PDM image was calculated using the plugin Image Correlation Analysis of MBF ImageJ software. *Scale bar = 10 μm.* **Panel C:** Endosome were isolated from PC12 cells as described in materials and methods and analysed for USP8, EEA1 and TrkA by Western blotting. NS: extracts from un-stimulated PC12 cells; S15: extracts from PC12 cells stimulated with 50ng/ml of NGF for 15 minutes; *ET:* total extracts; *IP*: Immunoprecipitates.
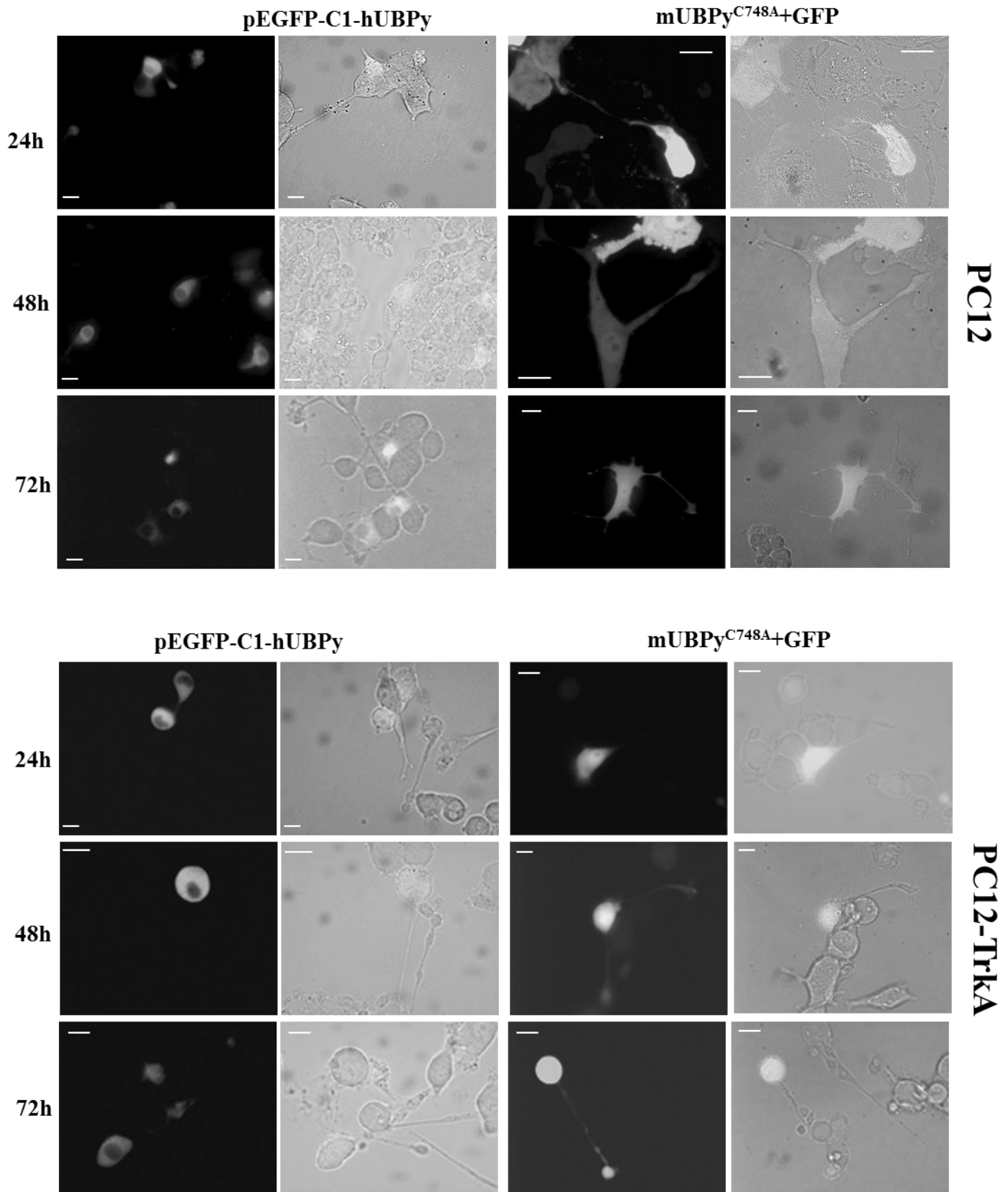
USP8 overexpression blocks NGF-induced PC12 and PC12-TrkA cells differentiation.

