

Thermal proteome profiling for unbiased identification of direct and indirect drug targets using multiplexed quantitative mass spectrometry

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The direct detection of drug-protein interactions in living cells is a major challenge in drug discovery research. Recently, we introduced an approach termed thermal proteome profiling (TPP), which enables the monitoring of changes in protein thermal stability across the proteome using quantitative mass spectrometry. We determined the intracellular thermal profiles for up to 7,000 proteins, and by comparing profiles derived from cultured mammalian cells in the presence or absence of a drug we showed that it was possible to identify direct and indirect targets of drugs in living cells in an unbiased manner. Here we demonstrate the complete workflow using the histone deacetylase inhibitor panobinostat. The key to this approach is the use of isobaric tandem mass tag 10-plex (TMT10) reagents to label digested protein samples corresponding to each temperature point in the melting curve so that the samples can be analyzed by multiplexed quantitative mass spectrometry. Important steps in the bioinformatic analysis include data normalization, melting curve fitting and statistical significance determination of compound concentration-dependent changes in protein stability. All analysis tools are made freely available as R and Python packages. The workflow can be completed in 2 weeks.

INTRODUCTION

The comprehensive identification of a drug's targets is one of the biggest challenges in current-day drug discovery¹. Recently, Pär Nordlund and colleagues at the Karolinska Institute developed the cellular thermal shift assay (CETSA)^{2,3}; this method can assess whether a protein binds a drug in cell extracts, living cells or *in vivo*. The cornerstone of the CETSA method is the long-known fact that a protein complexed to a ligand tends to become more resistant against heat-induced unfolding⁴. This phenomenon has been used for more than a decade to systematically screen recombinant proteins against potential inhibitors in a thermal shift assay⁵. Compounds that alter the melting point (T_m) of a protein are considered binders of the protein under investigation. However, the binding of a compound to a purified protein is not a fail-proof predictor of target engagement in cells, in which the cell permeability of the compound, as well as many other factors such as the target's interactions with other proteins^{6,7} and cofactors⁸, have important roles. The ability to assess drug-protein interactions in a physiologically relevant setting by determining changes in the thermal stability of a protein upon compound treatment in living cells using CETSA, and then subsequently determining compound potency by an isothermal dose-response (ITDR)² experiment, is regarded as a major step forward in drug discovery research⁹. The initial readout from CETSA and ITDR experiments was restricted to antibody-based detection by western blotting. Although this enables verification of the engagement and potency of predefined targets, it lacks the potential for detecting unexpected targets.

Recently, we combined the concepts of CETSA and ITDR with multiplexed quantitative mass spectrometry^{10,11} and performed the first large-scale unbiased TPP in a human cell line¹².

We found that TPP performed over the temperature range (TPP-TR) of 37–67 °C and at a fixed compound concentration enabled an unbiased assessment of the proteins targeted by a drug on a proteome-wide scale. Initial experiments were performed using the pan-kinase inhibitor staurosporine, which has a large number of known protein targets. We were able to detect target engagement for many of these, as well as for several hitherto unknown non-kinase targets—e.g., ferrochelatase, in human K562 cell extracts.

We also discovered that treatment of living K562 cells with the ABL inhibitor dasatinib led to altered thermal stability not only of the drug's direct targets but also of indirect targets—for example, the v-crk avian sarcoma virus CT10 oncogene homolog-like protein (CRKL), a well-characterized member of the BCR-ABL pathway¹³, which is permanently switched on in K562 cells, showed a substantial T_m shift. TPP experiments that analyze the compound concentration-dependent stabilization or destabilization of a protein at fixed temperatures (TPP-compound concentration range (CCR))^{2,12} revealed a dasatinib concentration required to elicit the T_m shift in CRKL that was consistent with the concentration required for the inhibition of cell growth. Notably, the thermal stability of the large BCR-ABL fusion protein was not affected by dasatinib, indicating that this protein is not stabilized by the drug.

However, the thermal stability of the BCR-ABL fusion protein in untreated K562 cells was markedly different from the thermal stability of the ABL1 protein profiled in untreated human Jurkat cells that do not have the BCR-ABL fusion, indicating that thermal profiling could be used to identify protein fusions¹².

The TPP methodology therefore has the potential to identify new (off-)targets and indirect targets of drugs in living cells that can be further validated by using orthogonal biochemical and functional assays.

