RESEARCH ARTICLE

Proteomic analysis of endosomes from genetically modified p14/MP1 mouse embryonic fibroblasts

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The p14/MP1 scaffold complex binds MEK1 and ERK1/2 on late endosomes, thus regulating the strength, duration and intracellular location of MAPK signaling. By organelle proteomics we have compared the protein composition of endosomes purified from genetically modified $p14^{-/-}$, $p14^{+/-}$ and $p14^{rev}$ mouse embryonic fibroblasts. The latter ones were reconstituted retrovirally from $p14^{-/-}$ mouse embryonic fibroblasts by reexpression of pEGFP-p14 at equimolar ratios with its physiological binding partner MP1, as shown here by absolute quantification of MP1 and p14 proteins on endosomes by quantitative MS using the Equimolarity through Equalizer Peptide strategy. A combination of subcellular fractionation, 2-D DIGE and MALDI-TOF/TOF MS revealed 31 proteins differentially regulated in $p14^{-/-}$ organelles, which were rescued by reexpression of pEGFP-p14 in $p14^{-/-}$ endosomes. Regulated proteins are known to be involved in actin remodeling, endosomal signal transduction and trafficking. Identified proteins and their *in silico* interaction networks suggested that endosomal signaling might regulate such major cellular functions such as proliferation, differentiation, migration and survival.

Received: April 19, 2010
Revised: May 28, 2010
Accepted: June 1, 2010

Keywords:
Absolute quantification / Cell biology / Organelle proteomics / Scaffold complex / Signaling endosomes / Subcellular fractionation

1 Introduction

Protein signaling complexes, which are organized by scaffold and adaptor proteins, in specific subcellular locations, coordinate cellular functions such as proliferation, differentiation, apoptosis and migration. We have shown previously that the mitogen-activated protein kinase scaffold protein 1 (MP1) is localized to late endosomes by the adaptor protein p14 (MP1-interacting protein) [1]. The p14 protein forms a stable heterodimeric complex with the MP1 [2], which binds MEK1, ERK1 and ERK2, recruits the entire complex to the late endosome and thus facilitates signal transduction through the MAPK (mitogen-activated protein kinase) cascade on this specific subcellular location. P14/MP1complexes regulate the EGFR/MAP kinase pathway by providing critical spatial and temporal specificity [3]. Using conditional gene disruption of p14 in mice we demonstrated that the p14/MP1 complex regulates the late endosomal traffic and cellular proliferation [4]. Recently, we described a novel human immunodeficiency syndrome caused by a

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Abbreviations: CE, crude endosomes; **HB,** homogenization buffer; **LBPA,** lysobisphosphatidic acid; **MAPK,** mitogen-activated protein kinase; **MEFs,** mouse embryonic fibroblasts; **MP1,** mitogen-activated protein kinase scaffold protein 1; **PNS,** post-nuclear supernatant

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genetic deficiency of the p14 and, therefore, aberrant lysosomal signaling function [5].

Previously, we have identified organelle-specific targets of EGF receptor signaling on endosomes by functional organelle proteomics, which suggested signaling specification through intracellular organelles [6]. Here, we describe the organelle proteome, differentially regulated in endosomes after genetic ablation/reconstitution of the late endosomal p14 protein. In these cells p14 is missing and its binding partner MP1 significantly down regulated, as previously described [4, 5, 7]. This proteomic approach identified more than 30 proteins, with a p14-dependent endosomal association or differential protein processing/modification. Regulation of all of them was confirmed by reexpressing pEGFP-p14 in $p14^{-/-}$ cells at equimolar ratios with its physiological binding partner MP1 on endosomal membrane. Regulated proteins are involved in endosomal signal transduction, endosomal trafficking and actin remodeling.

2 Materials and methods

2.1 Cell lines

$P14^{+/-}$ and $p14^{-/-}$ mouse embryonic fibroblasts (MEFs) were generated as described previously [7]. $P14^{rev}$ cell line was established by reexpressing pEGFP-p14 in $p14^{-/-}$ MEFs. The pEGFPp14 fusion protein was constructed by inserting p14 in a pEGFP-C1 Vector (Clontech) and subcloned in a pLib-MCS2-iresPURO vector [8]. Retroviruses were generated in phoenix cells and used to infect 70% confluent $p14^{-/-}$ MEFs. Single cell clones were derived from the infected MEFs by selection with 15 μg/mL puromycin. After selection, cells were kept at 5 μg/mL puromycin. Clones of pEGFPp14 MEFs were tested for expression of the retrovirally transduced protein and a clone, where expression levels matched the levels of the endogenous proteins in control cells was selected.

MEFs were cultured in high glucose DMEM, supplemented with 10% FBS (GIBCO Life Technology), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were cultivated on 150 mm tissue culture plastic dishes (Sarstedt) at 37°C in 5% CO_2 and 90% humidity. For experiments the cell lines were cultured for 3 days till they reached a confluency of 80–90%.

2.2 Subcellular fractionation

Crude endosomal fractions (CE) were isolated according to our previously described procedure [9] with the following modifications. Cells were washed twice with ice-cold PBS, scraped, and pelleted at 200 × *g* for 5 min (Fig. 1). The pellet was resuspended in homogenization buffer (HB: 250 mM sucrose, 3 mM imidazole buffer pH 7.4, 5 μg/mL aprotinin, 0.5 μg/mL pepstatin, 5 μg/mL leupeptin, 50 mM NaF, 1 mM Na_3OV_4). Cells were transferred to a 15 mL tube and centrifuged at 1300 × *g* for 10 min. The cell pellet was resuspended in HB buffer (three times the volume of the cellular pellet) supplemented with 0.03 mM cycloheximide (Sigma), 1 mM EDTA, 10 μg/mL aprotinin, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 50 mM NaF, 1 mM Na_3OV_4 and homogenized by seven passes through a G25 needle, attached to a 1 mL syringe (both from Braun Melsungen AG). Post-nuclear supernatant (PNS) was obtained by centrifugation at 2000 × *g* for 10 min. For the isolation of crude endosomes the sucrose concentration of the PNS was adjusted to 40.6% and loaded on the bottom of an SW41 ultracentrifuge tube (Beckman, Vienna, Austria), overlaid with 35% sucrose in 3 mM imidazole buffer pH 7.4, HB (8.6% sucrose) was added up to the top of the tube, and centrifuged at 210 000 × *g* for 3 h at 4°C. CE were collected from the interphase between 35% sucrose and HB buffer, diluted 1:1 in 3 mM imidazole, pH 7.4, 1 mM EDTA to reduce the sucrose concentration and centrifuged for 1 h at 100 000 × *g*. The organelle pellets were resuspended in the same buffer or in the 2-D DIGE sample buffer.

