Single Cell Chemical Proteomics (SCCP) Interrogates the Timing and Heterogeneity of Cancer Cell Commitment to Death

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Abstract

Chemical proteomics studies the effects of drugs upon cellular proteome. Due to the complexity and diversity of tumors, the response of cancer cells to drugs is also heterogeneous, and thus proteome analysis at single cell level is needed. So far, single cell proteomics techniques have not been quantitative enough to tackle the drug effects on the target proteins. Here, we developed the first single cell chemical proteomics (SCCP) technique and studied with it the time-resolved response to anticancer drugs methotrexate, camptothecin and tomudex of individual adenocarcinoma A549 cells. For each drug SCCP identified and quantified 1,000-2,000 proteins across >150 single cells in a time course of treatment up to 48 h, revealing the early emergence of cellular subpopulations committed and uncommitted to death. SCCP represents a novel approach to exploring the heterogeneous response to drugs of cancer cells.

Keywords: TMT labeling; Drug sensitivity; Resistance to drugs; Cell death; Methotrexate; Camptothecin; Tomudex

1. Introduction

Chemical proteomics studies the effects of drugs on cellular proteomes with the purpose of deciphering the targets and mechanisms of action (MOAs) of these molecules. [1–3] When sensitive cells are treated with toxic compounds for extended period of time, mechanistic target proteins become significantly regulated, and their profiling provides the first hint on the compound's targets and MOA.[4,5] This approach has been employed in functional identification of target by expression proteomics (FITExP)[6], which laid ground for the online chemical proteomics ProTargetMiner tool.[1] In FITExP, cells are treated at a LC_{50} concentration for 48 h, by which time half of the cells die. The dying cells detach from the substrate (for adherent cell types) and are found floating on the flask surface. In the remaining (surviving) cells, the drug target's expression level is significantly and specifically regulated up or down, which serves a basis for drug target identification in FITExP. As an example, when cancer cells undergo treatment with methotrexate (MTX), the target protein dihydrofolate reductase (DHFR) becomes highly upregulated before the cells undergo programmed cell death.[7–9] Interestingly, while the proteomes of the dying and surviving cells are very different (lending support to the notion that cell death is the ultimate case of cell differentiation), the drug target behaves in a similar manner in both types of cells. [2]

The adherent cells usually start losing their attachment to the surface after 24 h of treatment at LC_{50} concentration, but the decision to survive or become dying must be made by the cell well before that.[1,10,11] The intricate details of this decision making process are of great scientific interest, as they possibly hold keys to drug resistance mechanisms.[1,6] These decision making processes can only be studied at the single cell level, while all so far reported chemical proteomics studies relied on bulk cell analysis.[12] Cellular heterogeneity is currently analyzed routinely by single cell transcriptomics (SCT),[13] with mRNA levels assumed to be proportional to the protein expression levels. However, at any given moment the concentration of both

mRNA and proteins reflects the balance between their corresponding expression and degradation, and while mRNA transcription and protein expression are linked together rather well, the degradation processes for mRNA and proteins are completely decoupled. As a result, in the biological processes driven mostly by protein expression, mRNA levels provide excellent proxy for protein concentrations, but this correlation seems to break down already at steady states of the cell.[14] In cell death processes mediated by protein degradation (*e.g.,* via caspase proteases), a correlation between mRNA and protein levels cannot be presumed. Therefore, cell heterogeneity in death-related processes can best be studied with single cell proteomics (SCP).

Compared to the rather well developed SCT approaches, SCP methods are still emerging. While some targeted antibody-based immunoassays have been applied to characterize proteins in single cells,[15,16] these approaches are limited to a few dozen proteins per experiment and exhibit strong bias in quantification. Mass spectrometry (MS)-based proteomics can in principle overcome these limitations, but lacking the benefit of PCR, MS proteomic analysis at a single cell level is very challenging due to the extremely low amounts of proteins (ca. 0.2 ng in a mammalian cell), the high dynamic range of protein expression (7 orders of magnitude versus 3-4 orders for mRNAs),[17,18] and the inevitable sample loss during protein extraction, digestion and chromatographic separation of the peptide digest.[19] Consequently, despite the introduction of such ground-breaking SCP methods as SCoPE-MS[19], SCoPE2[20,21] and nanoPOTS[22] that have been able to analyze between 500 and 2000 proteins in diverse cell lines, SCP has not been sufficiently quantitative to apply the techniques of chemical proteomics, such as FITExP. Thus, we have embarked on a painstaking enterprise of solving this issue to develop Single Cell Chemical Proteomics (SCCP).

Most SCP studies so far have considered two different types of cells (*e.g.,* monocytes *vs* macrophage cells or Jurkat *vs* U-937 cells)[19,21] with vastly different proteomes. Separation

of these cells by SCP was relatively straightforward as it could be done using a few most abundant proteins. In contrast, in cells influenced by a drug the most significantly regulated proteins (drug targets) are seldom highly abundant, being frequently found in the abundance-sorted list below the 1000th position. Therefore, our SCCP-development efforts were directed to achieving the following two intermediate objectives. First, average protein abundances in a homogeneous cell population measured by SCP must correlate with the abundances in bulk proteome analysis. This goal was achieved by starting from analyzing as bulk a relatively high number of cells and gradually reducing this number down to single cells, monitoring the correlation with the bulk analysis and systematically troubleshooting when this correlation broke down. A number of issues have been found and resolved related to protein extraction, digestion, labeling with isobaric regents, LC separation, MS acquisition and statistical analysis. At the end, satisfactory correlations between SCP and bulk proteomics results were consistently obtained. The second intermediate goal objective was to detect with SCP the known strong regulation of the drug targets, as in FITExP, with high statistical significance. This again required systematic studies and optimizations.