Overview of the protocol

The aim of this protocol article is to give step-by-step instructions for performing TPP experiments, including data analysis with open-source software. The key requirement for TPP experiments is multiplexed mass spectrometry^{10,11}. Bioinformatics analysis tools have been specifically designed for mass spectrometry experiments in which multiple biological samples are combined after labeling with isobaric mass tags¹⁴ and then analyzed in a single mass spectrometry experiment. The advantage of doing this is that the user performs fewer mass spectrometry experiments (less instrument time) and that the variation between the sample workup and instrument conditions is reduced¹⁴. The software tools are also necessary for determining the relative changes in concentration for each peptide or protein (the fold change) as a function of temperature and drug treatment.

This protocol starts from the point at which compound treatment and heat treatment of cells have been performed as described in the Jafari *et al.*³ protocol for either the CETSA or the ITDR experiment. Although we do not reiterate the experimental steps from the previous protocol³ (**Supplementary Methods**), we highlight necessary considerations for making the samples amenable for mass spectrometry analysis. The protocol describes the experimental and analysis steps for performing TPP-TR and TPP-CCR experiments. Two software packages are made available: isobarQuant, written in Python; and TPP, written in R. The isobarQuant package, in combination with the widely used, commercially available search engine, Mascot¹⁵, provides a workflow for identifying and quantifying proteins from mass spectrometry samples labeled with isobaric mass tags¹⁶. The R package TPP provides the functionality needed to analyze the quantified proteins from TPP experiments across different temperatures (the TR workflow), as well as different compound concentrations at a fixed temperature (the CCR workflow). We use the treatment of K562 cells with the histone deacetylase (HDAC) inhibitor panobinostat^{6,17} as an example. This experiment demonstrates the *in situ* effect of panobinostat on several HDAC targets in living cells, but it also shows a hitherto unknown effect of panobinostat treatment on the tetratricopeptide repeat protein 38 (TTC38).

Applications of the method

The key feature of the TPP methodology is the ability to measure the target occupancy of drugs by assessing thousands of proteins in parallel. As outlined in both original publications, this can be used to identify targets of drugs in cell extracts, living cells^{2,12} and also in tissues². Whereas the use of antibodies to quantify proteins can provide evidence for ligand binding and target engagement, as well as enable primary screening for selected cognate targets, quantitative mass spectrometry makes it possible to obtain such data for entire cellular proteomes. This is not limited to proteins directly binding the ligand, but it also allows the identification of indirect targets of drug treatment in living cells (**Box 1**)¹². Therefore, TPP is an ideal tool for addressing issues and challenges throughout all steps of drug discovery. For example, the recent renaissance of phenotypic screening¹⁸ requires

additional methods to determine both individual proteins and entire pathways targeted by the identified bioactive molecules. The relatively low amount of cell material required for these experiments makes TPP also applicable to primary cells and tissues. The approach should be beneficial to support efforts to reduce late-stage failure of compounds in clinical development either because of a lack of target engagement and therefore efficacy, or because of adverse effects caused by drug interaction with unexpected targets that mediate toxic effects^{19,20}. We believe that application of TPP in early-stage drug discovery will lead to reduced costs, as it could identify some of these obstacles well ahead of costly commitments. Conventional screening of a small number of target panels is not adequate, because most drug candidates or even approved drugs have multiple physiological targets, and it is often not possible to predict what these will be^{21–23}. These additional targets might also be essential to achieve the therapeutically relevant efficacy. The use of this beneficial ‘polypharmacology’ effect has been an emerging concept in recent years; a good example is that the kinase inhibitors used in cancer therapy perform their drug functions by acting on multiple kinases²⁴. The TPP methodology could serve as an important approach to help understand and rationally design multitarget ligands, as well as supporting the identification of additional therapeutic opportunities for both candidate molecules and drugs that are already approved for use.

Unbiased detection of altered protein states, resulting from the activation of specific pathways, could also be applied to the detection of pathway activation caused by nonpharmacological events, such as genetic or epigenetic changes in cells—e.g., the effects of the BCR-ABL fusion protein in Philadelphia chromosome-containing cell lines¹². In addition, the TPP methodology could be used to study the effect of mutations on thermal stability of the proteins²⁵, as well as for investigating the thermal stability differences of proteomes in different organisms.

Experimental design

TPP-TR workflow. The experimental design of CETSA using an antibody-based readout has already been thoroughly described³. Here we focus on the combination of the CETSA concept with quantitative mass spectrometry, which enables the thermal profiling of the proteome (see **Fig. 1a** for an overview of the general procedure), and on the implications this different readout has for the experimental design.