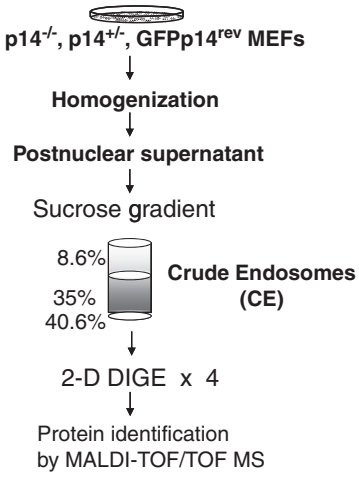

Figure 1. Flowchart of organelle proteomics approach used in this study

2.3 2-D DIGE

Endosomes isolated by subcellular fractionation were solubilized in DIGE sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, buffered to pH 8.5. Thirty micrograms of protein (according to Coomassie Plus Protein Assay, Pierce, Rockford, IL) in 30 μL of sample buffer was labeled with CyDye DIGE Fluor minimal dyes (GE Healthcare, Vienna, Austria) according to the manufacturer's instruction.

2-D DIGE was performed as described previously [6]. For IEF the samples were loaded by rehydration on immobilized 18 cm pH 3–11 NL gradient strips (GE Healthcare). For the

second dimension, samples were separated on 9–16% gradient polyacrylamide gels with the PROTEAN System (Bio-Rad Laboratories, Vienna, Austria). After electrophoresis, gels were scanned using a Typhoon 9410 Imager at a resolution of 100 dpi (GE Healthcare). The images of the gels were analyzed with the DeCyder^{TM} 2D 6.5 software (GE Healthcare). Two 2-D DIGE experiments were performed to analyze four independently purified endosomal samples ($4\times$ $p14^{+/-}$ *versus* $4\times$ $p14^{-/-}$ and $4\times$ $p14^{rev}$ *versus* $4\times$ $p14^{-/-}$) and statistically analyzed using DeCyder Biological Variation Analysis software (GE Healthcare). The ratiometric normalization using the internal standard is provided in software package as a default setting and a log transformation is used on the standard abundance to stabilize the variance. To decrease the number of missing values manual landmarking, correction of mis-matches and removing the artifact spots by manual editing were performed. Student's *T*-test and ANOVA were used as statistical analysis tools. We did not discard protein spots containing missing values, hence all selected by software regulated spots were confirmed manually.

2.4 Mass spectrometry

Differentially expressed protein spots were excised from gels with Ettan Spot Picker (GE Healthcare) and in-gel digested with modified trypsin (sequence grade, Promega, Madison, WI, USA) as described in [10]. The in-gel digests were concentrated and desalted using microZipTipC18 (Millipore, Billerica, MA, USA) by elution of peptides with the ACN solution containing the CHCA (Fluka, Buchs, Switzerland) as a matrix directly onto the target. Mass spectra were acquired using a MALDI-TOF/TOF Ultraflex instrument (Bruker Daltonics, Bremen, Germany). The N_2 laser (337 nm wave length) was used at a 50 Hz frequency. Calibration was done externally using Peptide Calibration Standard II (Bruker Daltonics). Flex Control 2.4 was utilized for data acquisition, and further data processing was carried out using Flex analysis 2.4 and BioTools 2.2 software packages provided by the manufacturer. PMF were interpreted with the on-line search engine MASCOT (http://www.matrixscience.com/) against Swiss-Prot database (Version 57.6, *Mus musculus*, 16 129 sequences). A peptide mass tolerance was set to 0.1 Da and one missed cleavage was allowed.

2.5 Absolute quantification of MP1-p14 in endosomes

Twenty microliter purified CE at a concentration of 1 mg/mL were lysed with a final concentration of 1% Triton-X 100. The protein was precipitated by addition of four volumes of ice-cold acetone and subsequent incubation at $-20^{\circ}C$ overnight. After centrifugation at $5000\times g$ at $4^{\circ}C$ the protein pellet was washed three times with 80% acetone. The protein pellet was then air-dried for 5 min at room temperature and re-dissolved with 40 μL digest buffer containing 500 mM TEAB and 0.1% RapiGest. Alternatively, CE were lyzed with a final concentration of 1% RapiGest SF (Waters, Milford, MA) by the addition of 20 μL of a solution containing 1 M TEAB and 2% RapiGest SF to 20 μL of CE. Both preparations were digested for 16 h at $43^{\circ}C$ with 1 μg Trypsin Gold (Promega). About 10 μL of the digested CE was labeled with half of a unit mTRAQ light reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. After 1 h samples were diluted 5-fold with 1% TFA. Before analysis, the RapiGest lyzed CE was incubated for 30 min at $37^{\circ}C$ and centrifuged at $20\,000\times g$ for 15 min to hydrolyze and remove the surfactant. Prior to LC-MS analysis 0.43 μg digested and labeled CE were spiked with 14.8 fmol of the internal standard peptides (equilibrated with the equalizer peptide ASGVNTIAR) and methionines were oxidized with a final concentration of 0.5% hydrogen peroxide. Preparation of the internal standard peptides and analysis on the 4000 QTRAP and the Orbitrap instrument was performed as described in [11].