To evaluate a possible role of USP8 in the cellular pathways activated by NGF we analyzed the effects of its overexpression on the differentiation of PC12 cells. Normally NGF signaling through the receptor TrkA causes differentiation in this cell line (Chao and Hempsted 1995). As USP8 deubiquitinate TrkA receptor it could influence the trafficking and availability of the receptor and could influence the PC12 differentiation induced by NGF. PC12 and PC12- TrkA cells (Hempsted et al. 1992) were transfected with USP8-GFP construct (hUBPy fused to GFP) or co-transfected with USP8/UBPy^{C748A} and GFP; cells were then starved and stimulated with NGF (50ng/ml). Analyzing cells at different times (Fig. 6 A, B and Supplementary Fig. S2) we found that the over-expression of USP8 inhibits the NGF induced differentiation either in PC12 and in PC12-TrkA cells.
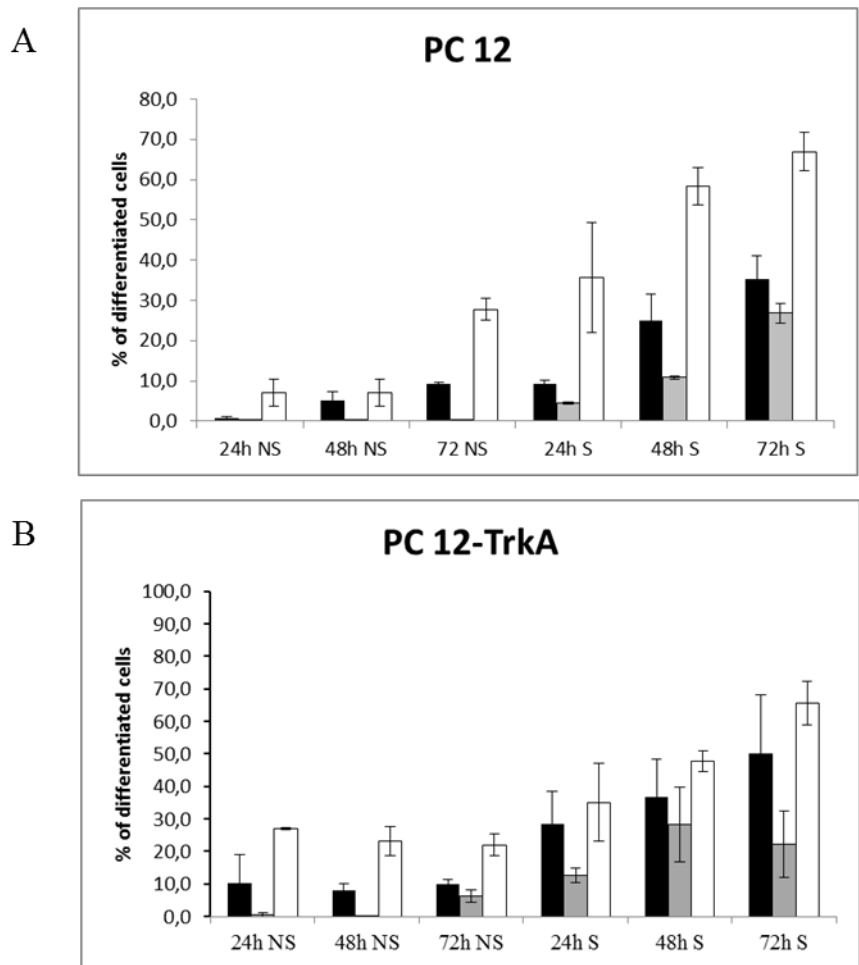
Figure 6

Figure 6. Neurite outgrowth induced by USP8 or USP8^{C748A} in PC12 cells. Graphic representation of the percentage of cells with neurites. PC12 (black bar) or PC12 transfected with pEGFP-C1-hUBPy (gray bar) or cotranfected with pME18S-FLAG-mUBPy^{C748A} and pEGFP-C1 (white bar) let unstimulated or stimulated with 50 ng/ml of NGF for 24, 48 and 72 hrs. About 200 cells were counted for each experiment; cells were considered positive for neurite outgrowth if one or more neuritis exceeded two cell body diameters in length. Bars represent Standard Error Of the mean.