Both samples of cultured cells treated with a drug and vehicle-only controls are divided into aliquots that are heated to a range of temperatures, as previously described³ (**Fig. 1a** and **Box 2**). This induces denaturation, ‘melting’ of proteins and subsequently aggregation. After extraction from cells, the aggregates are removed by centrifugation³, and the remaining soluble proteins at each temperature condition are analyzed by mass spectrometry¹². As discussed in both original publications^{2,12} membrane proteins are not accessible using the current detergent-free cell extraction conditions. Exploring the use of mild detergents for assessing membrane protein stability will be the subject of future work.

For a standard experiment, we recommend using ten heating temperatures for both the compound-treated and vehicle conditions, with 37 °C as the lowest temperature and 67 °C as the highest temperature (**Fig. 1a** and **Box 2**). Depending on the actual melting temperature of proteins of interest, this range

Box 1 | Distinguishing direct from indirect targets

In addition to detecting thermal shifts caused by direct physical interaction between a target protein and a ligand, TPP can also detect altered melting behavior of proteins connected to changes of the proteotype of a cell caused by the drug. Such indirect effects on the thermal stability of a protein can be mediated, e.g., by post-translational modifications leading to altered conformation and changes in the ability to bind other ligands such as other proteins, cofactors or metabolites. As indirect effects are less likely to manifest themselves in cell extracts than in living cells, we suggest the following strategy, which we have used in our previous work¹², for distinguishing between direct and indirect drug effects.

Additional material

Cell extracts should be prepared using the freeze/thaw cycle, as previously described¹². If K562 cells are used, we recommend taking 3×10^6 cells per data point, which yields 50 µg of protein. If other cells are used, the number will have to be adjusted depending on the cell size. Prepared cell extracts can be stored at -80°C .

Procedure

1. Perform a TPP-TR experiment using both vehicle and compound treatment on living cells (each at least in two independent replicates).
2. Repeat the experiment using the corresponding cell extracts (each at least in two independent replicates). All experimental conditions including the TR and heating times should be the same as for intact cells.
3. Analyze the TPP-TR experiments on intact cells and in cell extracts separately.
4. Identify the subset of proteins with significant shifts in the intact cell experiment that is not shifted in the cell extract experiment.
5. Finally, TPP-CCR experiments picking temperatures of newly identified cellular targets could be done to confirm the findings and to obtain quantitative data that allow, e.g., the direct comparison with existing cell-based potency data.

As is the case for direct binding, the sensitivity for detecting an indirect effect is increased when there are large or even better stoichiometric changes in the protein state. Using the above-described procedure, we could demonstrate that CRKL, an adapter protein that is phosphorylated directly by the kinase BCR-ABL and that can be used as a surrogate marker for BCR-ABL activity *in vivo*, is a perfect example of an indirect target¹².

In the panobinostat experiment exemplifying the TPP workflow, we detected a significant melting point shift for the histone protein H2AFV or H2AFZ (the two variants could not be distinguished; **Fig. 6i**), which is probably an indirect effect of panobinostat. The difference in the melting behavior could be explained by changes of the acetylation pattern of the histone protein after HDAC inhibition⁶.

might need to be adjusted; for example, analyses including the quinone reductase NQO2 might need an extended or shifted TR (60 – 80°C)²⁶. However, the vast majority of proteins ($>90\%$) have a melting point within the 37 – 67°C range¹².

We suggest that the Jafari *et al.*³ protocol be followed up to the point when centrifugation is performed in order to pellet aggregated proteins and cell debris after the heat treatment (Step 14 in the Jafari *et al.*³ protocol). In the subsequent centrifugation step, an important difference to Step 15 of the protocol by Jafari *et al.*³ is the change of the centrifugation speed after the heat treatment. Application of 20,000g for centrifugation turned out to produce a low signal-to-noise ratio when the samples were analyzed by mass spectrometry. Instead, we transferred the cell extracts to polycarbonate thick-wall tubes and performed an ultracentrifugation step at 100,000g.