Here we present the developed SCCP workflow applied to studying in a time-course manner the proteome effects of anticancer drugs MTX, camptothecin (CPT) and tomudex (TDX), also known as raltitrexed. These drugs were applied at LC_{50} concentration to A549 human lung adenocarcinoma cells, leading half of the cells to death in 48 h. Our workflow comprises the isolation of cells using fluorescence-activated cell sorting (FACS), minimal sample preparation including tryptic digestion, tandem mass tag (TMT) isobaric labeling for protein quantification, incorporation of a carrier proteome (CP) to boost the MS signal, chromatographic separation at a low flow rate, MS/MS data acquisitions and SCCP-optimized data processing (see **Figure 1**). The goal of the study was to identify the time scale of the decision-making dying/surviving process, *i.e.*, to reveal at what time the homogeneous cell

106 population started to differentiate under the influence of a drug into cells committed to
107 surviving or dying.

108
109 ## 2. Results

110 ### 2.1. Improving critical steps of the SCCP workflow

111 To establish a robust analytical method, a human lung cancer cell line A549 was selected as a
112 cellular model because of its easy cultivation and handling. The cell size (ca. 10 µm in diameter)
113 permitted isolation of single cells by FACS without using surface markers for gating. The cells
114 treated with MTX (LC_{50} = 1.15 µM), CPT (LC_{50} = 3 µM), TDX (LC_{50} = 50 µM) and vehicle
115 (DMSO) exhibited in FACS homogeneous populations that only changed shape when drug was
116 applied (**Figure S1**). We consider FACS homogeneity (single population) an important element
117 of any SCP study that justifies single cell analysis, as otherwise cell subpopulations can be
118 counted, isolated by FACS and conventionally analyzed as bulk.

119 All drugs were known to strongly regulate their targets after 48 h incubation in A549
120 cells.[1] Since cell lines are known to "drift" with time, we corroborated the regulation of DHFR,
121 topoisomerase 1 (TOP1) and thymidylate synthase (TYMS) as the targets of MTX, CPT and
122 TDX, respectively, in our cellular model using bulk proteome analysis.

123 The sample preparation optimization started from the SCoPE-MS strategy with
124 TMT10plex labeling and 200 cells in the CP channel,[19] and was adapted to maximize the
125 number of detected proteins and the precision of their quantification. Among the optimized
126 parameters was the volume of buffer to capture cells on plate. Smaller volumes give higher
127 protein concentration but are more difficult to handle, which potentially leads to larger losses.
128 The results were comparable for volumes in the 1-5 µL range (16,000-20,000 peptides and
129 2200-2400 proteins quantified), and for practical reasons 5 µL was chosen for all subsequent
130 experiments.

We found that the property of the ABIRD device to significantly (2-3 times) reduce the chemical noise was especially beneficial in SCP, and utilized this device in all experiments. The flow rate across the EASY-Spray™ C18 analytical columns was reduced from the nominal ~300 nL/min to 100 nL/min to increase the signal abundance. Further reduction in the flow rate reflected negatively on spray stability. We also increased the accumulation time in the Orbitrap for MS/MS above the recommended maximum 86 ms to 150 ms. These measures allowed us to identify and quantify over 2000 proteins in a stable way across 64 cells.

Significant improvements were required in data analysis. PCA of raw SCP data gave no separation between the treated and untreated cells; the data clustered instead according to the TMT sets. To address this inherent for SCP batch effect, we employed the batch-compensation function in the *SVA* package using empirical Bayesian approach. We also found that it was important to normalize the peptide and protein abundances by the median abundance in a given cell. Using these approaches, we started to obtain treated-untreated group separation in PCA.

2.2. Introducing split semi-bulk CP

In order to verify that the separation between the cell groups in PCA was related to biology and was not an uncompensated artefact, we introduced a CP in two separate channels. In general, CP channel is commonly used in TMT-SCP to increase the identification rate and reduce the risk of a catastrophic sample loss during sample preparation.[19,23,24] Most often, 200 cells or their equivalent are used for CP, and since its effect on ion signal is additive[25], we split the CP between two channels, with one channel carrying 100 treated cells and another one – 100 untreated cells. The expectation was that the average (or median) protein abundances in single treated cells will correlate with the protein abundances in treated CP, and the untreated cells will correlate with untreated CP.

Failure to observe such correlation between single cell and bulk proteome data in our first such experiments made us reconsider the way how bulk proteome is obtained. In previous experiments, the bulk proteomic analysis was performed in a conventional proteomics way with the use of detergents, S-S bond reducing and alkylating agents, *etc*., which significantly differed from SCP sample preparation. Then we prepared CPs the same way as single cell proteomes, calling such CPs semi-bulk. We used semi-bulk CPs for analysis of 84 MTX-treated and 84 vehicle-treated single cells in 24 TMT11plex sets. Each of these sets these sets carried 7 single cells (3 or 4 for each condition), an untreated semi-bulk CP in the channel 131C and MTX-treated semi-bulk CP in the channel 131N, while two channels (130N and 130C) remained empty. In total, 13,142 peptides were quantified belonging to 1825 proteins. A satisfactory correlation (R=0.64) was obtained between the log2 values of the protein treated-control regulations in the semi-bulk CPs and the median SC data (**Figure 2A**), which was similar to the correlation between the semi-bulk CPs and the bulk-analyzed CPs prepared in the SCP manner from large number of cells (R=0.81, data not shown). In an OPLS-DA model where treated CP, control CP, treated single cell and untreated single cell data were pooled in four respective groups, single cell data clustered together with their corresponding CPs (data not shown). When only the top 25, 200, 900 most abundant proteins as well as all 1825 proteins were used for OPLS-DA, the Q^2 quality coefficient increased with the number of proteins, proving that RIAs from most proteins in single cells were responsible for the observed separation and not just a few abundant molecules. This was an encouraging message for SCCP, as in Chemical proteomics most drug targets have medium abundance.

Importantly, the SCCP dataset contained the MTX target DHFR that was found on the 6th position from the top among the most significantly upregulated proteins (**Figure 2B**). These results gave us confidence that the SCP data can be used in Chemical proteomics for drug target determination and monitoring. In subsequent experiments we continued to use for carrier and

bulk proteomes the SCP protocol – that is, all subsequent CPs and bulk proteomes are "semi-bulk". Also, in subsequent experiments we obtained CPs by sorting 100 or 200 cells into one well, thus ensuring that CP cells undergo the same FACS treatment as single cells.