2.6 Antibodies and Western blotting

SDS-PAGE was conducted on 20 μg endosomal and PNS proteins using 14% polyacrylamide gels. Western blotting was performed as published previously [12]. Polyclonal anti-p14 and -MP1 antibodies were described previously [3]. Anti-p18 (C11-ORF59) were purchased from Atlas Antibodies, anti-ATP6V0d1 was from Abnova, anti-GFP was from Abcam, anti-actin antibody was obtained from Chemicon, anti-phospho-ERK1/2 was obtained from Cell Signaling, anti-HSP90 was from StressMarq, anti-CDC42 was from Transduction Laboratories, anti-Rab8 and anti-cathepsin B were from Santa Cruz. Anti-Rab5 and anti-Rab7 antibodies were kind gifts from Dr. Angela Wandinger-Ness (University of New Mexico, Albuquerque, USA).

2.7 Immunofluorescence microscopy

pEGFP$p14^{rev}$ MEFs were fixed with 4% formaldehyde (freshly depolymerized from paraformaldehyde) and labeled for late endosomes/lysosomes with the primary anti-LBPA (lysobisphosphatidic acid) antibody (a kind gift from J. Gruenberg, University of Geneva, Geneva, Switzerland). As secondary antibody Alexa-647 anti-mouse (Sigma) was used. Staining with DAPI (Sigma) was used for visualization of cell nuclei. GFP fluorescence marked the p14 protein. Images were captured on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Image de-convolution and re-construction were performed with Hygens Professional Version 3.4 deconvolution software (Scientific

Volume Imaging) followed by image processing with ImageJ software.

2.8 Bioinformatic analysis

For functional annotation analysis using gene ontology terms and Biological interaction networking between the identified proteins PathwayStudio 7.0 software (Ariadne Genomics, Rockville, MD, USA) was used.

3 Results and discussion

3.1 Purification of endosomes from MEFs

To address which endosomal proteins were regulated by the presence or absence of p14 on endosomes we analyzed $p14^{-/-}$ and $p14^{+/-}$ MEFs, previously generated in our laboratory by organelle proteomic analyses. The procedure used here is summarized as flowchart in Fig. 1. The CE were purified from MEFs by floatation gradient centrifugation in a discontinuous sucrose gradient. The previously described procedure of subcellular fractionation [9] was optimized here for MEFs (Section 2). The subcellular fractionation procedure used here allowed us to reveal protein amounts (approximately 10 μg of organelle proteins from a standard 150 mm plastic dish) sufficient for subsequent 2-D DIGE analyses and was highly reproducible.

Additionally, the $p14^{rev}$ stable MEF cell line was established by retroviral re-expression of pEGFP-p14 in $p14^{-/-}$ MEFs. The analysis of $p14^{rev}$ MEFs allowed us to get highly confident results in our proteomic approach by restoring differentially regulated proteins in $p14^{rev}$ cells. According to Western blotting, p14 protein expression levels in $p14^{rev}$ MEFs were very close to the levels of the endogenous protein, only two times higher than p14 in control $p14^{+/-}$ MEFs (Fig. 2A). As one would expect, MP1 was not detectable by Western blotting in $p14^{-/-}$ endosomes, confirming our previous data that p14 is absolutely required to localize MP1 to late endosomes [3]. Interestingly, the recently discovered p14 anchor protein p18 [13] was significantly downregulated in $p14^{-/-}$ endosomes (Fig. 2A). Importantly, reconstitution of p14 in $p14^{-/-}$ MEFs rescued the protein levels of both the MP1 and p18 partner proteins. $P14^{rev}$ MEFs reverted $p14^{-/-}$ cell morphology and proliferation rate [3] to very similar ones of $p14^{+/-}$ MEFs (data not shown).

Importantly, pEGFP-p14 protein specifically localized to late endosomes, as demonstrated by confocal laser scanning immunofluorescence microscopy and immunoelectron microscopy. The pEGFP-p14 localized in the perinuclear region to the cytoplasmic side of late endosomal vesicles (Fig. 2B and Supporting Information Fig. S1) of circular shape, labeled with anti-LBPA, a specific late endosomal/lysosomal phospholipid marker.

3.2 Absolute quantification of p14/MP1 in Endosomes

In order to characterize in more details the stoichiometry of the p14/MP1 complex on endosomes we measured absolute concentrations of both proteins by quantitative MS using the recently published Equimolarity through Equalizer Peptide (EtEP) strategy to generate an equimolar mixture of internal standard peptides [11]. MP1 and p14 proteins were absolutely quantified in CE using three internal standard peptides for MP1 and two for the p14 protein (Fig. 2C, Supporting Information Fig. S2). Absolute quantification based on proteotypic (*i.e.* specific, unique for corresponding proteins) peptides gave the concentrations of 4.99 ± 0.49 fmol/μg CE protein of MP1 (DGVPVIK) and 5.54 ± 0.27 fmol/μg CE protein of p14 (ETVGFGMLK) in the acetone precipitated CE. Therefore, the ratio MP1 to p14 is 1:1.11 in control $p14^{+/-}$ MEFs (Table 1), which was only slightly increased to 1:1.51 in $p14^{rev}$ cells (Table 1 and Fig. 2C). The p14 expression levels in re-constituted $p14^{rev}$ endosomes were increased 1.59-fold in comparison to $p14^{+/-}$ MEFs, rescuing MP1 to almost endogenous expression (1.15-fold increase compared to $p14^{+/-}$ MEFs). As absolute quantification was based on only one proteotypic peptide per protein, we tested whether we could also include the remaining three non-proteotypic peptides (FLYK, LGLSK, and VAITR) for quantification to increase the confidence of the measurement. We therefore measured these peptides in CE of $p14^{-/-}$ cells, which served as a p14/MP1 depleted negative control. Measurements showed no (FLYK) or only a minor (LGLSK, VAITR) contamination from non-p14/MP1proteins, *i.e.* proteins containing these peptides were removed almost entirely in the process of CE enrichment. Inclusion of these peptides in the measurements of p14/MP1 in CE of $p14^{+/-}$ and $p14^{-/-}$ cells confirmed the results obtained using the proteotypic peptides only (Table 1 and Fig. 2C). Furthermore, we compared two different sample preparation methods for absolute quantification of p14/MP 1 in endosomes. In the first protocol CE were lysed with 1% Triton X-100 followed by an acetone precipitation of the proteins. In the second protocol CE were lysed with 1% RapiGest and directly subjected to LC-SRM analysis without protein precipitation. Both sample preparations were performed in a similar way, giving comparable protein ratios (Table 1). The 30–50% decrease in absolute concentration of the same peptide after acetone precipitation probably reflected some expected losses of the proteins due to the protein precipitation procedure itself.