The relative soluble amounts of a given protein across the different heating conditions are used to infer the protein's thermal stability (**Fig. 1a** and **Box 2**). To quantify the soluble protein complement of cells after the different heating steps, we use the recently developed neutron-encoded isobaric mass tagging reagents, which in conjunction with high-resolution mass spectrometry enable multiplexing of ten temperature conditions in a single experiment (i.e., TMT10)¹¹. The soluble fractions corresponding to each temperature are individually labeled with different isobaric tags after trypsin digestion; the labeled peptides are then combined so that each temperature series is analyzed as a single sample. Consequently, one biological replicate of a TPP-TR experiment requires two

mass spectrometry experiments: one for the TMT10-labeled compound-treated sample and one for the TMT10-labeled vehicle-treated sample. To obtain in-depth proteome coverage, samples are fractionated offline before liquid chromatography/tandem mass spectrometry (LC-MS/MS) using reversed-phase chromatography at a pH of 12 (ref. 27). The resulting fractions are separated by liquid chromatography and online electrosprayed into a Q Exactive mass spectrometer²⁸. Protein identification and quantification is performed using both the isobarQuant package provided with this protocol and Mascot¹⁵. The analysis of the protein quantification data from the compound- and vehicle-treated samples is performed using the TR functionality of the TPP package. We strongly suggest performing at least two biological replicates of the TPP-TR experiments to avoid false-positive target identifications.

In the protocol, we follow the example of an experiment using living cells. However, the biochemical workflow, mass spectrometry and data analysis described in this protocol would be identical for a TPP-TR experiment performed on cell extracts^{2,12}.

TPP-CCR workflow. The importance of inferring the concentration of the ligand at which 50% of the total stabilizing effect has been observed (EC_{50}), in order to rank the potency of the different protein targets, has been discussed in the original publications and shown for a broad range of target classes^{2,12}. This is achieved by performing TPP-CCR. A TPP-CCR experiment is conducted at a single temperature over a range of concentrations of the test compound including a vehicle control

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(Fig. 1b and Box 3). The selection of the temperature is the most crucial parameter. TPP-CCR experiments are typically conducted slightly above the melting temperature of the protein(s) of interest (without compound treatment), such that the protein will just have largely disappeared in the absence of the stabilizing compound, but it is easily detectable if the compound is added³. By using this strategy, the maximum absolute difference in protein abundance between the vehicle and the compound treatment conditions can be achieved for proteins that are stabilized by the ligand. Analogously, for proteins that are destabilized by the compound, the temperature above the melting temperature observed in the presence of a saturating excess of compound should be chosen. By using this strategy, even proteins that do not reveal a significant thermal shift under TPP-TR conditions might show stabilization under TPP-CCR

conditions. This was demonstrated for the kinases GAK and CSK upon treatment with dasatinib in our previous study¹².

We suggest that the Jafari *et al.*³ protocol be followed up to the point before centrifugation is performed in order to pellet aggregated proteins and cell debris after the heat treatment (Steps 18–30A(iii) in the Jafari *et al.*³ protocol). The subsequent biochemical workflow and mass spectrometry analysis including protein quantification are the same as for the TPP-TR experiment. The analysis of the protein quantification data is performed using the CCR functionality of the TPP package.

A standard TPP-CCR experiment using TMT10 reagents will enable the comparison of nine different compound concentrations and a vehicle control in one mass spectrometry experiment. The high multiplexing rate makes it possible to choose the range of compound concentrations in such a way that the maximum