2.3. TMTpro™ in SCCP workflow

The recently introduced 16-plex TMTpro™ offers the obvious advantage of multiplexing more single cells into one TMT set (12 vs. 7 in TMT11plex™). However, this advantage might come with a price paid in form of a lower single cell reporter ion signal in MS/MS, as it is distributed over larger number of channels. To test whether the trade-off is acceptable for SCCP, we analyzed 96 single A549 cells treated with MTX and 96 vehicle-treated cells with split CPs (in channels 126 and 127N) and obtained somewhat lower number of protein IDs but still comparable with the TMT11plex™ analysis. In total, 1515 proteins were TMTpro-quantified with 8870 peptides in single cells with 133 significantly regulated proteins, and 1874 proteins with 12,317 peptides in the CPs with 326 regulated proteins. The correlation between SC data and CPs was satisfactory (R=0.71), and significant up-regulation of DHFR was also detected for both SCs and CPs. This result confirmed that SCP can be performed using the TMTpro labelling, but as 11-plex TMT provided somewhat deeper proteome coverage, we continued to use it for SCCP purposes. To accommodate more single cells in one TMT11plex set, for single cell analysis subsequently we used one CP (unless specified otherwise), composed of a 1:1 mixture of treated and untreated cells, while separately analyzing these proteomes for calculating the correlation with single cell data.

2.4. Time-course SCCP analysis

The goal of the experiment was to determine the time point at which the attached cells make the decision to die, so that their proteome becomes altered to resemble that of the end-point detached (dying) cells rather than the end-point attached (surviving) cells. For that purpose, the

cells were treated with MTX for 3, 6, 12, 24 and 48 h at LC_{50} concentration. The attached cells at each time point and the detached cells at 48 h were collected, and FACS-isolated 96 cells of each type were analyzed with SCCP using a single CP representing a mixture of the 48 h attached and 48 h detached cells. The bulk proteomes of 48 h detached and attached treated cells were analyzed separately. On average, over 1500 proteins and 10,000 peptides were identified and quantified in single cells at each incubation time. **Figure 3** shows how the attached treated and untreated cell populations, being almost indistinguishable on a PCA plot at 3 h treatment, become gradually separated with time, achieving nearly full separation at 12 h.

In order to identify at each timepoint the cell subgroup that was committed to death and the one committed to survival in the attached treated population, a hierarchical cluster analysis of protein abundances was performed (**Figure S2**). It was assumed that the two most abundant cell clusters represent the subgroups of the future surviving and dying cell. The hypothesis was that, being put on a PCA plot together with the 48 h attached and detached cells representing the two ultimate cell destinies, the two subgroups will reveal their identities by being closer to the respective destiny type. For time points earlier than the commitment event, cell clustering into the two subgroups will be random, and thus both subgroups would end up in the middle of the OPLS-DA plot close to each other.

Both these predictions were confirmed when the median abundances of all 1170 quantified proteins and 100 most abundant proteins in group 1 (G1) and group 2 (G2) separated by clustering analysis of attached cells were used for building an OPLS-DA model. The model also included the data on 48 h attached cells and 48 h detached cells, which represented the final destinations of the survival and dying subpopulations (**Figure 4A**). For 3 h and 6 h treatments, there was an overlap of the dots representing the G1 and G2 clusters, with a separation between them in the direction of the destiny points at 12 h and longer treatment

231 times. G1 was thus acquiring a proteome profile corresponding to the dying fate, while G2
232 represented the surviving subpopulation. As expected, the OPLS-DA separation between these
233 two subpopulations grew with time. Similarly, the number of proteins with significantly
234 changed abundances between the vehicle- and MTX-treated populations increased with time
235 from 32 and 15 proteins at 3 and 6 h to 38, 121 and 134 proteins at 12, 24 and 48 h, respectively.
236 Therefore, the A549 cell commitment to death occurs between 6 and 12 h past MTX treatment.
237 This time scale is consistent with the earlier reports on dynamic proteomics measurements in
238 cells treated with a drug at LC_{50} – in the first hours past treatment, the cells try to overcome the
239 encountered difficulty, activating survival pathways, and only commit to death after such an
240 attempt fails.[1]
241 Interestingly, at 12 h more separation was seen for the whole proteome, while at 24 h the 100
242 most abundant proteins showed bigger separation. This observation agreed well with the notion
243 that the cell path to death starts with the inner mechanism altering lower-abundant mechanistic
244 proteins first, followed by the altering household proteins that change cell morphology.
245 Consistent with this scenario, when the main OPLS-DA coordinates of the individual cells were
246 plotted on a scale normalized such that the attached cells treated for 48 h had x = 1 and the
247 corresponding detached cells had x = -1, the obtained distributions of G1 and G2 cells were
248 separated in 12 h for the full proteome, but less so for 400 most abundant proteins and not at
249 all for top 100 proteins (**Figure 4B**). At the same time, for 24 h treatment the G1 and G2
250 proteomes gave broad distributions separated more for highly abundant proteins, suggesting
251 that cell morphology alteration is well underway.
252 Pathway analysis of 179 proteins with significantly different abundances in G1 versus G2 at 12
253 h past MTX treatment revealed that they preferentially belong to metabolic, carbon metabolism,
254 ribosome- and proteasome-related pathways (**Figure S3)**.
255

2.5. SCCP with camptothecin (CPT) and tomudex/raltitrexed (TDX)

Similar results as with MTX were obtained with CPT and TDX, with the targets TOP1 (downregulated) and TYMS (upregulated) emerging among the top proteins in the respective areas of the volcano plot (**Figure 5**). While these drugs have different MOAs and targets, the A549 cells have clearly formed two well separated clusters in PCA.

