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A novel IEF peptide fractionation method reveals a detailed profile of N-terminal Acetylation in chemotherapy responsive and resistant ovarian cancer cells

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Abstract

Although acetylation is regarded as a common protein modification, a detailed proteome wide profile of this posttranslational modification may reveal important biological insight regarding differential acetylation of individual proteins. Here we optimised a novel peptide IEF fractionation method for use prior to LC-MS/MS analysis in order to obtain a more in depth coverage of N-terminally acetylated proteins from complex samples. Application of the method to the analysis of the serous ovarian cancer cell line OVCAR-5 identified 344 N-terminally acetylated proteins, 12 of which are previously un-reported. The protein peptidyl-prolyl cis-trans isomerase A (PPIA) was detected in both the N-terminally acetylated and un-modified forms, and was further analysed by data independent acquisition in Carboplatin responsive parental OVCAR-5 cells and Carboplatin resistant OVCAR-5 cells. This revealed a higher ratio of un-acetylated to acetylated N-terminal PPIA in the parental compared to the Carboplatin resistant OVCAR-5 cells, and a 4.1-fold increase in PPIA abundance overall in the parental cells relative to Carboplatin-resistant OVCAR-5 cells ($P = 0.015$). In summary, the novel IEF peptide fractionation method presented here is robust, reproducible, and can be applied to the profiling of N-terminally acetylated proteins. All mass spectrometry data is available as a ProteomeXchange repository (PXD003547).

KEYWORDS: N-terminal Acetylation, Ovarian cancer, carboplatin resistance, chemoresistance, peptidyl-prolyl cis-trans isomerase A, PPIA

Introduction

Ovarian cancer is the seventh most common cancer and the eighth leading cause of cancer-related deaths among women, responsible for approximately 250, 000 new cases and 150,000 deaths each year worldwide [1]. Due to delayed presentation and diagnosis, over 70% of women with ovarian cancer are diagnosed with stage III/IV disease. The standard treatment for stage III/IV diagnoses involves debulking surgery followed by combination chemotherapy with the drugs carboplatin and paclitaxel. Despite an 80% initial response rate, the majority of women exhibit disease relapse and die from recurrent Carboplatin-resistant disease [2]. It is therefore of great importance to investigate mechanisms and markers that predict ovarian cancer resistance to standard chemotherapy regimens, in order to save patients from damaging, ineffective treatments, and determine if alternative regimens and novel drug combinations would be more beneficial.

The chromosome centric human proteome project (C-HPP) aims for the characterisation of the ~20,300 proteins expressed by the protein-coding genome with respect to gene location on each chromosome, cellular distribution, and quantification [3, 4, 5]. Moreover, the C-HPP also plans to map the major classes of post-translational modifications (PTM) for all of the identified proteins in disease related contexts [6]. N(alpha)-terminal acetylation is one of the most common covalent protein modifications to occur in eukaryotes, with ~80% of soluble human proteins predicted to be N-terminally acetylated [7]. N-terminal acetylation is an irreversible process that typically occurs during protein synthesis and involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of a protein's first amino acid, as catalysed by N-terminal acetyltransferases (NATs) [8]. The functional changes induced by N-terminal acetylation can be highly variable but can be broadly categorized into subcellular localization, protein interactions and complex formation, protein folding, and perhaps the

most well-known, protein degradation with regard to the N-end rule [9, 10, 11]. The N-end rule pathway targets proteins for ubiquitin-mediated degradation based on their N-terminal residue and the PTM status of this residue. These degradation signals are known as N-degrons.

Initially N-terminal acetylation was shown to block ATP-dependent ubiquitin-mediated degradation [12], however it has also been shown to promote sequence specific protein degradation [13]. In eukaryotes there are two N-end rule pathways that form part of the ubiquitin system that target the majority of cellular proteins for degradation; the Ac/N-end and the Arg/N-end rule pathways. In the Ac/N-end rule pathway, proteins containing N-terminally acetylated residues are targeted for degradation. Conversely, the Arg/N-end rule pathway targets specific un-acetylated N-terminal residues (Arg, Phe, Lys, Leu, His, Tyr, Trp, and Ile) and N-terminal Asn and Gln which have been deaminated and then Arginylated [14]. Un-acetylated N-terminal Met followed by a hydrophobic residue are also targeted for degradation by the Arg/N-end rule pathway [15]. Protein N-terminal acetylation is thought to be relevant to cancer development with NATs acting as tumour suppressors in healthy tissues and oncoproteins in cancerous cells [16].

The aims of this study were to characterise the N-terminal acetylation profile of the ovarian cancer cell line OVCAR-5 in order to identify novel acetylation sites and help determine if the occurrence of N-terminal acetylation differs in chemoresistant cells and therefore can be used as putative markers for chemotherapeutic resistance in ovarian cancer. In order to comprehensively profile the N-terminal acetylation sites and develop spectral libraries, a novel IEF Sephadex flatbed gel method for peptide fractionation of up to 10 mg was developed and applied prior to nano-LC-ESI-MS/MS. The novel method was first applied to

trypsin digested mouse brain lysates to show the technique is capable of separating and improving the coverage of highly complex peptide mixtures. Following this the method was then applied to OVCAR-5 cell lysates, enabling the detection of 344 N-terminally acetylated proteins, 12 of which are previously un-reported. This stands in contrast to the 189 N-terminal acetylation sites detected in the unfractionated OVCAR-5 sample.

To determine if variable N-terminal acetylation may play a role in the development of chemoresistance, the results from the IEF fractionation were searched for N-terminally acetylated proteins present in both the modified and un-modified forms that could be analysed further in non-resistant (parental) and carboplatin resistant (CBPR) OVCAR-5 cells. The protein peptidyl-prolyl cis-trans isomerase A (PPIA), also known as Cyclophilin A, was identified and selected for further analysis given the protein's potential involvement in chemoresistance [17] and given the protein was detected in both the N-terminally acetylated and un-acetylated forms. PPIA was quantified using data independent acquisition methods in the parental and CBPR OVCAR-5 cells. This revealed a higher ratio of un-acetylated to acetylated N-terminal PPIA in the parental compared to the CBPR cells, and a 4.1-fold increase in PPIA abundance overall in the parental cells relative to CBPR OVCAR-5 cells ($P = 0.015$).