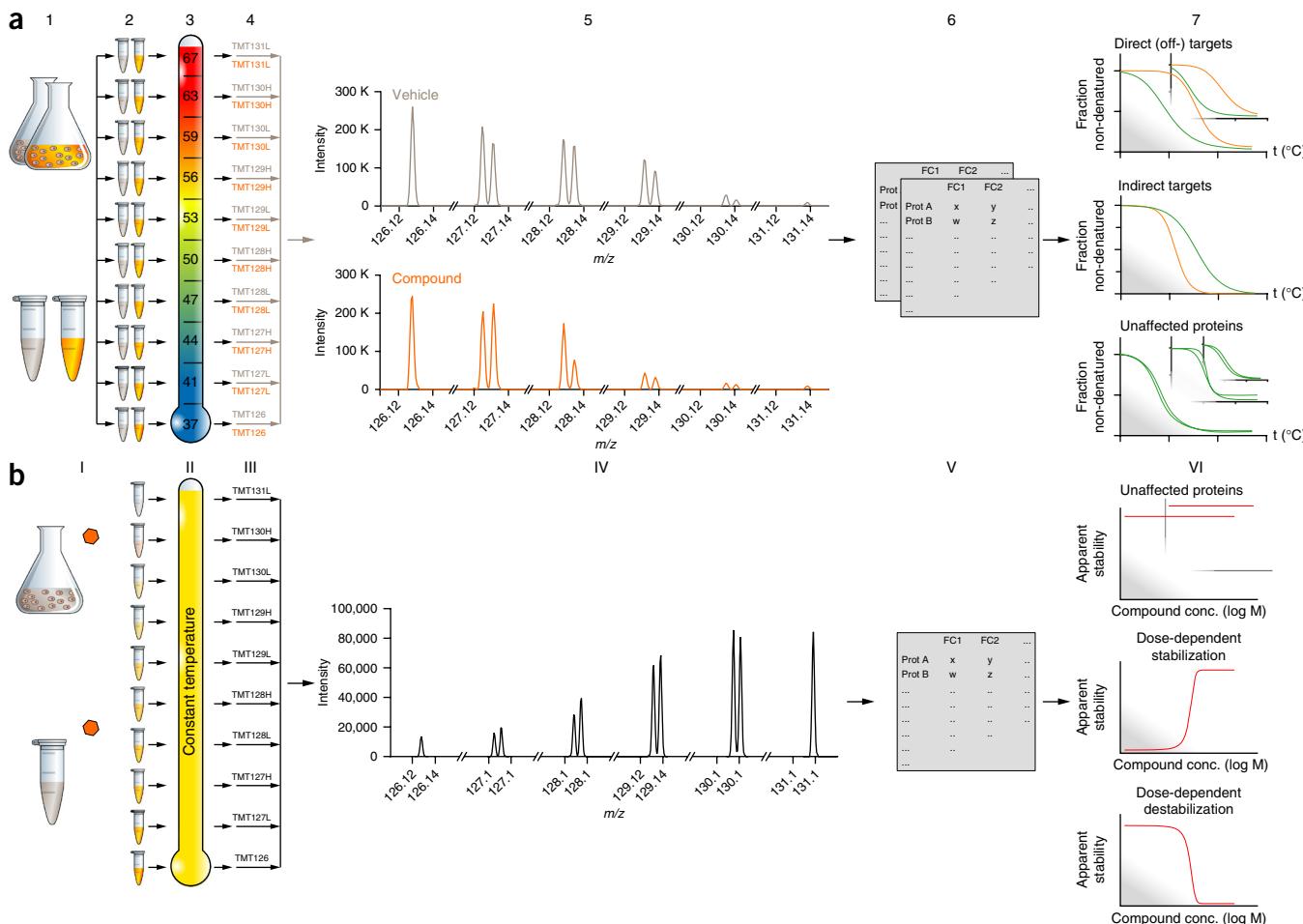


Figure 1 | Schematic representation of TPP experiments. (a) TPP-TR experiment: cells are treated with vehicle or drug. In an alternative method, the cells are extracted first and the extracts are incubated with vehicle or drug (1). For each of the two conditions, the cell or cell extract samples are divided into ten aliquots (2). Aliquots are subjected to heating at the indicated temperatures. Samples of intact cells are subsequently extracted with PBS (3). Each sample is digested with trypsin and labeled with a different TMT10 isotope tag (4). Samples from each condition are mixed (4) and analyzed by LC-MS/MS (5). Protein identification and quantification is performed using Mascot and the isobarQuant software package (6). Melting curves are fitted and the melting temperatures, T_m are calculated for all proteins in the vehicle- and drug-treated condition, and the proteins with significantly altered T_m as a result of drug treatment are identified using the TR workflow of the TPP R package (7). (b) TPP-CCR experiment: cells are treated with vehicle or drug over a range of nine concentrations (I). All ten samples are subjected to heating at the same temperature, which is chosen to fit a subset of proteins. Samples of intact cells are subsequently extracted with PBS (II). Each sample is digested with trypsin and labeled with a different TMT10 isotope tag (III). The ten labeled samples are mixed (III) and analyzed by LC-MS/MS (IV). Protein identification and quantification is performed using Mascot and the isobarQuant software package (V). Dose-response curves are fitted and pEC_{50} values are calculated for proteins whose thermal stability is affected by the drug using the CCR workflow of the TPP R package (VI).

Box 2 | Analysis of TPP-TR experiments

In a TPP-TR experiment, ten cell or cell-extract aliquots from either a compound- or vehicle-treated sample are heated to a range of temperatures (we typically use the following ten temperatures: 37, 41, 44, 47, 50, 53, 56, 59, 63 and 67 °C). After analysis by mass spectrometry, protein fold changes are computed at different temperature points relative to the protein abundance at the lowest temperature. These fold changes represent the relative amount of non-denatured protein at the corresponding temperature. After a global normalization procedure¹², melting curves are fitted to the fold changes of each individual protein according to the formula

$$f(T) = \frac{1 - \text{plateau}}{1 + e^{-(\frac{a-b}{T})}} + \text{plateau}$$

Where T is the temperature and a , b and plateau are constants. Several parameters are derived that are relevant for the subsequent significance analysis:

- The coefficient of determination R^2 , which indicates how well the fold changes fit the melting curve.
- The melting point (T_m) of the protein under the corresponding condition, which is given by the temperature at which the value of the melting curve is 0.5.
- The slope of the curve at its steepest point (i.e., the inflection point).
- The plateau of the fitted melting curve, which is given by its lower horizontal asymptote.