2.6. Target percolation by OPLS-DA of MTX, CPT and TDX data

The ultimate goal of a Chemical proteomics experiment is drug target identification, which can be obtained by contrasting a specific treatment against all other treatments and controls. While designing ProTargetMiner[2], we found that on average it takes 30-50 contrasting treatments to identify ("percolate") the target uniquely among thousands of proteins in the proteome as the most specifically up- or down-regulated protein. Here, we merged the MTX, CPT and TDX SCCP data (treatment *vs.* untreated control) at 48 h of treatment and contrasted one drug against the other two (**Figure S4**). For MTX, the target DHFR was 4th most specifically upregulated protein; for CPT, TOP1 was 15th most specifically downregulated protein; and for TDX, TYMS was 10th most specifically upregulated protein. These results demonstrate that SCCP has the potential for unique drug target identification, provided enough contrasting treatments are obtained.

3. Discussion

Considering that cell to cell heterogeneity is a fundamental property of highly complex cellular systems[16], the analysis of proteomes at a single cell level is essential for understanding the complex diseases, such as cancer, where diverse phenotypes contribute to the survival and progression,[26] as well as for studying the mechanisms of cell resistance to anticancer treatment.

Here we demonstrated that SCP can be sufficiently quantitative for enabling Chemical proteomics approaches for drug target identification and monitoring. One important milestone was to achieve significant correlation between the average protein regulation factors in SCCP between the treated and untreated cells with those obtained with conventional bulk proteomics of these cells. Another important milestone was to demonstrate similar behavior of the drug target proteins in SCCP as in bulk Chemical proteomics. Finally, the possibility to "percolate" by a contrasting OPLS-DA analysis the drug targets has a paramount importance for the use of such a powerful drug target deconvolution method as ProTargetMiner.

While these achievements were the SCCP proofs of principle, time course analysis provided new biologically relevant information, confirming that cell commitment to death can now be studied at a proteome level for individual cells. Between 6 h and 12 h past treatment, a large group of attached drug-treated cells already committed to detach and form a floating dying population. Importantly, these changes were detected among the lower-abundant proteins, while highly abundant proteins remained at that point unaffected. It was even possible to determine the pathways and parts of cell machinery participating in the decision-making process.

4. Conclusions

After the quantitative aspect of single cell proteomics has been improved, chemical proteomics at the level of single cells became reality. The detailed profiling with SSCP of the heterogeneity of cancer cell response to drugs or treatments and the mechanistic analysis with cellular resolution of resistance to therapy is now possible. Moreover, the FITExP method of chemical proteomics is now applicable to single cells. The remaining challenges are however vast. For example, SCCP needs to provide deeper proteome analysis, targeting the benchmark of 5,000 proteins quantified with ≥2 peptides. A great achievement would be if complementary tools of

chemical proteomics, such as proteome-wide integral solubility alteration (PISA) assay[27] could be implemented for single cells. With PISA, one could monitor the protein target engagement of the drug molecule. This, however, requires significant efforts in improving the methods of handling and analyzing ultra-small protein amounts.

5. Methods

5.1. Cell culturing treatment

Human A549 lung adenocarcinoma cells obtained from ATCC (Manassas VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM – Lonza, Wakersville MD) supplemented with 10% FBS superior (Biochrom, Berlin, Germany), 2 mM L-glutamine (Lonza) and 100 U/mL penicillin/streptomycin (Thermo, Waltham MA) at 37°C in 5% CO_2.

The LC_{50} values for the drugs (MTX, CPT and TDX) were determined by CellTiter-Blue® cell viability assay (Promega). Cells were seeded into 96 plates at a density of 3000 cells per well, and after 24 h of culture they were treated with serial concentrations of the respective drug: MTX (0 – 100 μM), CPT (0 – 100 μM) and TDX (0 – 100 μM). After 48 h, the media were discarded and replaced with 100 μL of fresh culture media. In each well, 20 μL of resazurin (CellTiter-Blue® Cell Viability Assay kit – Promega) were added to perform the viability assay. After 4 h of incubation at 37°C, fluorescence of wells was measured in Infinite F200 Pro fluorometer (Tecan) by detecting the ratio between the excitation at 560 nm and emission at 590 nm. The LC_{50} values were determined from the dose-response curves by calculating the concentration causing the 50% fluorescence reduction compared with the untreated control.

Cells were then cultured and treated with MTX, CPT and TDX at LC_{50} concentrations in 75 cm^2 flasks for 3, 6, 12, 24 and 48 h (for CPT and TDX only 12, 24 and 48 h treatment were performed). Control cells were treated with the vehicle (10 mM dimethyl sulfoxide - DMSO).

After each incubation time point, the supernatant was collected and the attached cells were disconnected from the surface with TrypLE (Gibco) for 5 min, after which they were harvested by centrifugation at 1000 rpm for 3 min. Both types of cells (detached and adhered) were washed twice with cold 1X phosphate buffered saline (PBS).

5.2. Isolation of single cells by FACS

Collected attached cells were subjected to FACS analysis in FACSAria™ Fusion (BD Biosciences), in which cells were sorted based on the forward and side scatter (FSC/SSC) parameters only. Individual singlet cells were collected in a 96-well Lo-Bind plate (Eppendorf, Hamburg, Germany) containing 5 µL of 100 mM triethylammonium bicarbonate (TEAB) per well. A total of 96 single cells were sorted for each condition/drug (untreated and treated cells) using separate plates. In addition, a third plate was prepared, being dedicated only to CP. On that plate, 200 cells (100 treated plus 100 control) were collected per well in the first two rows. Altogether, 24 wells of CP cells were collected for each treatment and time point.

5.3. Protein extraction and digestion

Proteins from single cells and CPs were extracted in four freeze – thaw cycles. Plates were frozen for 2 min in liquid nitrogen and immediately heated at 37°C for 2 min. Proteins were denatured by heating the plates at 90°C for 5 min. The resulted protein solutions were centrifuged at 1000 rpm for 2 min to spin down all the volume present in the wells. Finally, 1 µL of 25 ng/µL sequencing grade trypsin (Promega, Madison WA) in 100 mM TEAB was dispensed using a MANTIS® automatic dispenser (Formulatrix, Bedford MA). In the case of CP, digestion was achieved with 2 µL of trypsin solution added. Plates were incubated at 37°C overnight (16 h).