Materials and Methods

Preparation of tryptic peptides from mouse brain samples

Freshly dissected P30 mouse brain was lysed at room temperature under denaturing conditions in 10mL of lysis buffer (8 M urea (Merck, Darmstadt, Germany), 20 mM HEPES (ICN Biochemicals, Ohio, USA), pH 8, complete protease inhibitors (Roche, Basel, Switzerland), 10mM NaF (Sigma-Aldrich, St. Louis, USA), 1mM Na_3VO_4 (Sigma-Aldrich), 1 mM beta-glycerophosphate (Sigma-Aldrich), 2.5 mM sodium pyrophosphate (Sigma-Aldrich)) with 6 titrations through an 18G needle (Becton Dickinson, Franklin Lakes, USA), followed by 6 titrations through a 21G needle (Becton Dickinson), followed by 3 x 15 second pulses with a 300W sonicator (Branson, Danbury, USA) at 50% amplitude. Lysates were centrifuged at 20,000 x g for 15 minutes at 20°C to remove insoluble material and protein concentrations were estimated using an EZQ protein assay (Life Technologies, Carlsbad, USA). The samples were reduced with 5 mM DTT (Roche) for 45 min at room temperature and alkylated with 10 mM iodoacetamide (IAA) (GE Healthcare, Little Chalfont, UK)) for 30 minutes at room temperature in the dark. Lysates were then diluted to 2 M urea with 20 mM HEPES, pH 8, and digested overnight at 25°C with trypsin-TPCK (Worthington Biochemicals, Lakewood, USA) at an enzyme to substrate ratio of 1:50. Following digestion, samples were acidified with formic acid (FA) (Sigma-Aldrich) and subsequently desalted using 500 mg C18 Sep-Pak SPE cartridges (Waters, Milford, USA). C18 cartridges were conditioned with 5 mL of 100% Acetonitrile (ACN) (Merck), followed by 5 mL of 50% (v/v) ACN, 0.1% FA, and finally 20 mL of 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). Sample was loaded onto the conditioned C18 cartridge, washed with 15 mL of 0.1% TFA, and eluted with 6 mL of 50% ACN, 0.1% FA. Desalted samples were dried to completeness overnight in an Alpha 1-2 freeze drier (Martin Christ, Osterode am Harz, Germany). The

peptides were then resuspended in 10% ACN to a concentration of 10mg/mL as estimated by the initial protein amount before digestion.

Peptide fractionation by IEF Sephadex gel flatbed electrophoresis

335 mg of Sephadex G-100 SF (GE Healthcare) was mixed with 3 mL 10% (v/v) Acetonitrile (ACN) (Merck) and rehydrated for 72 hours at room temperature. Subsequently, the required volume of mouse brain tryptic digest for 1 mg or 10 mg peptides (0.1 mL or 1 mL, respectively), 180 µL Pharmalyte 3-10 (GE Healthcare) and 50 µL pI-marker mixture (14) were added. Thereafter, 10% (v/v) ACN (Merck) was added to a total volume of 4.5 mL and mixed carefully, to not introduce air-bubbles, with a 5 mL pipette (Eppendorf, Hamburg, Germany). The mixture was pipetted into a lane (Dimensions: (10 x 1) cm^2) in a HD-PE tray, a layer of six Whatman 3MM Chr paper (GE Healthcare) was wetted in the respective cathode (100 mM NaOH (Merck)) or anode solution (100 mM H_2SO_4 (Merck)) and applied to the ends of the lane. The mixture was then distributed homogenously in the lane by short, rapid movements of the HD-PE tray. IEF was conducted using a Multiphor II apparatus (GE Healthcare) at 4 °C, with the voltage limited to 1500 V and current limited to 0.1 mA/ cm^2 lane area until 1,000 Vhrs (1mg peptides) or 1,300 Vhrs (10 mg peptides) were reached. Following separation, 20 fractions were collected into 1.5 mL 0.22 µm cellulose acetate spin-filter-tubes (Corning, New York, USA) using a spatula. Sephadex fractions were eluted with 3 x 200 µL 10% (v/v) ACN using a bench-top centrifuge. Elution of peptides was deemed complete at this point as no pI-marker was left visible in the Sephadex gel. In the case of the 1 mg of fractionated mouse brain peptides, the eluate was concentrated using a vacuum centrifuge until approximately 100 µL solution was left and diluted 1:10 in 2% (v/v) ACN, 0.1% (v/v) formic acid in 97.9% water (FA2). For the 10 mg of the fractionated mouse brain

peptides, the vacuum concentration step was omitted and the eluate was directly diluted 1:10 in FA2.

OVCAR-5 sample preparation and IEF fractionation

For fractionation by IEF the OVCAR-5 cells were lysed using a Precellys 24 bead beater. Briefly, the cells were placed in Precellys bead beating tubes (Bertin Technologies, Montigny-le-Bretonneux, France) in 1% (w/v) SDS (GE Healthcare) and homogenised using a Precellys 24 (Bertin Technologies) at 6,300 revolutions per minute for 3 x 1 minute cycles. The samples were centrifuged at 20,000 x g for 30 minutes, the supernatant was recovered and DNA was subsequently sheared using a Bioruptor (Diagenode, Seraing, Belgium) set to "high" output for 6 x 30 second cycles with one minute breaks between each cycle. Sonication was carried out in ice-cold water. Protein concentration was quantified using an EZQ protein assay (Life Technologies). Protein extracts were digested with a modified FASP method as previously described by Wisniewski *e.t al.*(2009) [18]. Briefly, samples were made up to a volume of 200 µL with 7M urea (Merck), 100mM ammonium bicarbonate (U-AmBic) (Merck) and reduced by adding a final volume of DTT to 50 mM (Roche) followed with incubation at 20°C for 1 hour. Vivacon ultrafiltration spin columns (Sartorius Vivacon 500, 10,000 MWCO HY) were pre-rinsed at 14,000 x g for 10 minutes with 100 µL U-AmBic to remove traces of glycerine. Samples were loaded into the spin columns and centrifuged at 14,000 x g for 10 minutes. 100 µL of 55 mM IAA (GE Healthcare) in U-AmBic was added to the spin columns and incubated in the dark for 20 minutes at 20°C. Samples were centrifuged at 14,000 x g for 10 minutes and washed with 100 µL of U-AmBic twice, followed by one wash with 100 µL of 50 mM U-AmBic. Samples were digested overnight at 37°C with trypsin gold (Promega, Madison, WI, USA) at an enzyme to substrate ratio of 1:50 in 100 µL of 5 mM U-AmBic. 1 mg tryptic peptides from the OVCAR-5 cells

was desalted, dried to completeness, re-suspended in 100 µl 10% (v/v) ACN (Merck), and IEF was carried out as described above until 1,000 Vh were reached. Once separated the sample was collected into 20 fractions and prepared for nano-LC-ESI-MS/MS as described above.