A change in the thermal stability of a protein is indicated by a compound-induced shift of its melting curve, which becomes apparent in a difference between the melting points derived under vehicle- and compound-treated conditions $T_m(\text{treatment}) - T_m(\text{vehicle})$.

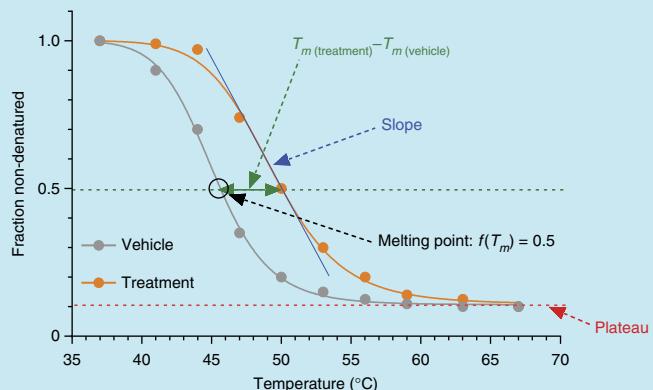
For a protein to be considered in the statistical evaluation, we require the R^2 of both the vehicle- and the compound-treated melting curves to be >0.8 and the plateau of the vehicle curve to be <0.3 in our analysis.

The slope of the melting curve has a substantial impact on the reproducibility of the melting point difference. The shallower a curve is, the greater the influence of small variations in individual fold changes on the derived melting point, meaning in turn that more reproducible melting points can be obtained from steeper curves. Thus, the slope has a central role in our data quality-dependent significance assessment. We divide the considered set of proteins into bins, starting with the shallowest slope, so that each bin contains at least 300 proteins. Then, we use z -tests to assess the statistical significance of melting point differences for the proteins one bin at a time, before performing the Benjamini-Hochberg correction for multiple testing on the full data set.

To obtain reliable results, we strongly recommend performing experiments in two independent replicates (two pairs of vehicle- and compound-treated experiments). For a compound-induced change in a protein's thermal stability to be regarded as significant, it is required to fulfill the following criteria:

- One of the P values for the two replicate experiments is <0.05 and the other one is <0.1 .
- The melting point shifts in the two vehicle versus treatment experiments have the same direction (i.e., the protein was either stabilized or destabilized in both cases).
- Both melting point differences in the two pairs of control versus treatment experiments are greater than the melting point difference between the two vehicle controls.
- The minimum slope in each of the control versus treatment experiments is <-0.06 .

It is important to keep in mind that although we found these thresholds to be practical for our purposes it is prudent to manually examine cases of T_m shifts that fall just outside these criteria. Equally, one should critically examine and visualize cases that pass the criteria. A TPP-CCR experiment (Box 3) is an ideal validation strategy for the findings in the TPP-TR experiments.



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compound effect on either stabilization or destabilization is achieved at two concentrations (**Box 3**) for the main targets of interest.

As the last step of the TPP-CCR analysis, we perform a sigmoidal curve fit (**Box 3**), which yields a pEC_{50} value (negative logarithm to base 10 of the half-maximal effective concentration) as a quantitative result. In our previous work, these pEC_{50} values

reproduced very well, and they were in line with data obtained from various other assays, suggesting that they provide a reliable measure for target occupancy in the cell¹². We recommend performing two biological replicates of TPP-CCR experiments and accepting pEC_{50} values that are consistent between both replicates—i.e., within a range of $0.5 \log_{10}$ units.

Box 3 | Analysis of TPP-CCR experiments

In TPP-CCR experiments, cell or cell extract aliquots are treated with a compound at a range of different concentrations and subsequently heated to a single defined temperature. This temperature should be chosen so that for the proteins of interest it maximizes the offset between the vehicle- and compound-treated melting curves in the corresponding TPP-TR experiment (green arrows). The range of compound concentrations should reach from 0 (vehicle) to a saturating concentration—i.e., one should aim at having at least two compound concentrations at which one would expect to get a maximum effect of the compound on the proteins of interest. After analysis by mass spectrometry, fold changes are computed at the different compound concentrations, relative to the protein abundance at the lowest compound concentration (vehicle). These fold changes represent the protein's apparent stability at the corresponding compound concentration.