5.4. TMT labeling

Both TMT10plex™ (or TMT11plex™ including channel 131C) and 16-channel TMTpro™ (ThermoFisher Scientific, Rockford IL) were used in this study. Unless specified, each TMT10plex™ set contained 4 control cells and 4 treated cells with tags interspaced, as well as a single channel with CP (200 cells in channel 131). TMT 130N was not used because of the cross contamination with the CP channel. Peptides were TMT-labeled by dispensing 1 µL of the respective TMT reagent dissolved in dry acetonitrile (ACN) at a concentration of 10 µg/µL using the MANTIS robot. Plates were incubated at room temperature (RT) for 2 h and then the reaction was quenched by adding 1 µL of 5% hydroxylamine (also with automatic liquid handler), following incubation at RT for 15 min. In the case of TMTpro™ labeling, each set contained 6 control cells and 6 treated cells with the tags interspaced. The CP was split in two channels, 126 and 127N, one composed of 100 control cells and the other one 100 treated cells. Channels 127C and 128N were left empty to prevent cross-contamination from CP channels. The labeled samples were pooled together using a 10-µL glass syringe (VWR, Japan), starting always with the CP samples in each TMT set in order to minimize sample loss during the pooling.[19] Samples were pooled into MS-sample vials with glass insert (TPX snap ring vial from Genetec, Sweden) and dried in a speed vacuum concentrator (Concentrator Plus, Eppendorf). Dry peptides were resuspended in 7 µL of 2% ACN, 0.1% formic acid (FA) prior to LC-MS/MS analysis.

5.5. Bulk proteome analysis

Unless specified, $1x10^6$ were harvested and washed twice with PBS. To each cell pellet, 150 µL of 8M urea in 50 mM ammonium bicarbonate (AmBic) pH 8.5 were added, tubes were sonicated in bath at 4°C for 5 min and probe-sonicated for 40 s with a Vibra-cell™ ultrasonic liquid processor (VCX 130, Sonics & Materials Inc., Newtown CT) using pulse 2/2 with amp

20. Each cell lysate was transferred to a new tube, while the original tube was washed with 150 µL of 50 mM AmBic pH 8.5 and the wash was added to the new tube. Samples were centrifuged for 10 min at 13,000 *g* and 4°C. Protein concentration was measured with BCA protein assay. Proteins were reduced by adding 20 mM dithiothreitol in 50 mM AmBic and incubated at 37°C for 1 h, followed by alkylation with 40 mM iodoacetamide at RT for 30 min in the dark. Finally, proteins were digested by adding trypsin in a 1:50 ratio and incubating overnight at 37°C. The resulting peptides were cleaned-up on HyperSep™ filter plate (ThermoFisher Scientific, Rockford IL) then dried in a speed vacuum concentrator. Dried samples were dissolved in 100 mM TEAB and labeled with TMT10plex™ reagents that were dissolved in dry ACN and then mixed with the peptide solutions and incubated at RT for 1 h. The reaction was quenched by adding 11 µL of 5% hydroxylamine and incubating at RT for 15 min. Finally, samples with different TMT labels were pooled into a single tube, cleaned-up on HyperSep™ filter plate and dried in a speed vacuum concentrator prior to LC-MS/MS analysis.

5.6. RPLC-MS/MS analysis

Peptide samples were separated on a Thermo Scientific™ Ultimate™ 3000 UHPLC (ThermoFisher Scientific) using a 10 min loading at 3 µL/min flow rate to a trap column (Acclaim™ PepMap™ 100, 2 cm x 75 µm, 3 µm, 100 Å - ThermoFisher Scientific). The separation was performed on an EASY-Spray™ C18 analytical column (25 cm x 75 µm, 1.9 µm, 300 Å – ES802A, ThermoFisher Scientific). A constant flow rate of 100 nL/min was applied during sample separation achieved in a linear gradient ramped from 5%B to 27%B over 120 min, with solvents A and B being 2% ACN in 0.1% FA and 98% ACN in 0.1% FA, respectively. LC-MS/MS data were acquired on an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (ThermoFisher Scientific, San José CA), using nano-electrospray ionization in positive ion mode at a spray voltage of 1.9 kV. To reduce atmospheric background and enhance

406 the peptide signal-to-noise ratio, an Active Background Ion Reduction Device (ABIRD - ESI
407 Source Solutions, LLC, Woburn MA) was used on the nanospray ion source. Data dependent
408 acquisition (DDA) mode parameters were set as follows: isolation of top 20 precursors in full
409 mass spectra at 120,000 mass resolution in the *m/z* range of 375 – 1500, maximum allowed
410 injection time (IT) of 100 ms, dynamic exclusion of 10 ppm for 45 s, MS2 isolation width of
411 0.7 Th with higher-energy collision dissociation (HCD) of 35% at resolution of 50,000 and
412 maximum IT of 150 ms in a single microscan. The mass spectrometry proteomics data are
413 deposited to the ProteomeXchange Consortium via the PRIDE partner repository[28] with the
414 data set identifier PXD025481.
415
416 **5.7. Data analysis**
417 Raw data from LC-MS/MS were analyzed on Proteome Discoverer v2.4 (ThermoFisher
418 Scientific), searching proteins against SwissProt human database (release July 30, 2019 with
419 20,373 entries) and known contaminants with Mascot Server v2.5.1 (MatrixScience Ltd, UK)
420 allowing for up to two missed cleavages. Mass tolerance for precursor and fragment ions was
421 10 ppm and 0.05 Da, respectively. Oxidation of methionine, deamidation of asparagine and
422 glutamine, as well as TMTpro or TMT6plex lysine and N-termini were set as variable
423 modifications. Percolator node[29] in Proteome Discoverer was set to target false discovery rate
424 (FDR) at 1% with validation based on *q*-value.
425 The TMT reporter ion abundances (RIA) at a peptide level were extracted from the search
426 results. The subsequent analyses were performed in the RStudio (version 1.3.1073)
427 programming language environment, the software for multivariate data analytics SIMCA (v.
428 15.0.2.5959, Sartorius) and Perseus software platform.[30] Peptides from single cells with RIAs
429 exceeding 10% of the abundance values for the respective carrier channel were filtered out,
430 being considered a result of co-isolation or other interferences, resulting in about 30% of the