Generation of carboplatin resistant OVCAR-5 cells

The human serous ovarian cancer cell line, OVCAR-5 was obtained from Dr. Thomas Hamilton (Fox Chase Cancer Centre, Philadelphia, PA) and cultured under conditions as previously described [19]. OVCAR-5 carboplatin resistant cells were derived from the parental OVCAR-5 cells by continuous exposure to carboplatin (Hospira Australia Pty Ltd). OVCAR-5 cells were cultured in T-75 flasks and treated with 25µM of carboplatin for 24 hours followed by a recovery phase (cells in normal growth media) for 72 hours. The 25µM dose of carboplatin was chosen because the inhibitory concentration (IC_{50}) carboplatin results in the OVCAR-5 cell senescence and delayed cell growth. The OVCAR-5 cells exposure to carboplatin and recovery phase cycle was repeated for 8 cycles and development period was performed within 8-10 weeks. Cell survival was calculated using a MTT assay, as per the manufacturer's instructions (Sigma-Aldrich), as previously described [19]. The (IC_{50}) of carboplatin was calculated from three independent experiments performed in triplicate using exponential regression curve fitting. We observed a 2.3 fold increase in IC_{50} of the OVCAR-5 CBPR cells (IC_{50} = 232 µM) compared to the parental OVCAR-5 cells (IC_{50} = 98 µM). For data independent acquisition mass spectrometry 300 ng the parental OVCAR-5 and OVCAR-5 CBPR cell lysate was analysed. These cells were prepared as described in the previous section, without the use of the IEF fraction step.

Nano -LC-ESI-MS/MS

Nano-LC-ESI-MS/MS was performed using an Ultimate 3000 RSLC system (Thermo-Fisher Scientific, Waltham, USA) coupled to an Impact HD™ QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) via an Advance CaptiveSpray source (Bruker Daltonics). Approximately 1 µg from each of the 20 mouse brain and OVCAR-5 fractions, and 1 µg from the un-fractionated mouse brain and OVCAR-5 digests were analysed. Peptide samples were pre-concentrated onto a C18 trapping column (Acclaim PepMap100 C18 75 µm × 20 mm, Thermo-Fisher Scientific) at a flow rate of 5 µL/ min in 2% (v/v) ACN 0.1% (v/v) TFA for 10 minutes. For all IEF fractionated samples peptide separation was performed using a 75 µm ID C18 column (Acclaim PepMap100 C18 75 µm × 50 cm, Thermo-Fisher Scientific) at a flow rate of 0.2 µL/ minutes using a linear gradient from 5 to 45% B (A: 5% (v/v) ACN 0.1% (v/v) FA, B: 80% (v/v) ACN 0.1% (v/v) FA) over 130 minutes, followed by a 20 minute wash with 90% B, and a 20 minute equilibration with 5% A. MS scans were acquired in the mass range of 300 to 2,200 *m/z* in a data-dependent fashion using Bruker's Shotgun Instant Expertise™ method. This method uses IDAS (intensity dependent acquisition speed) to adapt the speed of acquisition depending on the intensity of precursor ions (fixed cycle time), and RT^2 (RealTime Re-Think) to exclude previously selected precursor ions from undergoing re-fragmentation unless the chromatographic peak intensity of the ion has increased by a factor of 5. Singly charged precursor ions were excluded from acquisition. Collision energy ranged from 23% to 65% as determined by the *m/z* of the precursor ion.

Nano-LC-ESI-MS/MS data analysis

All spectra collected using data dependent acquisition (DDA) were analysed using the MaxQuant software (version 1.5.2.8) with the Andromeda search engine [20] against the UniProt human database (downloaded on the 13th of October 2015, containing 20,204 entries)

for the OVCAR-5 cell line and against the UniProt mouse database (downloaded on the 13th of October 2015, containing 16,785 entries) for the mouse brain lysates. For the OVCAR-5 data, all novel N-terminal acetylation and methionine cleavage site identifications were also checked against the UniProt human database containing all isoforms to ensure matching was genuine and not caused by protein isoforms. The standard Bruker QTOF settings in MaxQuant were used with a mass error tolerance of 40 ppm. The variable modifications of oxidation of methionine and N-terminal acetylation and the fixed modification of carbamidomethyl of cysteines were specified, with the digestion enzyme specified as trypsin. The protein false discovery rate (FDR) and peptide spectrum match FDRs were both set to 1% using a target decoy approach, with a minimum peptide length of 7 amino acids [20]. Only unique and razor peptides were used when reporting protein identifications. For the status of N-acetylation, the neXtProt [21] (02-2016) and PRIDE databases were consulted.

Further data analysis was carried out using R (Version 3.0.1, The R Foundation for Statistical Computing) [22] and additionally the ggplot2 v1.01 [23] package. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD003547.

Quantification of PPIA by DIA

Nano-LC was performed as described above on 300 ng of the CBPR and un-treated parental OVCAR-5 cells using an Ultimate 3000 RSLC system coupled to an Impact HD™ QTOF mass spectrometer set to acquire data using Bruker's Middle Band CID™ method. This data independent acquisition (DIA) method scans a mass range of *m/z* 375 to 1,206 in 26 Da increments and CID is performed with increasing collision energies of 20 to 36. The acquired data was analysed using Skyline (Version 3.1.0.7382) [25] against a spectral library generated

from the results of the OVCAR-5 IEF fractionation experiment. Analysis of PPIA abundance in the parental compared to CBPR OVCAR-5 cells (n=2) was carried out using the following spectral library peptides for quantification; V[+42]NPTVFFDIAVDGEPLGR (2+), VNPTVFFDIAVDGEPLGR (2+), VSFELFADK (+3), SIYGEKFEDENFILK (3+), FEDENFILK (+2), KITIADC[+57]GQLE (+2), and ITIADC[+57]GQLE (+2). The Skyline peptide and transition settings were as follows: trypsin was specified as the cleavage enzyme with a maximum of 1 missed cleavage, precursor charge states 2 and 3, ion charges 1 and 2, ion types y and b from ion 2 to ion 6, an ion match tolerance 0.1 *m/z*, a MS/MS filtering DIA isolation scheme from *m/z* 375 to 1,206 (26 Da windows), retention time window of 5 minutes, and a resolution of 10,000. For analysis of the un-modified vs acetylated N-terminal peptide of PPIA in the parental compared to CBPR OVCAR-5 cells (n=3), the % of the un-acetylated VNPTVFFDIAVDGEPLGR relative to the acetylated V[+42]NPTVFFDIAVDGEPLGR peptide was calculated. To calculate the relative abundance of PPIA overall, the area intensities of the detected peptides was summed. For all results the standard error of the mean and *P* values using un-paired *t*-tests were calculated using GraphPad Prism 6 v008.

Results

Validation of IEF Sephadex gel flatbed electrophoresis method using mouse brain

Fractionation of the mouse brain sample prior to nano-LC-ESI-MS/MS analysis significantly increased the total number of peptide and protein identifications obtained. The number of unique protein identifications with an FDR of 1% increased from 1,984 in the unfractionated sample (1 μg of digest analysed) to 3,716 and 3,546 in the 1 mg and 10 mg IEF fractionated mouse brain samples, respectively (both 1 μg of digest analysed per fraction). The peptide fractionation was deemed satisfactory, as 70% of the peptides were detected in 2 fractions or less from a total of 20 fractions (Figure 1A). Increasing the peptide load from 1 mg to 10 mg did not alter the quality of peptide separation, as in the case of the 10 mg load, 75% of peptides were identified in 2 fractions or less (Figure 1B). A median of 4 additional peptides were identified per protein in the 1 mg of fractionated sample as compared with the unfractionated sample, with additional peptides identified for 86% of the proteins overall (Figure 1C). For the 10 mg IEF fractionated sample the median increase in the number of peptides identified per protein was 3, with additional peptides identified for 78% of proteins (Figure 1D). The mean additional sequence coverage obtained by the IEF fractionation was 15% for the 1 mg sample and 10% for the 10 mg sample (Figure 1E, F).