Figure S2. *USP8 overexpression blocks NGF-induced PC12 and PC12-TrkA cells differentiation*

PC12 and PC12-TrkA cells were transfected with pEGFP-C1-hUBPy or pME18S-FLAG-mUBPy^{C748A} and pEGFP-C1. 24h after transfection cells were maintained for 24, 48 or 72 h in basal medium with 0.5% Horse Serum with or without NGF (50 ng/ml). After different period of stimulation cells were observed with a Nikon Eclipse C600 fluorescence microscope and photographed with a Leica DG350F CCD camera. *Scale bar = 10 μm.*

On the other hand cells expressing the dominant negative, catalytically inactive USP8^{C748A} differentiate at a higher degree in comparison with the control cells. These data suggest that USP8 over-expression might inhibits cells differentiation likely by limiting in some way the signaling generated by TrkA and that this process required the deubiquitinating activity. To verify if USP8 regulates the TrkA receptor stability it has been performed a turn-over experiment; PC12 cells were transiently transfected with control Stealth siRNA duplex (Nonspecific) (scr) or USP8-specific Stealth siRNA duplex (siUSP8). For each of the two type of transfection were prepared 6 plates. Cells were then starved and stimulated with 50 ng/ml of NGF for different times as indicated in Fig.7A. As it is shown in figure 7A and B, in control cells expressing only control Stealth siRNA duplex (scr) TrkA receptor protein level diminishes after 15 minutes from NGF stimulus while in cells depleted for USP8 it decreases after 60 minutes. So, the knockdown of USP8 increases TrkA half-life.
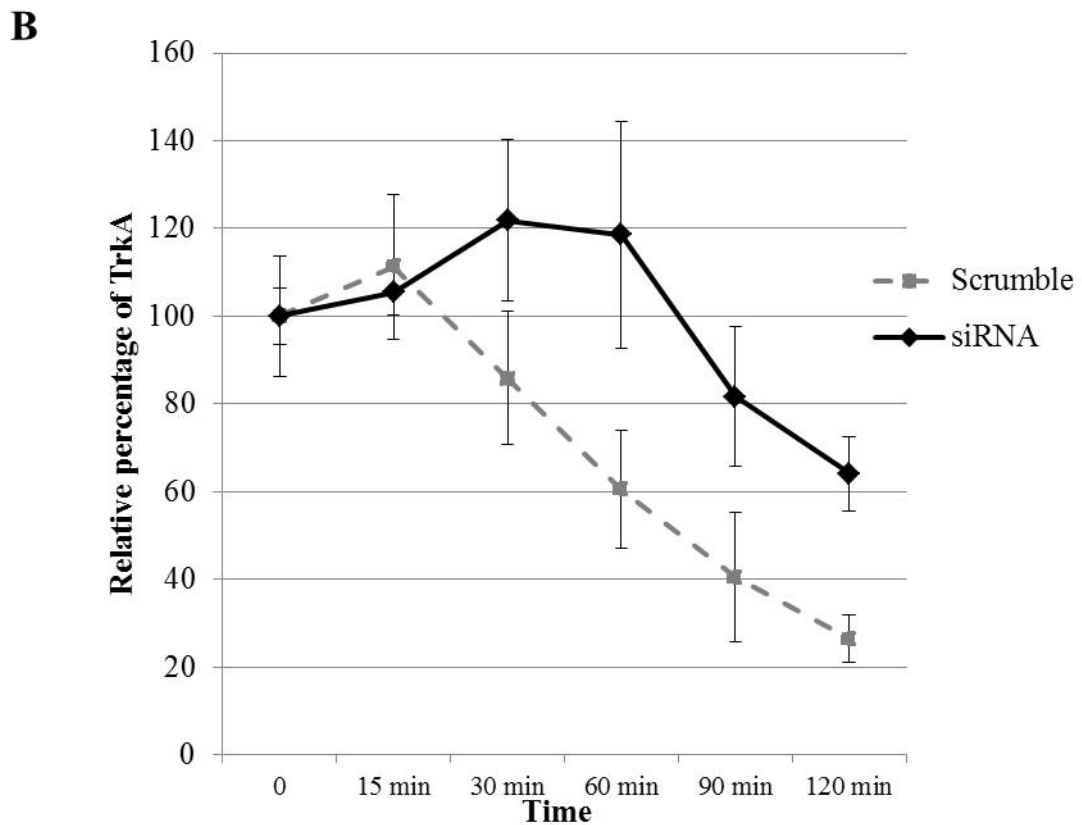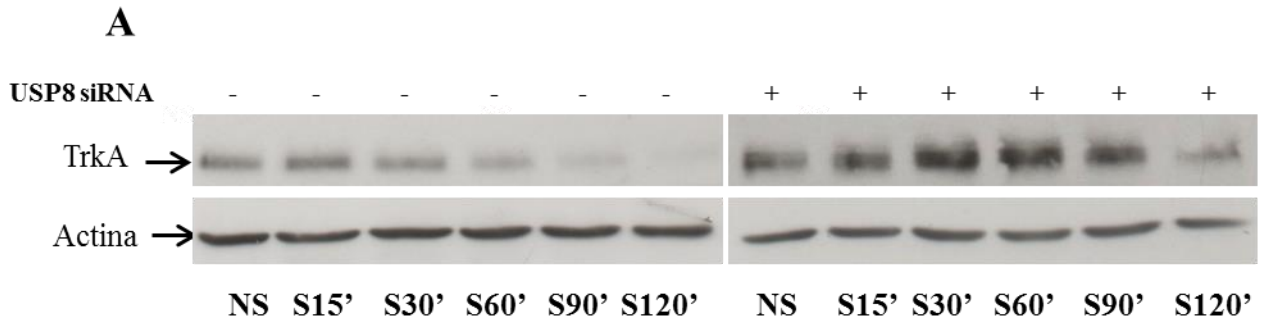
Figure 7. Effects of UBPy/USP8 depletion on TrkA stability. **Panel A:** PC12 cells were transiently transfected as described in Materials and methods with Control Stealth siRNA duplex (Nonspecific) (scr) or UBPY/USP8-specific Stealth siRNA duplex (siUSP8). After starvation cells were stimulated for the time indicated in figure and then harvested and lysed in Lysis buffer. Total protein extracts were quantified and 30 μg of total protein extracts (ET) were then analysed by Western blot. Filters were probed with anti-TrkA (C14) (Santa Cruz Biotechnology) antibodies and then revealed with anti-mouse HRP secondary antibodies and ECL system. Anti-actin antibodies were used to normalize samples. *NS*: un-stimulated; *S*: stimulated.