peptides discarded for further analysis. After filtering, the remaining RIAs were arranged into a matrix of peptide IDs (rows) *vs.* single cells (columns). All RIAs were log2-transformed and the data were normalized in columns by subtracting their median values computed, ignoring the missing values. Peptides quantified in less than ten cells were discarded (usually <0.05% peptides per dataset). Protein-level quantification was achieved by attributing each unique peptide to its respective top ranked protein within a protein group. As protein relative abundance, the median RIA value among the peptides belonging to that protein was taken. The new relative abundance matrix (protein IDs *vs.* single cells) was again normalized by calculating the median value for each column (or single cell), and then, subtracting the median value calculated to each abundance on the respective column. Missing values in the resulting matrix were imputed based on the normal distribution of valid values (method available in Perseus software platform[31]), using a width of 0.3 standard deviations of the Gaussian distribution of the valid values and a downshift of 1.8 standard deviations. Finally, the batch effects across the TMT sets were corrected by applying an empirical Bayesian framework in the SVA package[32].

The obtained matrix of relative protein abundances was used for statistical analysis. Principal component analysis (PCA) was performed to determine the separation degree between the control and drug-treated cells and to identify the outliers (single cells outside the limits of the PCA diagram with $p < 0.05$), which were removed from subsequent analysis. Resulting data were analyzed by orthogonal partial least squares discriminant analysis (OPLS-DA) and clustering analysis, and the fold changes were presented as volcano plots.

6. Acknowledgements

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7. Authors contributions

RAZ conceptualized the study. AV and JER contributed to the conception and design of the study. AV and JER performed experiments and data analysis. RAZ wrote the manuscript and all authors contributed to the article and approved the submitted version.

8. Competing interest statement

The authors declare no competing interest.

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10. Figure legends

Figure 1. SCCP workflow. The workflow developed for SCCP included cell culturing and treatment with drugs, isolation of individual cells by FACS, protein extraction and digestion, TMT labeling of thus obtained tryptic peptides followed by multiplexing, LC-MS/MS and statistical data analysis. All steps are optimized for achieving the desired proteome depth and quantitative correlation with bulk analysis. In the figure, split carrier proteome occupies two channels (131N and 131C) in a TMT11plex set, with two other channels (130N and 130C) remaining empty (doted lines). Identification of peptides is achieved via matching masses of sequence-specific fragments, and quantification is performed by the abundances of the low-mass TMT reporter ions.

Figure 2. Benchmarking SCCP data. A) Correlation between the protein regulations in the semi-bulk CPs (y-axis) and the median SC data (x-axis). B) Volcano plot of protein regulations obtained from SCCP data showing MTX target DHFR to be among the most significantly upregulated proteins. The curved lines separate the areas with significantly regulated proteins.

Figure 3. Time-course results upon treatment with MTX. PCA plots of SCCP data as a time course demonstrating the emergence of separation between the MTX-treated and untreated attached cells with incubation time, and the corresponding volcano plots of regulated proteins showing the emergence of DHFR among the top regulated proteins.

Figure 4. Statistical analysis of single cells treated with MTX. A) OPLS-DA analysis of SCCP data on median protein abundances in G1 and G2 cell groups from MTX treated cells at different time points together with bulk CP abundances for the total proteome (1170 proteins)

and top 100 most abundant proteins. B) Distribution of the main OPLS-DA coordinates of G1 and G2 groups of MTX-treated attached cells at 12 h and 24 h past treatment for total proteome, top 400 and top 100 proteins. The x-coordinates were normalized such that the coordinates of the attached and detached cell bulk-analyzed proteomes after 48 h treatment are +1 and -1, respectively.

Figure 5. Time-course results upon treatment with CPT and TDX. PCA plots of SCCP data as a time course demonstrating the emergence separation between the untreated cells and the attached cells treated with A) CPT and B) TDX with incubation time, and the corresponding volcano plots of regulated proteins showing the emergence of the known drug target among the top regulated proteins.