The absolute quantification of p14/MP1 produced the same results independently on from the mass spectrometer used. The abovementioned results were obtained using the 4000 QTRAP instrument operated in SRM-mode. However, the same samples analyzed using an LTQ-Orbitrap instrument resulted in very similar values (Supporting Information Table S1), *e.g.* MP1:p14 in control $p14^{+/-}$ MEFs was 1:1.15 (1:1.11,QTRAP), in $p14^{rev}$ cells the ratio was increased to 1:1.48 (1:1.51, QTRAP). An equimolar ratio of proteins in

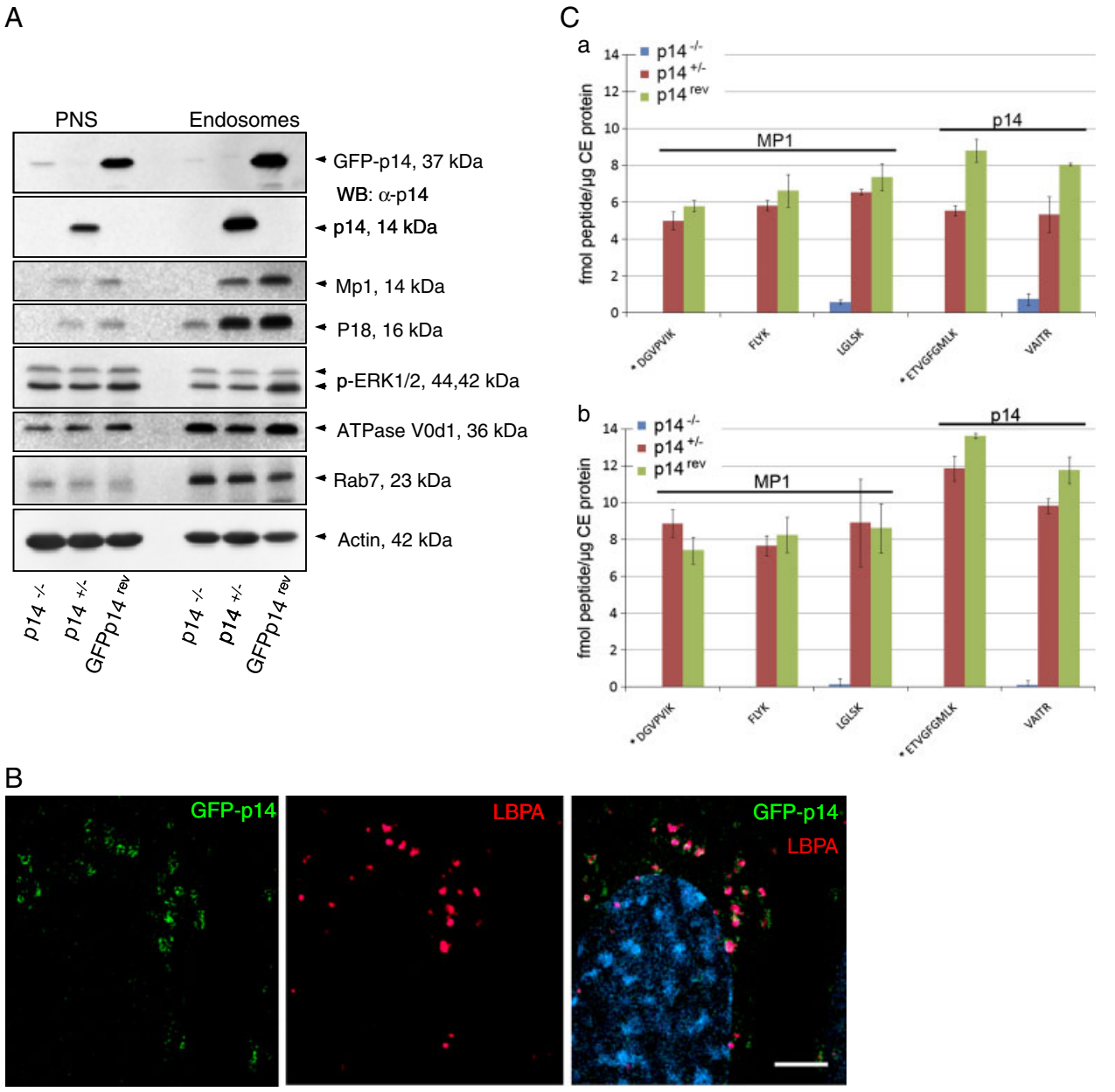

Figure 2. (A) A stable reconstitution of pEGFP-p14 (p14^{rev}) in p14 $^{-/-}$ rescued endogenous protein level of p14 as well as interacting partners MP1 and p18. Post-nuclear supernatants (PNS) and Crude Endocomes (CE) purified by subcellular fractionation were analyzed by Western blotting using specific antibodies against endosomal marker proteins. (B) Reconstituted pEGFP-p14 co-localizes with the late endosomal marker LBPA, confocal laser scanning microscopy images of p14^{rev} MEFs, scale bar 5 µm. (C) MP1-p14 was absolutely quantified using three internal standard peptides for MP1 and two for the p14 protein. Data are mean ± S.D. of duplicate measurements. The proteotypic (*i.e.* specific) peptides DPVPVIK (for MP1) and ETVGFGMLK (for p14) are indicated with an asterisk. (a) CE were lysed with 1% Triton followed by an acetone precipitation of the proteins. (b) CE were lysed with 1% RapiGest and directly subjected to LC-SRM analysis without protein precipitation.

p14/MP1 complex was shown previously after complex reconstitution *in vitro* [2]. Hence, here we demonstrate for the first time predicted equimolar ratios of the endogenous proteins on endosomes, as well as on p14$^{-/-}$-reconstituted organelles, by absolute quantification of MP1 and p14 proteins.