N-terminal acetylation analysis of the IEF fractionated ovarian cancer cell line OVCAR-5

Following validation of the peptide IEF fractionation, the method was applied to the ovarian cancer cell line OVCAR-5. In total 4,305 unique proteins were identified from the OVCAR-5 cells (protein FDR of 1%) of which 2,067 were exclusively detected in the 1 mg IEF fractionated sample (Supporting Table 1a), as compared to 2,282 proteins detected in the 1 μg of unfractionated sample (44 exclusively identified) (Supporting Table 1b). Between the IEF fractionated and un-fractionated analyses there was an overlap of 2,238 proteins

identified in both experiments. The total number of unique peptides identified in the IEF fractionated sample was 17,773 (peptide FDR of 1%) as compared to 7094 identifications in the unfractionated OVCAR-5 cells (peptide FDR of 1%). Additional peptides (median: 2) were identified for 90% of proteins and a mean additional protein sequence coverage of 9.1% was gained in the IEF fractionated sample as compared to the un-fractionated sample (Supporting Figure 1). The chromosomal locations of the corresponding genes for all of the identified proteins (IEF fractionated and unfractionated) are provided in Supporting Figure 2. All of the detected proteins had an evidence level of PE1 except for androglobin (Q8NX0), which had an evidence level of PE2. However, only one peptide from this protein was identified and hence it does not meet the strcit HPP criteria for the detection of low evidence level proteins, where a minimum of 2 unique peptides with a length of ≥9 amino acids is required.

The total number of identified N-termini peptides was 446 (acetylated and un-acetylated, Supporting Table 2a and 2b), of which 401 were detected in the IEF fractionation OVCAR-5 samples, and 215 were detected in the unfractionated OVCAR-5 sample. The overall number of identified N-terminal acetylation sites was 382 (85.6% of all N-termini detected), with an overlap between the IEF and un-fractionated samples of 170. In the IEF fractionated OVCAR-5 sample a total of 344 N-terminal acetylation sites were detected, 12 of which are previously un-reported in both the neXtProt and PRIDE databases. Annotated spectra exported from MaxQuant for these peptides are provided in Supporting Figure 3. This stands in contrast to 189 N-terminal acetylation sites identified in the unfractionated OVCAR-5 sample, 4 of which were novel, however 3 of these were also identified in the fractionated sample. The data was further analysed for initiator methionine cleavage. Forty-four proteins were detected to have undergone initiator methionine cleavage, none of which have been

previously reported. The acetylation status, initiator methionine presence or cleavage, and sample information for the detected novel N-terminal peptides is summarised in Table 1.

Of the detected N-terminally acetylated amino acid residues 51.6% were Ala, 23.3% were Met, 18.8% were Ser, 4.2% were Thr, 0.8% were Val, 0.5% were Cys, with Asp, Glu, and Gly each contributing 0.3%. Details for the detected N-terminally acetylated amino acid residues, un-modified N-terminal amino acid residues, and the following amino acid residues for all detected N-terminal peptides are provided in Supporting Table 3.

The N-terminus of 7 proteins from the IEF fractioned sample were detected with the initiator methionine present and also in the initiator methionine cleaved form (proteins Q96HQ2, Q14974, P62937, P56385, P49189, P46782, P31939). Only one of these proteins was detected in both forms in the unfractionated OVCAR-5 sample. However, both N-terminal forms (with and without initiator methionine) could be detected for 6 of the 7 proteins in 300 ng of the unfractionated OVCAR-5 sample when analysed by DIA using a spectral library generated from the IEF fractionated sample (Supporting Figure 4).

Quantification of PPIA in parental and CBPR OVCAR-5 cells

The IEF fractionated OVCAR-5 results were searched for N-terminally acetylated proteins present in both the modified and un-modified forms, which highlighted the protein peptidyl-prolyl cis-trans isomerase A (PPIA), also known as Cyclophilin A. In order to determine if the N-terminal acetylation of PPIA is altered in chemoresistant cells, CBPR and parental OVCAR-5 cells were analysed by DIA nano-LC-ESI-MS/MS. The level of N-terminal acetylation between the acetylated and un-modified N-terminal peptide of PPIA (VNPTVFFDIAVDGEPLGR) was analysed in the parental and CBPR OVCAR-5 cells (n=3)

revealing 24.4% ±3.3% un-acetylated to acetylated peptide in the parental OVCAR-5 cells compared to 9.4% ±4.2% in the CBPR OVCAR-5 cells, *P*=0.025 (Figure 2.A). This indicates a higher proportion un-acetylated N-terminal peptide in the parental cells as compared to the CBPR cells. Overall quantification of PPIA found the protein to be significantly increased in the parental OVCAR-5 cells as compared to the CBPR OVCAR-5 cells (n=2) 4.1 fold, *P* = 0.015 (Figure 2.C).

Discussion

In-line with the C-HPP, one aim of this study was to map novel N-terminal acetylation sites from proteins extracted from the ovarian cancer cell line OVCAR-5. In order to comprehensively characterise the human proteome, sample fractionation and analysis methods that allow for the detection of previously uncharacterised proteins and modifications are required. Isoelectric focusing of proteins using Sephadex as a matrix has been previously described by Radola *et al.* [26] and Görg *et al.* [27]: the protocol described here has been adjusted to enable the separation of peptides while being directly compatible with downstream analysis by nano-LC-ESI-MS/MS and allowed for the detection of 12 previously un-reported N-terminal protein acetylation sites.

Several protein and peptide pre-fractionation methods are available and are routinely used prior to MS analysis, most utilising strong cation exchange, strong anion exchange or some form of IEF. These methods have been shown to work with peptide amounts of up to one milligram [28], however the best resolution was achieved when peptide loads in the high microgram range were used [29, 30]. The variant of isoelectric focusing presented here allows for separation of peptide amounts of up to at least 10 mg while retaining the resolving power observed at lower loads. The high loading capacity of the technique is ideal for samples available in large quantities that also exhibit a high dynamic range of protein abundance, as it increases the chances of detecting very low abundance peptides, in particular peptides exhibiting PTMs. Further advantages of the method are that it is cost-effective, flexible in the number of fractions sampled, the separation distance is freely adjustable and the user has the ability to parallelize fractionations (e.g. our custom made HD-PE tray allows for simultaneous fractionation of up to 7 samples). Additionally, the method is easy to implement and requires only an electrophoretic chamber with cooling plate. The time required for the

fractionation is relatively short and can usually be finished within 6 hours, inclusive set-up and sampling time (excluding 72 h passive rehydration time required for the Sephadex gel). Therefore, this method is an interesting candidate for laboratories that want to establish their own, more comprehensive spectral libraries for data-independent mass spectrometry experiments (e.g. SWATH or middle-band CID), without the acquisition of additional, highly specialized and expensive equipment.