Figures

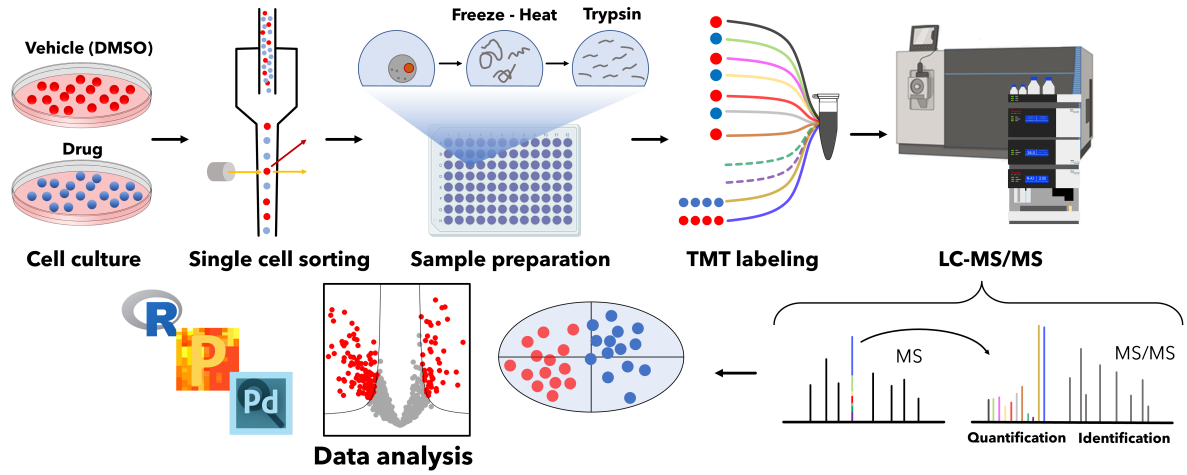
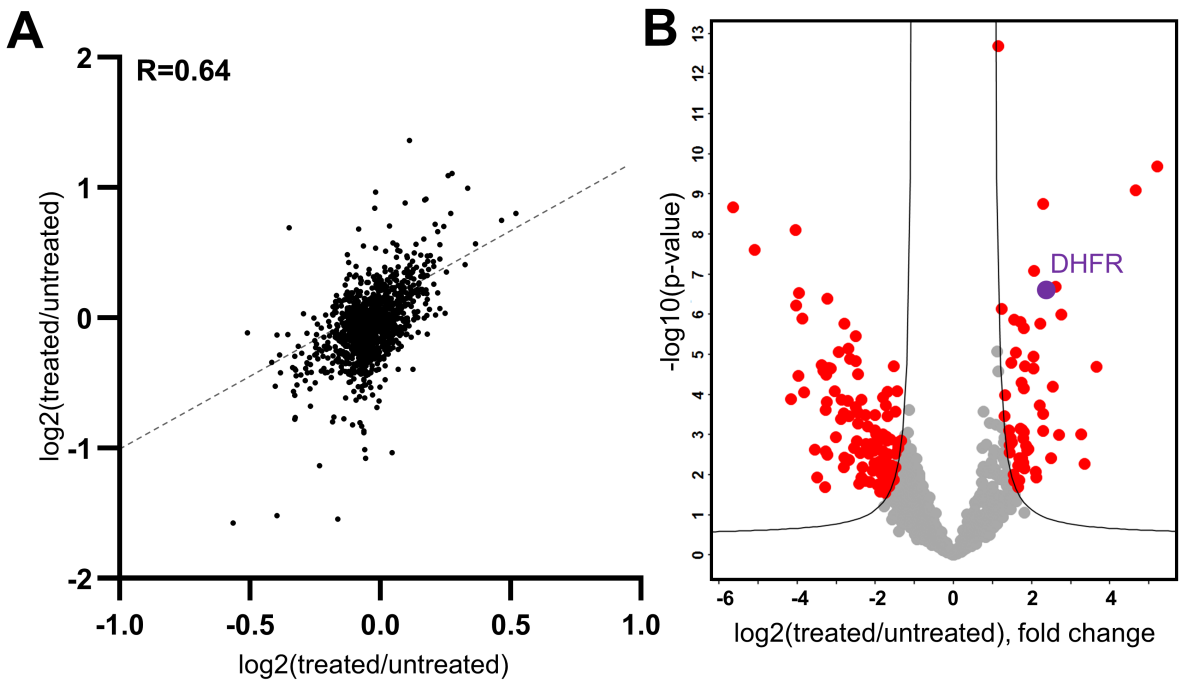


Figure 1.



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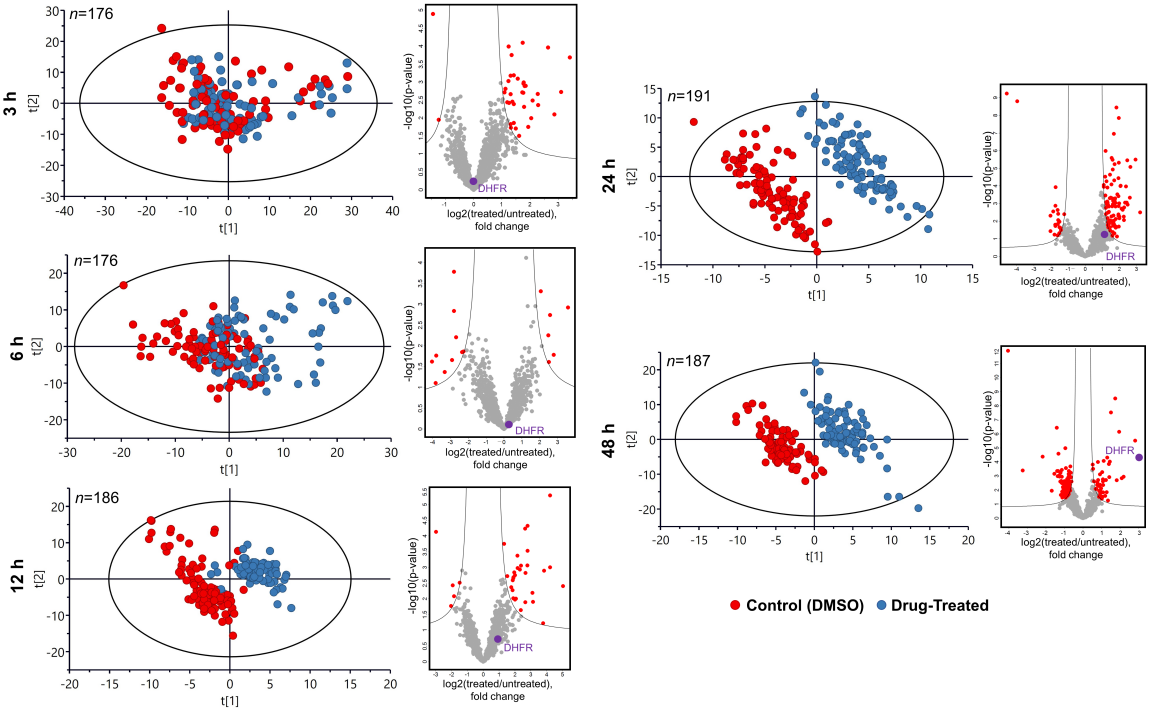

Figure 3.