3.3 Differentially regulated proteins in p14-null endosomes

All together 31 different proteins in 37 protein spots were found to be differentially regulated in endosomes by the absence/presence of the p14 scaffold protein and identified

Table 1. Average absolute concentrations ± standard deviation of MP1 and p14 in fmol *per* μg CE protein of duplicate measurements

	Based on proteotypic peptides only			Based on all measured peptides		
	MP1	p14	Stoichiometry	MP1	p14	Stoichiometry
A						
$p14^{-/-}$	0	0		0.19	0.37	
$p14^{f/-}$	4.99 ± 0.49	5.54 ± 0.27	1:1.11	5.79 ± 0.77	5.44 ± 0.14	1:0.94
$GPFp14^{rev}$	5.78 ± 0.30	8.80 ± 0.60	1:1.51	6.59 ± 0.79	8.43 ± 0.53	1:1.28
B						
$p14^{-/-}$	0	0		0.05	0.05	
$p14^{f/-}$	8.89 ± 0.74	11.86 ± 0.67	1:1.34	8.48 ± 0.70	10.84 ± 1.44	1:1.28
$GPFp14^{rev}$	7.42 ± 0.72	13.61 ± 0.14	1:1.83	8.09 ± 0.61	12.68 ± 1.31	1:1.57

Listed concentrations and p14/MP1 stoichiometries are calculated from SRM-based measurements of the proteotypic peptides (DGVPVIK, ETVGFGMLK) only (left row) and based on measurements of the entire set of internal standard peptides (right row), respectively. (A) CE were lysed with 1% Triton followed by an acetone precipitation of the proteins. About 0.43 μg dissolved CE protein was separated on a nano-LC and analyzed on a 4000 QTRAP instrument. (B) CE were lysed with 1% RapiGest and directly subjected to LC-SRM analysis without protein precipitation.

by MALDI-TOF/TOF-MS (Fig. 3, Table 2). Protein spots differentially expressed in $p14^{-/-}$ endosomes were considered for identification if they fulfiled four criteria: (i) *p*-values after *T*-test < 0.05 ($p14^{-/-}$ *versus* $p14^{+/-}$), (ii) differential regulation of a considered protein spot detected between $p14^{-/-}$ and $p14^{+/-}$ endosomal proteins was rescued in $p14^{rev}$ endosomes, (iii) *p*-values after *T*-test < 0.05 ($p14^{-/-}$ *versus* $p14^{rev}$), and (iv) corresponding ANOVA-test *p*-values in a combined analysis of all three types of samples ($p14^{+/-}$, $p14^{-/-}$ and $p14^{rev}$) < 0.05. Thirty-seven protein spots were found to fulfil those conditions. All the spots were picked and identified by PMF using MALDI-TOF-MS (Table 2).

Among the detected proteins, 27 spots were downregulated (Table 2), whereas other 10 spots were upregulated, suggesting p14-dependent association of soluble cytosolic and cytoskeleton proteins with endosomes. Interestingly in a previous organelle proteomics study [6] we have already observed such an EGFR signaling-dependent association. Indeed, 22 proteins according to Gene ontology (GO_GO_Cell-component) analysis were cytoplasmic proteins (Supporting Information Table S2), the major part of them, namely 11 were components of organelles (late endosomes, lysosomes and melanosomes), cytoplasm (9), CCT (6) and the cytoskeleton (5). Majority of the differentially regulated proteins in $p14^{-/-}$ endosomes were downregulated, which indicated dissociation of proteins from $p14^{-/--}$ organelles. Additionally, components of more stable protein complexes at normal conditions could be degraded because of a missing anchoring partner (*e.g.* MP1 in $p14^{-/-}$ endosomes). P18 downregulation in $p14^{-/-}$ MEFs suggested functioning of these three proteins MP1, p14 and p18 as one structural and signaling unit on endosomes.

According to the GO_Cell-biological-process analysis, the majority of regulated proteins belonged to a functional group of chaperons, to stress-responding and actin cytoskeleton organization proteins (all together 16 proteins, Supporting Information Table S2). Interestingly, we detected downregulation of seven subunits of a large heterooligomeric CCT, also known as TCP-1 ring complex, known to play a role in the folding of actin and tubulin, as well as in the organization/polymerization of the actin- and tubulin-based cytoskeletal systems [14]. Three other downregulated proteins were also chaperons (HSP90B, HSPA8 and DNAJA1), which could directly interact and regulate T-complex proteins (Supporting Information Fig. S3). Stip1, stress-induced phosphoprotein 1, is an adaptor protein that

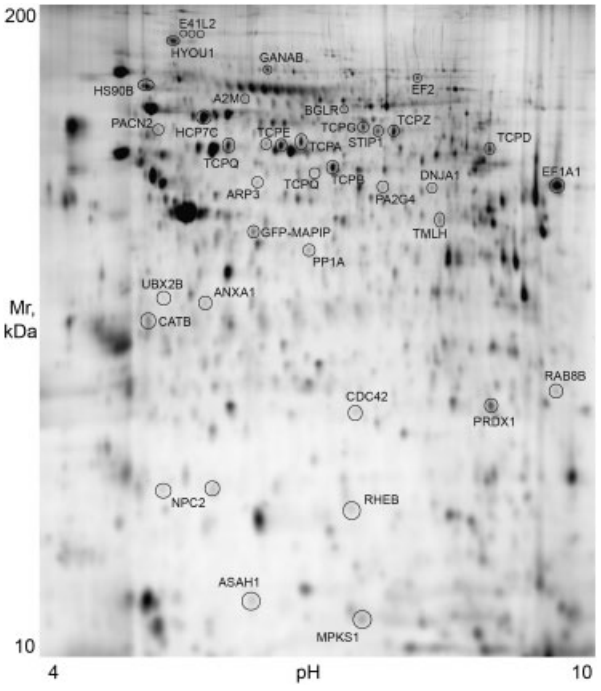

Figure 3. Proteins regulated on endosomes by p14 detected by 2-D DIGE and identified by MS.