Overall a total of 4,305 unique proteins were identified in the IEF fractionated OVCAR-5 sample as compared to 2,282 proteins in the unfractionated sample (protein FDR of 1%). From these results 379 proteins were detected as being N-terminally acetylated and 63 proteins were detected as being un-modified at the N-terminus. An interesting study by Lange *et al*. [31] profiled the N-terminal acetylation status of the naturally degraded proteome of human erythrocyte cells using the TAILS method [32]. The majority of the N-termini detected in the study mapped to positions within the protein sequence, revealing proteolytic processing in 64% of cases with a large amount of N-terminal acetylation. Lange *et al*. then analysed the frequency of the specific N-terminally acetylated amino acids observed and were able to define a stabilising N-end rule for N-termini generated by post translational proteolytic cleavage. The results showed Leu, Ile, and Phe to have the highest levels of acetylation, followed by Ala and Val, with little Ser and Thr N-terminal acetylation. These findings are in contrast to the data presented in this study, which found high levels of Ala, Met, and Ser N-terminal acetylation, a low level of Thr and Val N-terminal acetylation, and no Leu, Ile, and Phe N-terminal acetylation. The differences observed could be a reflection of N-terminal processing of original or intact protein N-termini as compared to Lange *et al.* which investigated new N-termini produced as a result of proteolytic cleavage.

The 379 proteins detected as being N-terminally acetylated in the IEF fractionated and unfractionated OVCAR-5 cells were mined for N-terminally acetylated proteins present in both the modified and un-modified forms. Such proteins were of interest for analysis in responsive parental and carboplatin resistant (CBPR) OVCAR-5 cells with the aim of determining whether variable N-terminal acetylation may be involved in, or altered during, the development of chemoresistance. The protein peptidyl-prolyl cis-trans isomerase A (PPIA), also known as Cyclophilin A or Cyclosporin A-binding protein, was detected using these parameters and selected for further analysis given the protein's potential involvement in chemoresistance [17]. When compared to the total amount of detected PPIA N-terminus, the relative abundance of the un-modified to acetylated form was 24.4% ±3.3% in the un-treated parental cells and 9.4% ±2.7% in the CBPR cells (P=0.025). This result suggests a trend towards more un-acetylated PPIA N-terminus in the cells that had not been treated with Carboplatin. Given the first amino acid of the PPIA N-terminus is Val (after cleavage of the initiator Met) using the Ac/N-end rule pathway, the presence of more un-acetylated N-terminus would suggest less degradation. Upon further investigation the overall relative abundance of PPIA was found to be significantly increased in the parental OVCAR-5 cells compared to the treated carboplatin resistant cells 4.1 fold, P=0.015.

PPIA is a peptidyl-prolyl cis-trans isomerase (PPIase), a family of proteins with molecular chaperone functions that also catalyse a rate-limiting protein folding process. PPIA falls directly under the transcriptional control of p53 and HIF1 alpha and has been implicated in a number of cancers with expression levels correlating to malignant transformation [33, 34]. Expression of PPIA is essential for the conformational maintenance of oncogenes, signaling proteins involved in cell proliferation, anti-apoptotic functions, transcription factors, and cell motility regulatory proteins [17]. The immunosuppressant drug cyclosporine A is known to

inhibit PPIA, a drug that has the capacity to sensitise Cisplatin and Carboplatin resistant ovarian tumours [35]. Given PPIA can protect cells against oxidative-stress induced apoptosis [36, 37] it has been hypothesised that cyclosporine A may act to reduce chemoresistance partially via the inhibition of PPIA. A study analysing gene expression prior to and following chemotherapy in ovarian tumour tissues found PPIA expression to be increased 2 fold following treatment, however the result was not significant [38]. A study analysing chemoresistance to the drug 3-bis (2-chloroethyl)-1-nitrosourea in malignant rat glioma cells found PPIA to be decreased in the drug resistant compared with responsive cells at the protein level [39], confounding the results observed here. Interestingly a study analysing paclitaxel chemoresistance in the ovarian cancer cell line SKOV3 found another PPIase, FKBP5, to be transiently up-regulated 100 fold at the mRNA level whilst chemoresistance was being established in the cells [40]. Long term expression of FKBP5 however, was decreased in the resistant cells compared to the responsive cells.

Conclusion

Further validation of the overall expression and variable N-terminal acetylation of PPIA in responsive versus chemoresistant ovarian cancer cell lines is required to determine if PPIA is involved in, or altered by, Carboplatin treatment. Ideally the expression and N-terminal acetylation status of PPIA would be analysed in patient samples prior to and following chemotherapy. Such an analysis is hampered only by the difficulty in collecting tissue samples following treatment, as patients rarely receive further surgery at this point. In summary the novel IEF peptide fractionation method presented here is a robust, reproducible, and a cost effective way of increasing the sequence coverage of protein digests and post-translationally modified peptides analysed by nano-LC-ESI-MS/MS. Although we did not conduct a direct comparison of our method to targeted N-terminomic methods like

COFRADIC [41] or TAILS [32], the data presented implies that our method is capable to cover N-terminally modified peptides in at least a complementary fashion, as indicated by their entries into the neXtProt database Therefore our method is of particular use when establishing spectral libraries for DIA analysis.