If a protein's fold change at the highest compound concentration is $>3/2$, we regard the protein to be potentially stabilized by the compound. Conversely, if a protein's fold change at the highest compound concentration is $<2/3$, we regard the protein to be potentially destabilized by the compound. Otherwise, we do not consider the protein for further analysis. Although a $<50\%$ difference does not rule out the possibility that the protein is affected by the compound, the range of fold-change variation is considered too narrow to yield reliable results. However, if a protein, based on the results of the TPP-TR experiment, is expected to be more than 50% altered in its thermal stability at the temperature chosen for the TPP-CCR experiment, but shows no dose-dependent behavior in the TPP-CCR experiment, it is likely that the result from the TPP-TR experiment is a false positive.

Subsequently, the fold changes are transformed so that with increasing compound concentration they range from 0 to 1 for stabilized proteins and from 1 to 0 for destabilized proteins. A sigmoidal curve according to the formula

$$Y = \frac{1}{1 + 10^{(\log EC_{50} - x) \text{ slope}}}$$

is fitted.

If the R^2 of the curve fit is >0.8 , we regard the observed stabilization or destabilization to be a compound-induced effect as opposed to random fluctuations. In that case, we derive the pEC_{50} as the compound concentration at which the value of the fitted curve is 0.5. If the derived value is below the lowest (nonvehicle) compound concentration (i.e., $pEC_{50} < \log_{10}$ (minimum applied compound concentration)), this is reported in the output and that pEC_{50} is not considered valid.

As for TPP-TR experiments, we strongly recommend performing at least two biological replicates and only considering pEC_{50} values for proteins that passed these criteria in both experiments and are within $0.5 \log$ units of each other.

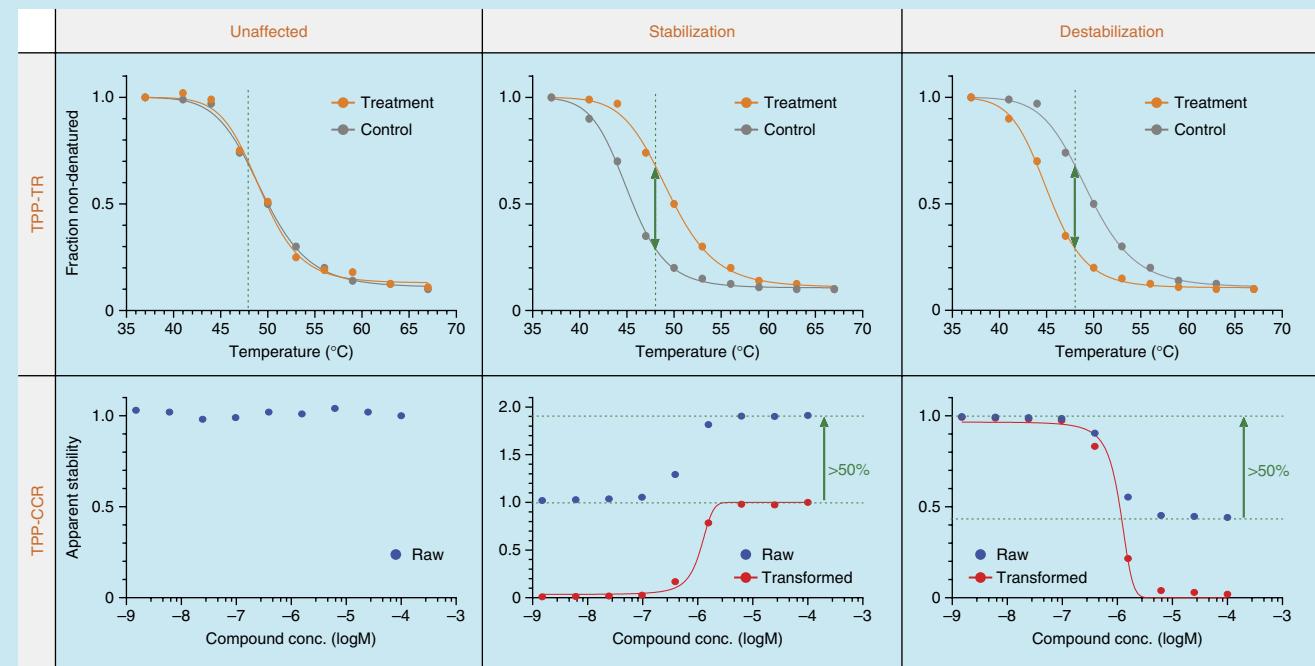
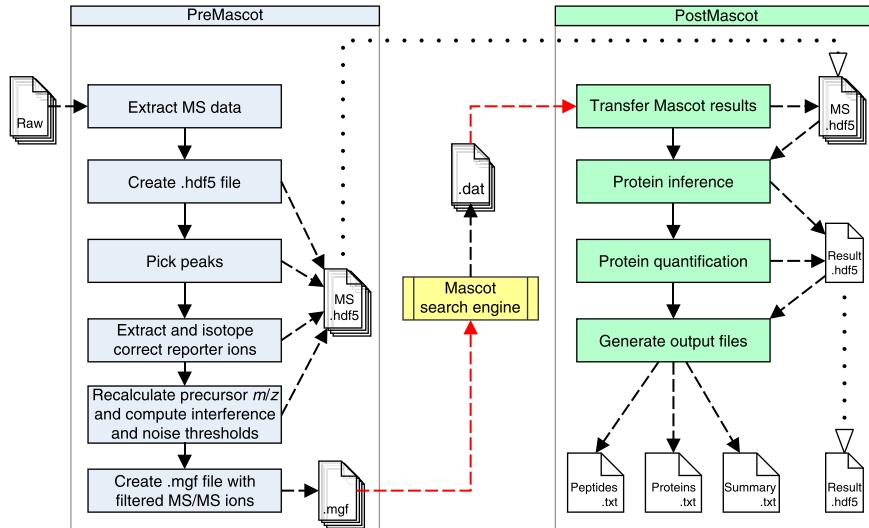