Table 2. Proteins differentially regulated on endosomes by p14 and identified by MALDI-TOF-MS

	Accession Swiss-Prot	Gene name	Protein name	Sequence coverage	Peptides matched	MASCOT score	$p14^{-/-}/p14^{+/-}$		$p14^{-/-}/GFPp14^{rev}$		1-ANOVA
							T-test	Av. ratio	T-test	Av. ratio	
1	O70318	Epb41l2	Band 4.1-like protein 2	25	19	72	0.0007	−2.12	0.0014	−2.21	0.0003
2	O70318	Epb41l2	Band 4.1-like protein 2	24	18	68	0.0460	−1.55	0.0087	−1.78	0.0096
3	O70318	Epb41l2	Band 4.1-like protein 2	26	20	74	0.0055	−1.97	0.0021	−2.18	0.0010
4	Q9JKR6	Hyou1	Hypoxia upregulated protein 1	34	40	298	0.0110	−1.78	0.0460	−1.45	0.0130
5	Q8BHN3	Ganab	Neutral α-glucosidase	49	46	360	0.0023	−1.52	0.0008	−1.44	0.0007
6	P58252	Eef2	Elongation factor 2	44	46	333	0.0006	−1.66	0.0013	−1.52	0.0003
7	P11499	Hsp90ab1	Heat shock protein HSP 90-β	45	34	292	0.0011	−1.67	0.0047	−1.51	0.0003
8	gil157954061[a)]	A2M	α-2-Macroglobulin [*Bos taurus*]	19	26	199	0.0300	1.63	0.0320	1.57	0.0290
9	P12265	Gusb	β-glucuronidase	37	22	209	0.0003	1.76	0.0018	1.5	0.0004
10	P63017	Hspa8	Heat shock cognate 71 kDa protein	54	33	303	0.0001	−1.55	0.0049	−1.4	0.0002
11	P80318	Cct3	T-complex protein 1 subunit gamma	57	33	280	0.0010	−1.77	0.0017	−1.62	0.0002
12	Q9WVE8	Pacsin2	PKC and casein kinase substrate protein 2	39	20	185	0.0035	−1.51	0.0110	−1.35	0.0029
13	Q60864	Stip1	Stress-induced-phosphoprotein 1	45	24	120	0.0001	−2.13	0.0018	−1.7	0.0000
14	P80317	Cct6a	T-complex protein 1 subunit zeta	55	29	253	0.0002	−2.11	0.0007	−1.86	0.0000
15	P11983	Cct1	T-complex protein 1 subunit α B	43	26	270	0.0006	−1.81	0.0030	−1.6	0.0002
16	P80316	Cct5	T-complex protein 1 subunit epsilon	45	24	169	0.0012	−1.9	0.0078	−1.65	0.0011
17	P42932	Cct8	T-complex protein 1 subunit theta	53	29	329	0.0013	−1.68	0.0007	−1.7	0.0003
18	P80316	Cct5	T-complex protein 1 subunit epsilon	50	34	240	0.0009	−1.85	0.0039	−1.66	0.0004
19	P80315	Cct4	T-complex protein 1 subunit delta	57	31	297	0.0004	−2.14	0.0008	−1.94	0.0001
20	P80314	Cct2	T-complex protein 1 subunit β	72	37	362	0.0011	−1.75	0.0037	−1.61	0.0004
21	P42932	Cct8	T-complex protein 1 subunit theta	25	13	95	0.0087	1.53	0.0004	2.09	0.0002
22	Q99JY9	Actr3	Actin-related protein 3	48	22	261	0.0000	−1.57	0.0016	−1.31	0.0000
23	P50580	Pa2g4	Proliferation-associated protein 2G4	52	27	223	0.0006	−1.57	0.0057	−1.33	0.0003
24	P63037	Dnaja1	DnaJ homolog subfamily A member 1	33	11	107	0.0008	−1.57	0.0016	−1.45	0.0002
25	P10126	Eef1a1	Elongation factor 1-α 1	44	21	186	0.0001	−1.74	0.0000	−1.75	0.0000
26	Q91ZE0	Tmlhe	Trimethyllysine dioxygenase	43	19	148	0.0000	−1.58	0.0000	−1.46	0.0000
27	P62137	Ppp1ca	Serine/threonine-protein phosphatase PP1-α	43	16	222	0.0003	−1.72	0.0016	−1.72	0.0003
28	P97429	Anxa4	Annexin A4	48	15	185	0.0013	1.68	0.0010	1.83	0.0005
29	P10605	Ctsb	Cathepsin B	39	14	107	0.0001	2.9	0.0150	1.48	0.0000
30	P61028	Rab8b	Ras-related protein Rab-8B	33	8	79	0.0008	1.44	0.0040	1.59	0.0023
31	P35700	Prdx1	Peroxiredoxin-1	78	19	296	0.0014	−1.31	0.0002	−1.54	0.0000
32	P60766	Cdc42	Cell division control protein 42 homolog	34	5	76	0.0000	−1.52	0.0080	−1.24	0.0000
33	Q9Z0J0	Npc2	Epididymal secretory protein E1	38	5	78	0.0000	2.13	0.0003	1.63	0.0000
34	Q9Z0J0	Npc2	Epididymal secretory protein E1	42	6	104	0.0000	3.35	0.0098	1.54	0.0000
35	Q921J2	Rheb	GTP-binding protein Rheb	38	8	118	0.0003	2.41	0.0040	1.8	0.0002
36	Q9WV54	Asah1	Acid ceramidase	32	8	92	0.0000	2.57	0.0001	1.9	0.0000
37	O88653	Mapksp1	Mitogen-activated protein kinase scaffold protein 1	61	7	134	0.0051	−1.9	0.0004	−2.29	0.0004

a) NCBInr database.

coordinates the functions and mediates the association of the two chaperons HSP90 and HSC70 (HSPA8) [15], identified here. Actin-related protein 3, component of the Arp2/3 complex, which nucleates actin filaments and organizes branched actin networks, is important for endocytic vesicle morphogenesis [16]. The changes in actin cytoskeleton organization could be responsible for endosomal trafficking regulated by the p14/MP1 complex that we observed previously [4].