Figures

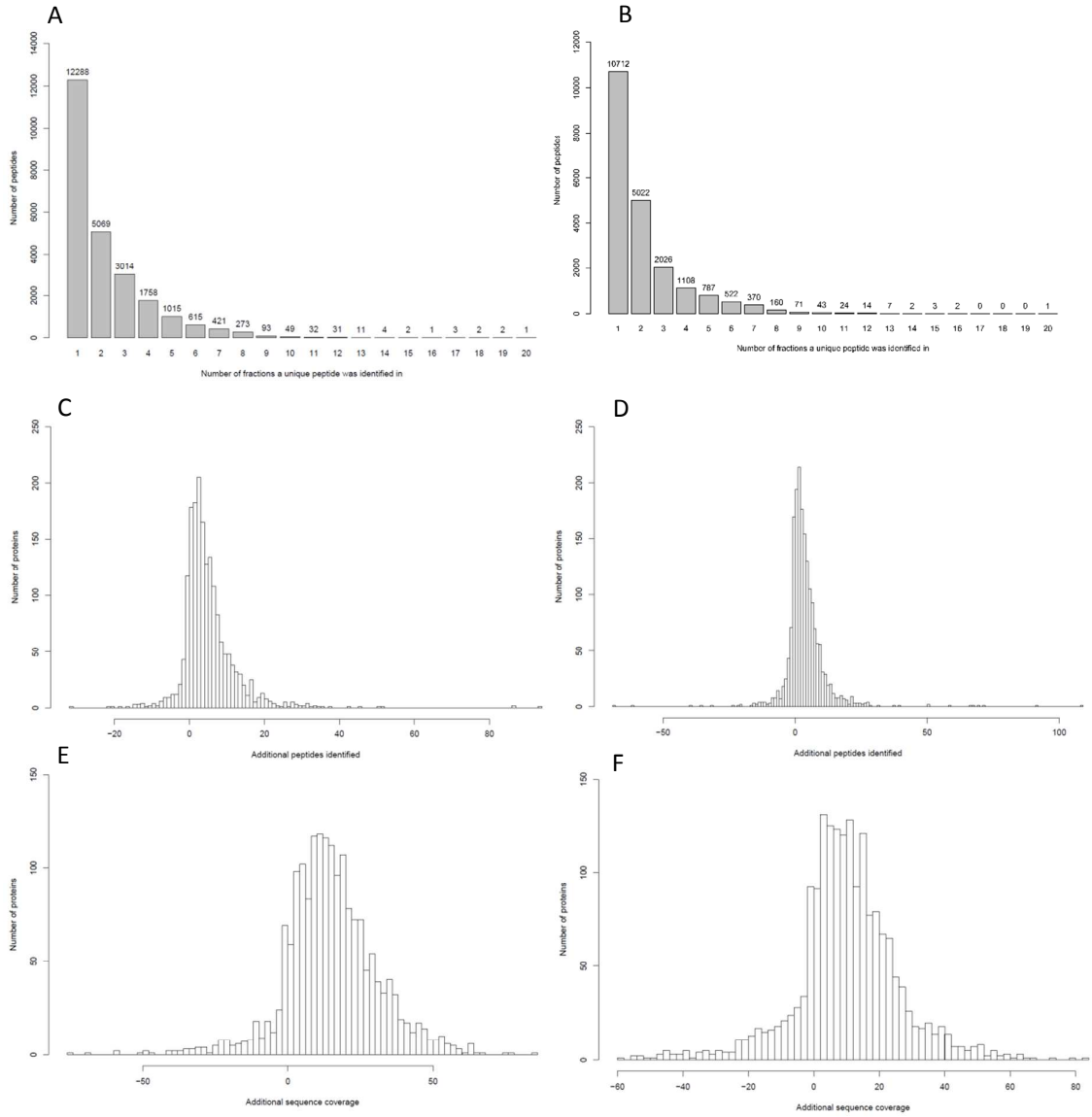
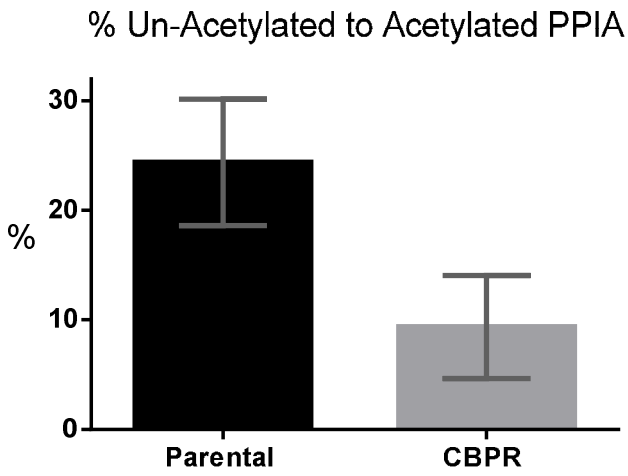
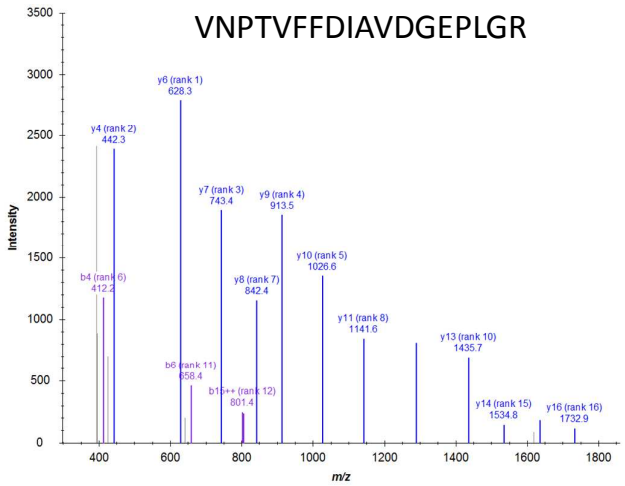


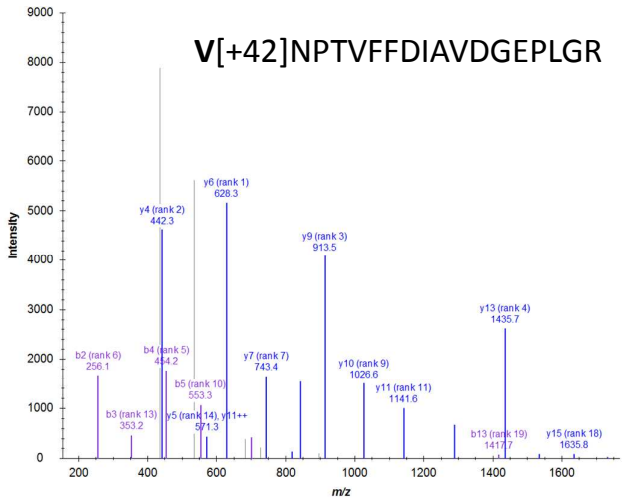
Figure 1. Verification of the IEF Sephadex gel flatbed electrophoresis method for peptides using mouse brain. The number of fractions a specific peptide was identified in (1mg mouse brain IEF (A) and 10 mg mouse brain IEF(B)). The number of additional peptides identified per protein in the 1 mg of mouse brain IEF fractionated sample as compared to the unfractionated sample. The median number of additionally identified peptides was 4. D) The number of additional peptides identified per protein in the 10 mg of mouse brain IEF fractionated sample as compared to the unfractionated sample. The median number of additionally identified peptides was 3. E) The additional protein sequence coverage in the 1 mg of mouse brain IEF fractionated sample as compared to the unfractionated sample, a mean increase of 14.86%. F) The additional protein sequence coverage in the 10 mg of mouse brain IEF fractionated sample as compared to the unfractionated sample, a mean increase of 10.02%.