Panel B: Relative percentage of TrkA receptor in control and in UBPy/USP8 depleted cells after NGF stimulus. The intensity of the TrkA immunoreactive bands were first quantified with Image J software and then normalized for the corresponding expression levels of actin. Data presented represent the relative amount of TrkA in respect of its un-stimulated sample. *Continuous black line:* PC12 cells transfected with UBPY/USP8-specific Stealth siRNA duplex (siUSP8); *broken grey line:* PC12 cells transfected with Control Stealth siRNA duplex (Nonspecific) (scr). Means ± SD are shown.

Discussion

There are many gaps regarding the trafficking of the TrkA receptor after addition of nerve growth factor; surely poly-ubiquitination and multiple mono-ubiquitination play a definite role in receptor sorting. TrkA is ubiquitinated at least by three different enzymes: the RING type ubiquitin ligases c-Cbl and TRAF6 and by the HECT type ubiquitin ligase Nedd4-2 (Arevalo et al. 2006; Takahashi 2011; Geetha et al. 2005). Both c-Cbl and Nedd4-2 promotes TrkA degradation. c-Cbl binds to autophosphorylated TrkA and ubiquitinate it; the receptor and the ubiquin ligase are found in a complex during prolonged NGF stimulation and are both degraded in lysosome (Takahashi et al. 2011). The ubiquitin ligase Nedd4-2 interacts with the C-terminal tail of the receptor and monoubiquitinate it on multiple sites; multi-monoubiquitination in absence of any stimulus is able to target TrkA receptors for lysosomal degradation (Georgieva et al. 2011). Furthermore TrkA is a substrate of the E3 ligase TRAF6; TRAF6 interacts with p75[NTR] (Khursigara et al. 1999) and interacts with the TrkA receptor through p62 (Wooten et al. 2001) catalyzing the formation of non-canonical K63-linked polyubiquitin chains on it. Both proteasomes and lysosomes are involved in the degradation of the internalized TrkA receptor, however, TrkA is deubiquitinated prior to its degradation in lysosomes (Geetha and Wooten 2008). In these work we have demonstrated that TrkA is deubiquitinated by the isopeptidase USP8, an enzyme that could process Lys-48- and Lys-63-linked polyubiquitin; the ubiquitination level of NGF-activated TrkA is reduced when cells are depleted of USP8. Moreover USP8 interacts with TrkA it in an NGF-dependent manner. Geetha (2008) (Geetha and Wooten 2008) and colleagues has reported that TrkA receptor, post-internalization, traffics from endosomes to lysosomes though a proteasome- intermediate step, which is coincident with the time frame where receptor deubiquitination occurred (Geetha and Wooten 2008). USP8 is normally localized at endosomal level and has been shown that knockdown of USP8 by RNA interference has multiple cellular effects that include the accumulation of ubiquitinated proteins on endosomes and an increase in number and size of multivesicular bodies (Row et al. 2006). Our results demonstrate that USP8 form a complex with TrkA receptor in a ligand dependent manner; moreover confocal analysis has confirmed that, after NGF stimulation, TrkA and USP8 partially co-localize. Using the early endosome marker EEA1 we have demonstrated that this co-localization take-place also in endosomes. This data was confirmed by biochemical assay in which, through endosomes immunoprecipitation, it has been demonstrated that TrkA and USP8 co-precipitate with endosome after NGF addition in PC12 cells. Results show that the overexpression of USP8 inhibited neuronal differentiation of

PC12 cells, while the overexpression of the catalytic inactive USP8/UBPy^{C748A} mutant had an opposite effect. Considering these results it could be supposed that, when USP8 is overexpressed, it interacts with TrkA, after NGF stimulus, at early endosomes and promotes TrkA deubiquitination, and prepare it for degradation. So the receptor signal is switched off faster and PC12 cells were not able to differentiate. This was indeed confirmed by the evidence that in cells expressing specific USP8 siRNA there is a decrement of TrkA level after 60 minutes from the stimulus and the receptor is still expressed at 120 minutes while in control cells, expressing a scramble siRNA TrkA amount diminish after 15 minutes. So, considering the data shown in this paper we can conclude that USP8 interacts with TrkA at endogenous level in a NGF-dependent manner, that this enzyme deubiquitinate the receptor and partially co-localize with in early endosomes after NGF treatment. Besides USP8, when is overexpressed, inhibits cell differentiation promoting TrkA degradation. Recently it has been demonstrated that CYLD, originally identified as cylindromatosis tumor suppressor gene, encodes a deubiquitinase specific for lysine63-linked polyubiquitin chains (Massoumi 2010) that interacts in an NGF dependent manner with TrkA and that deubiquitinate it in vivo. A study on p62, a CYLD-binding protein, implied that inhibition of CYLD leads to accumulation of proteins with lysine63-linked polyubiquitination in brains of p62$^{-/-}$ mice (Wooten et al. 2008). Recent findings have revealed that Parkin aggresomes contain Lys^{63}-ubiquitinated proteins (Olzmann et al. 2007) and that alteration in neurotrophins or Trk receptor levels have been documented in several neurodegenerative disorders (Dawbarn and Allen 2003; Beattie et al. 1996). In this point of view, considering that USP8 could process Lys-48- and Lys-63-linked polyubiquitin, it could be considered an important regulator that may exert important role in TrkA trafficking and in blocking the formation of ubiquitinated aggresomes.

Conclusions

These studies shows that the deubiquitinating enzyme USP8 interacts in a NGF-dependent manner with TrkA receptor, in fact USP8 and TrkA partially co-localize in early endosome after Nerve growth factor stimulus. Furthermore, as it happens for other Tyrosine kinase receptors (EGFR, Met…) USP8 promotes TrkA degradation inhibiting PC12 NGF-induced differentiation.

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