Another group of proteins are known to play a role in vesicle formation and transport. Pacsin2 downregulated on $p14^{-/-}$ endosomes is known to inhibit endocytosis and participate in rearrangements of actin networks during vesicle formation and transport [17]. Pacsins were suggested to be molecular links between membrane trafficking and cortical cytoskeleton dynamics, they associated with both dynamin and N-WASP, a potent stimulator of the Arp2/3 complex actin polymerization machinery [18]. Increased concentration of proteins from cell culture medium in endosomes (bovine α-2-macroglobulin, Table 2) supported the possibility of increased endocytosis in $p14^{-/-}$ MEFs. Alternatively, the presence of α-2-macroglobulin might reflect decreased activity of lysosomal enzymes in p14-deficient MEFs. Increased amounts of not active or unprocessed cathepsin B (Fig. 5) could also indicate an abnormal function of lysosomes as it was previously observed in p14-depleted specialized cells of the immune system (cytotoxic T cells, melanocytes and neutrophil granulocytes) in patients [5]. Interestingly, all three late endosomal/lysomal enzymes detected here (β-glucuronidase, cathepsin B and acid ceramidase) were significantly (2–3 fold) upregulated in $p14^{-/-}$ organelles.

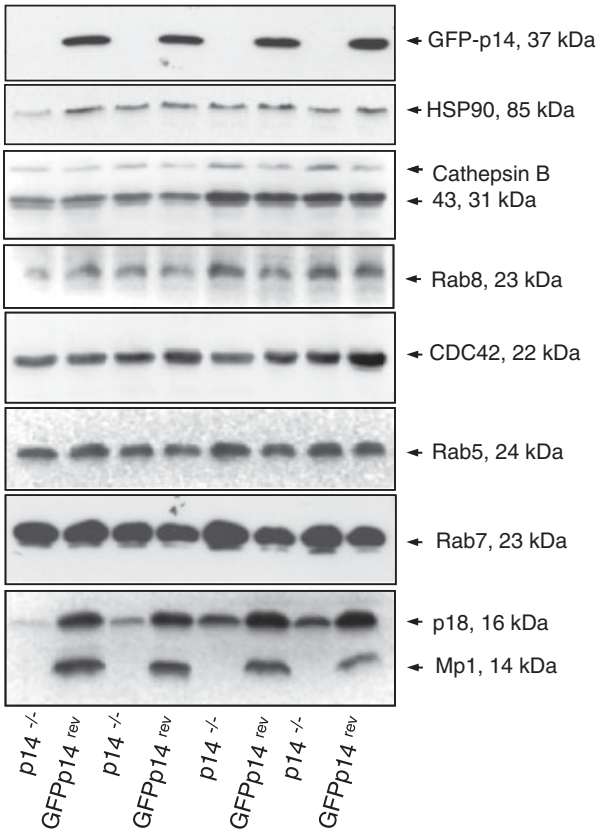

Figure 4. Regulation of selected endosomal proteins was confirmed by Western blot analysis with anti-HSP90, anti-cathepsin B, and anti-Rab8, anti-CDC42 anti-Mp1-specific antibodies.

Several proteins regulated in $p14^{-/-}$ endosomes are directly involved in vesicular trafficking, *i.e.* annexin A4, NPC2 and the small GTPases RAB8B, RHEB and CDC42. All of them, but not CDC42, were up-regulated in p14-depleted endosomes (Table 2). Annexin A4 belongs to the annexin family of calcium-dependent phospholipid binding proteins, which promotes membrane fusion and is involved in exocytosis [19]. The up-regulation of Rab8b, the marker of recycling endosomes, which mediate biosynthetic trafficking from the *trans*-Golgi network to the plasma membrane [20–22], also suggests a possible link of late endosomes with Rab8-recycling endosomes regulating dynamic actin containing structures [23]. This would be consistent with the aberrant distribution of recycling endosomes in $p18^{-/-}$ cells and the hypothesized lack of proper interaction or fusion with late endosomes [13]. NPC2, a soluble lysosomal glycoprotein that binds cholesterol, has a specialized function in lysosomal sterol transport. Niemann-Pick C disease, caused by a deficiency in NPC1 and NPC2, is a fatal neurodegenerative disorder characterized by an endolysosomal accumulation of cholesterol and other lipids [24]. NPC2 may act in an ordered sequence to effect sterol movement, and consequently, influence the process of intracellular vesicular trafficking [25].

Many of the proteins identified here are known to be involved in signal transduction. The MP1/p14/p18 protein complex regulates MAP kinase signaling [3, 13]. Small GTPases Rab8B, Rheb and CDC42, which cycle between an active GTP-bound and an inactive GDP-bound state and bind to a variety of effector proteins to regulate cellular responses. It was shown recently that late endosomes are essential for mTORC1 signaling, which regulates protein synthesis, cell growth and autophagy [26]. An upregulation of late endosomal Ras-related GTP-binding protein Rheb, a direct activator of mTORC1 [27], suggests an alteration of mTORC1signaling in $p14^{-/-}$ cells. The regulation of mTORC complex was also suggested by our bioinformatic analysis (Supporting Information Fig. S3). Serine/threonine-protein phosphatase 1 is essential for cell division [28] and has been shown to be recruited to endosomes by SARA and endofin scaffold proteins to regulate TGF-β [29] and BMP [30] signaling, respectively. PA2G4, proliferation-associated protein 2G4, also known as EBP1 tumor suppressor, is involved in growth regulation and plays a role in an ERBB3-regulated signal transduction pathways [31]. Peroxiredoxin-1 participates in the signaling cascades of growth factors by regulating the intracellular concentrations