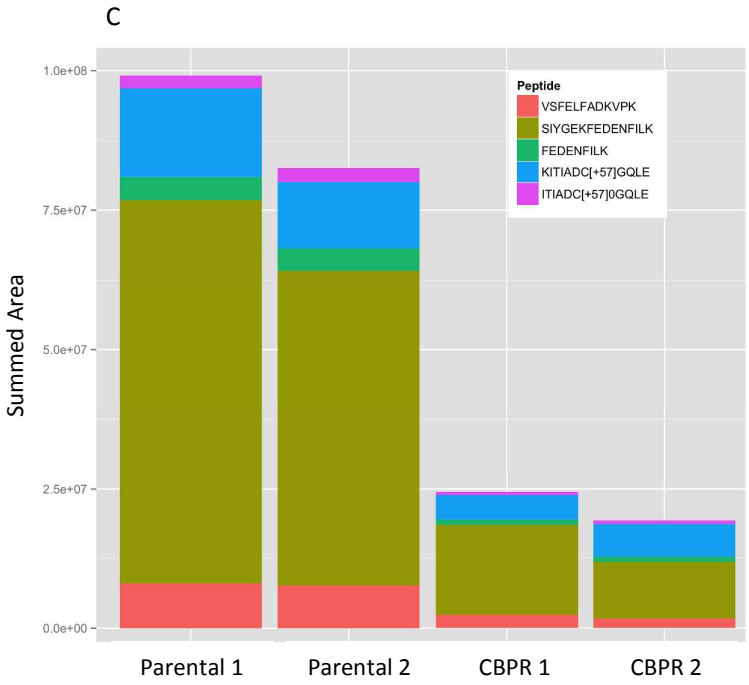
A

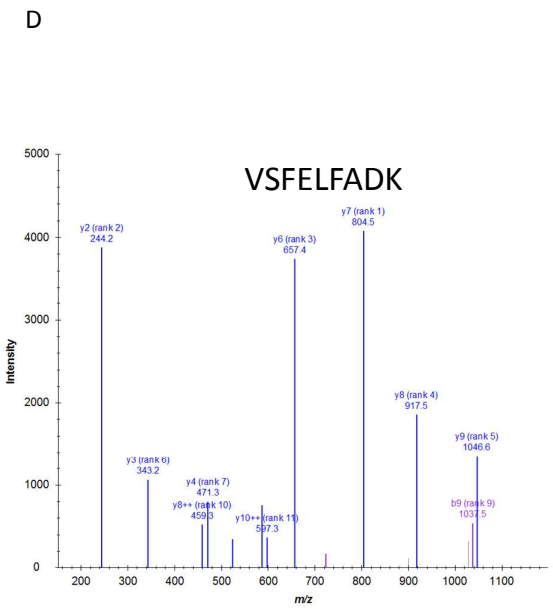

% Un-Acetylated to Acetylated PPIA
%
30
20
10
0
Parental
CBPR

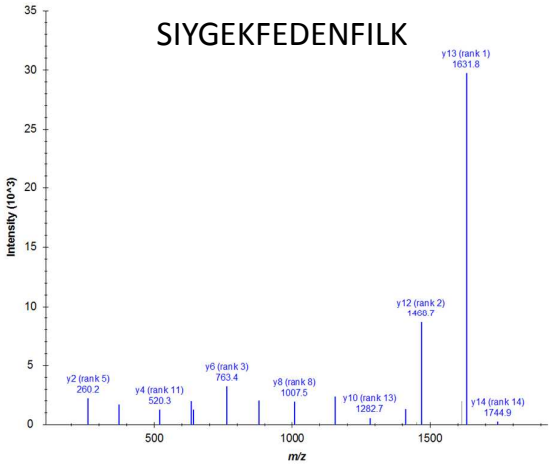
B

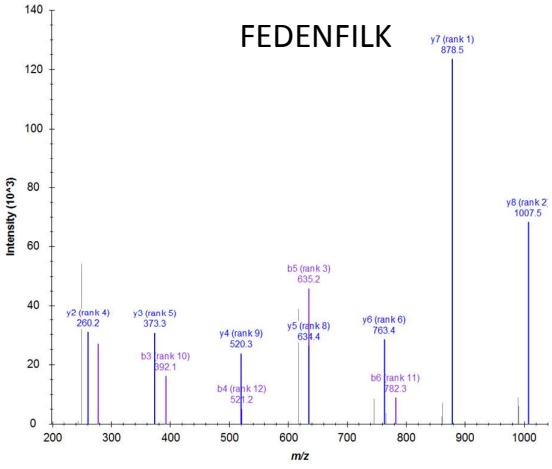

VNPTVFFDIAVDGEPLGR
Intensity
m/z
y6 (rank 1) 628.3
y4 (rank 2) 442.3
y7 (rank 3) 743.4
y9 (rank 4) 913.5
y10 (rank 5) 1026.6
b4 (rank 6) 412.2
y8 (rank 7) 842.4
y11 (rank 8) 1141.6
y13 (rank 10) 1435.7
b6 (rank 11) 658.4
b13++ (rank 12) 801.4
y14 (rank 15) 1534.8
y16 (rank 16) 1732.9

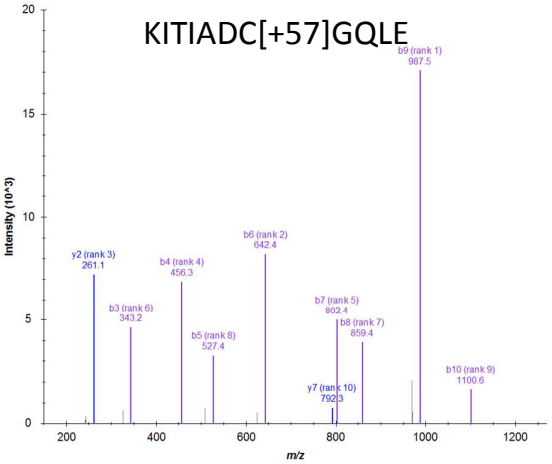

V[+42]NPTVFFDIAVDGEPLGR
Intensity
m/z
y6 (rank 1) 628.3
y4 (rank 2) 442.3
y9 (rank 3) 913.5
y13 (rank 4) 1435.7
b2 (rank 6) 256.1
b4 (rank 5) 454.2
y7 (rank 7) 743.4
y10 (rank 9) 1026.6
b5 (rank 10) 553.3
y11 (rank 11) 1141.6
b3 (rank 13) 353.2
y5 (rank 14), y11++ 571.3
b13 (rank 19) 1417.7
y15 (rank 18) 1635.8


C
Peptide
VSFELFADKVPK
SIYGEKFEDENFILK
FEDENFILK
KITIADC[+57]GQLE
ITIADC[+57]0GQLE
Summed Area
1.0e+08
7.5e+07
5.0e+07
2.5e+07
0.0e+00
Parental 1
Parental 2
CBPR 1
CBPR 2