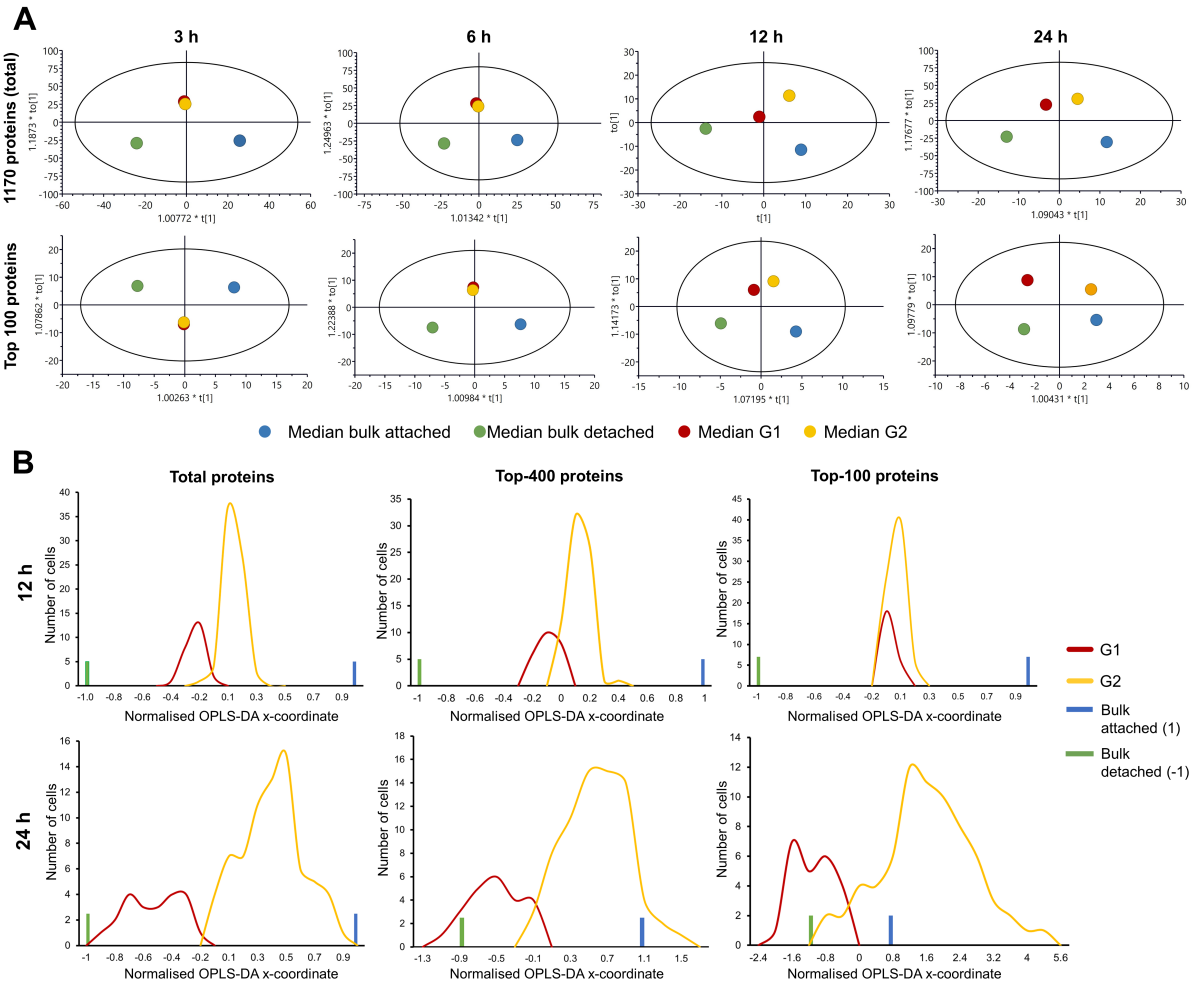

Figure 4.

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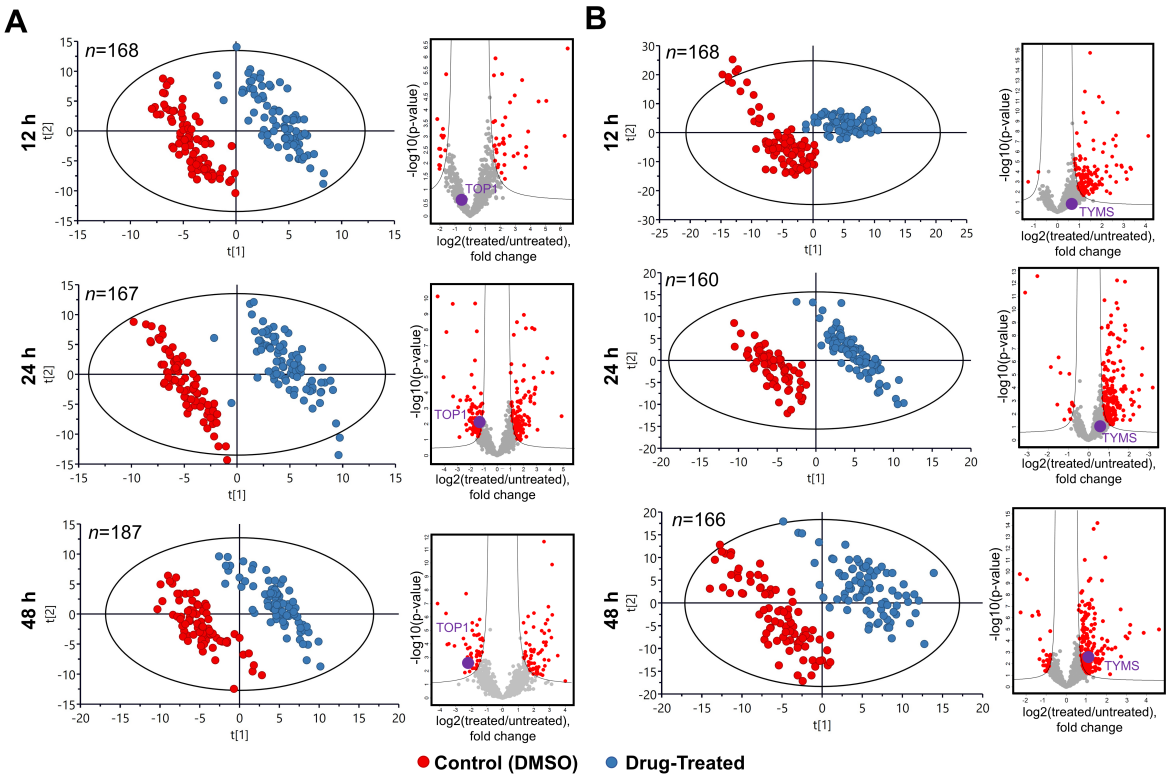

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616 **Figure 5.**
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