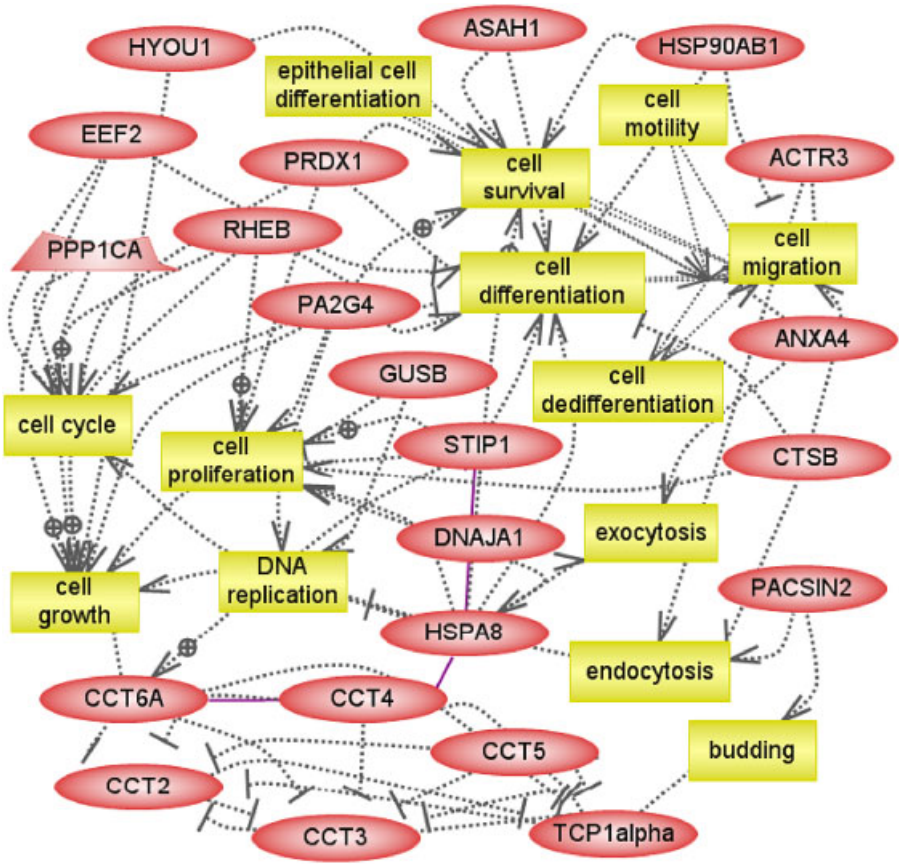

Figure 5. Biological interaction networking of p14-regulated endosomal proteins (Shortest pathway, Cell processes) identified using the PathwayStudio software.

of ROS. Recently, a subset of signaling endosomes, uniquely equipped with redox-processing proteins, termed redoxosomes, was suggested as responsible for subcellular compartmentalization of ROS signal transduction [32].

3.4 Confirmation by Western-blotting

To validate the proteomic results by Western blotting we used specific antibodies against selected proteins. As demonstrated in Fig. 4, anti-HSP90, anti-CDC42 and anti-MP1 antibodies confirmed downregulation of all three proteins in $p14^{-/-}$ endosomes detected by 2-D DIGE, whereas anti-cathepsin B, anti-Rab8 confirmed upregulation of corresponding proteins in the same samples. Interestingly, by Western blotting we could also detect upregulation of two forms of cathepsin B – the inactive 43 kDa proenzyme and the activated 31 kDa protein. The differences in protein expression detected by Western blotting were rather weak between $p14^{-/-}$ and reconstituted $p14^{rev}$ endosomes, but in line with the proteomics data and consistently found in four independently prepared endosome preparations (Fig. 5). Importantly, using $p14^{rev}$ endosomes instead of $p14^{+/-}$ ones as a control here, allowed us to avoid possible clonal artifacts, because p14 reconstitution was performed on the same $p14^{-/-}$ cell line. Western blotting using all five antibodies verified the 2-D DIGE pattern as well as further confirmed the protein spots identified by MS.

4 Concluding remarks

Organelle proteomics revealed clear differences in protein composition of endosomes purified from $p14^{-/-}$, $p14^{+/-}$ and $p14^{rev}$ mouse embryonic fibroblasts. Not only core proteins of MP1/p14/p18 scaffold complex were affected, but also many other proteins involved in signal transduction, endosomal trafficking and actin remodeling and regulating major cellular functions such as proliferation, differentiation, migration and survival (Fig. 5). Absolute quantification of MP1 and p14 proteins in endosomes using the Equimolarity through Equalizer Peptide strategy [11] demonstrated equimolar ratios of the endogenous proteins on membranes of control $p14^{+/-}$ endosomes, as well as on p14-reconstituted organelles and the absence of MP1 on $p14^{-/-}$ organelles. Downregulation of p18, the recently identified p14-anchor protein on late endosomes, in $p14^{-/-}$ MEFs suggests that cells tightly control concentration of scaffold and adaptor proteins in order to regulate signal transduction intensity and duration in specific subcellular locations. Even minor changes of scaffold

protein concentrations can regulate a level of MAPK activation (Fig. 2A and [33]). Detailed analysis of several intriguing findings lay out of the scope of this article and need to be further addressed.

The Austrian Proteomics Platform (APP) within the Austrian Genome Program (GEN-AU), Vienna, Austria, supports work in the Huber, Mechtler, and Bonn laboratories. Work in the Huber and Bonn laboratories is further supported by the Special research Program "Cell Proliferation and Cell Death in Tumors" (SFB021, Austrian Science Fund). Work in the Mechtler laboratory is further supported by the Special research Program "Chromosome Dynamics" (SFB-F3402). Work in the Hess laboratory is supported by grants P19486-B12 (Austrian Science Fund) and P11050 (Oesterreichische Nationalbank-Jubilaeumsfonds).

The authors have declared no conflict of interest.

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