D
VSFELFADK
Intensity
m/z
y2 (rank 2) 244.2
y3 (rank 6) 343.2
y4 (rank 7) 471.3
y6 (rank 3) 657.4
y7 (rank 1) 804.5
y8 (rank 4) 917.5
y9 (rank 5) 1046.6


SIYGEKFEDENFILK
Intensity (10^3)
m/z
y13 (rank 1) 1631.8
y12 (rank 2) 1460.7
y14 (rank 14) 1744.9


FEDENFILK
Intensity (10^3)
m/z
y7 (rank 1) 878.5
y8 (rank 2) 1007.5
b5 (rank 3) 635.2
y6 (rank 6) 763.4


KITIADC[+57]GQLE
Intensity (10^3)
m/z
b9 (rank 1) 987.5
b6 (rank 2) 642.4
y2 (rank 3) 261.1
b4 (rank 4) 456.3

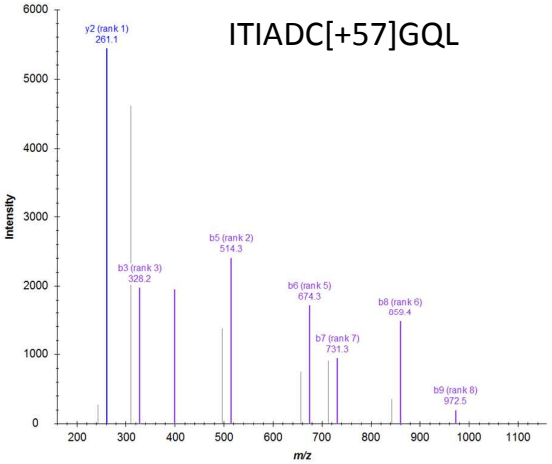

ITIADC[+57]GQL
Intensity
m/z
y2 (rank 1) 261.1
b5 (rank 2) 514.3
b3 (rank 3) 328.2
b6 (rank 5) 674.3
b8 (rank 6) 859.4
b7 (rank 7) 731.3
b9 (rank 8) 972.5

Figure 2. Relative abundance of PPIA in un-treated parental OVCAR-5 cells compared to CBPR OVCAR-5 cells as quantified by DIA nano-LC-MS/MS. A) The percentage of Un-Acetylated to Acetylated VNPTVFFDIAVDGEPLGR in the parental (24.4% ±3.3%) and CBPR (9.4% ±2.7%) OVCAR-5 cells (n=3), *P*=0.025. B) Peptide spectra of VNPTVFFDIAVDGEPLGR and V[+42]NPTVFFDIAVDGEPLGR used as library references for the relative quantification analysis performed in the software Skyline. C) Relative abundance of PPIA in the parental ($9.1x10^7$ ±1.2 $x10^7$) and CBPR ($2.2x10^7$ ±3.6 $x10^6$) OVCAR-5 cells (n=2) as quantified from the peptides VSFELFADK (+3), SIYGEKFEDENFILK (3+), FEDENFILK (+2), KITIADC[+57]GQLE (+2), and ITIADC[+57]GQLE (+2). PPIA was increased in the parental cells 4.1 fold as compared to the CBPR cells, *P*=0.015. D) Spectra for the reference library peptides used for the relative quantification of PPIA.

Table 1. Novel N-terminal peptides grouped via N-acetylation and initiator Methionine cleavage.

Group	Count[a]	N-acetylation[b]	Initiator M cleavage[c]	Initiator Methionine neXtProt[d]	N-acetylation neXtProt[e]	Novel N-terminus[f]	Novel N-Acetylation[g]	Count[h]	Novel N-terminus[i]	Novel N-Acetylation[j]
1	5	at Position 1	-	-	-	-	x	3	-	x
2	26	at Position 2	x	-	-	x	x	9	x	x
3	1	at Position 2	x	x	-	-	x	0	-	x
4	4	-	x	-	at Position 1	x	-	0	x	-
5	12	-	x	-	-	x	-	1	x	-
Total[k]	**48**	**-**	**-**	**-**	**-**	**44**	**32**	**13**	10	12
IEF total[l]	47	-	-	-	-	44	31	13	10	12
IEF exclusive[m]	42	-	-	-	-	40	27	12	10	11
Unfractionated total[n]	6	-	-	-	-	4	5	1	0	1
Unfractionated exclusive[o]	1	-	-	-	-	0	1	0	0	0
Overlap[p]	5	-	-	-	-	4	4	1	0	1

[a] Number of identified peptides not described in neXtProt

[b] Position of N-acetylation of identified peptide

[c] Cleavage of initiator methionine of identified peptide detected

[d] Initiator methionine removed (x) or alternation according to neXtProt

[e] Position of N-acetylation according to neXtProt

[f] N-terminus has not been described in the literature before according to neXtProt

[g] N-acetylation has not been described in the literature before according to neXtProt

[h] Number of identified peptides not described in neither neXtProt nor PRIDE

[i] N-terminus has not been described neither in neXtProt nor in PRIDE

[j] N-acetylation has not been described neither in neXtProt nor in PRIDE

[k] Total number of N-terminal peptides identified

[l] Total number of N-terminal peptides identified in IEF fractionated OVCAR-5 cell line

[m] Total number of N-terminal peptides identified exclusively in IEF fractionated OVCAR-5 cell line

[n] Total number of N-terminal peptides identified in unfractionated OVCAR-5 cell line

[o] Total number of N-terminal peptides identified exclusively in IEF fractionated OVCAR-5 cell line

[p] Total number of N-terminal peptides identified in IEF fractionated and unfractionated OVCAR-5 cell line

Supporting Information

Table S-1a. OVCAR-5 IEF fractionated nano-LC-ESI-MS/MS protein identification results.

Table S-1b. OVCAR-5 nano-LC-ESI-MS/MS protein identification results (unfractionated).

Table S-2a. N-terminal site identification results from the IEF fractionated and unfractionated OVCAR-5 cells.

Table S-2b. Novel N-terminal site identification results from the IEF fractionated and unfractionated OVCAR-5 cells

Table S-3. Number, percentage, and type of the detected acetylated and un-modified N-terminal amino acid residues. Number, percentage, and type of the amino acid following the N-terminal amino acid residue.

Figure S-1. OVCAR-5 IEF peptide fractionation results.

Figure S-2. The chromosomal locations of the corresponding genes for all of the OVCAR-5 identified proteins.

Figure S-3. Annotated spectra for all peptides identified with novel N-terminal acetylation or methionine cleavage sites.

Figure S-4. Detection of N-terminal peptides with and without initiator methionine in the unfractionated OVCAR-5 cells by DIA.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

CBPR, carboplatin resistance

COFRADIC, combined fractional diagonal chromatography

DIA, data independent acquisition

PPIA, peptidyl-prolyl cis-trans isomerase A

TAILS, terminal amine isotopic labeling of substrates

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