Figure 2 | Workflow for the isobarQuant Python package. Dashed lines indicate data flow to or from external files or data objects, continuous lines indicate program flow and dotted lines denote when the same files are used at different stages. Red lines represent required manual intervention. The workflow is grouped into the self-contained pre-Mascot and post-Mascot sections. Starting from Xcalibur raw files, the pre-Mascot section generates MS .hdf5 files and .mgf files (one each per input raw file). The .mgf files need to be manually submitted to the Mascot search engine to generate .dat files that then serve as input for the post-Mascot section together with the corresponding MS .hdf5 files. All results of the post-Mascot section are stored in the result .hdf5 file. The peptide and protein results are additionally written to two tabular .txt files, which can serve as input for subsequent analysis and summarized by basic statistics in the summary file.



Peptide and protein identification and quantification. As part of the protocol, we provide the isobarQuant software package. In combination with the commonly used search engine Mascot¹⁵, it provides an analysis pipeline that processes the raw files generated by an Orbitrap^{28,29} mass spectrometer and produces two tab-delimited files containing identified and quantified peptides and proteins, **Figure 2**. The software is designed to work with mass spectrometry data stemming from the analysis of TMT^{11,30}-labeled samples acquired on an Orbitrap instrument. The software can be adapted to other types of mass spectrometry data generated by an Orbitrap mass spectrometer in which isobaric mass tagging^{16,31} has been used for quantification, provided that the quantification is based on reporter ions in the tandem mass spectrometry (MS/MS) spectrum. Quantification strategies using MS3 spectra^{32,33} are currently not supported. Isobaric mass tag-based quantification approaches such as TMT are affected by the phenomenon of ratio compression due to co-fragmentation, which often leads to the underestimation of the true peptide or protein fold change³⁴. Our analysis package contains an implementation of a previously described method³⁵ to computationally address this problem by inferring and correcting for the extent of co-fragmentation for individual peptide MS/MS spectra. Briefly, the reporter ion containing the MS/MS spectra, which originate from precursor signals very close to the noise level (low precursor to threshold ratio, P2T) or contain a substantial amount of co-fragmented signals within the defined isolation window ($P2T < 4$, $S2I < 0.5$)^{36,37}, are discarded. This mild filtering step results in a $< 10\%$ loss in protein coverage³⁵. For the remaining data, the reporter ion area values are corrected by a simple algorithm³⁵ using the signal to interference measure, S2I, which has been shown to strongly reduce ratio compression due to co-fragmentation and to produce more accurate peptide and protein fold changes³⁵.

There are other software solutions³⁸ available for the processing of mass spectrometry data from TMT-labeled samples such as MaxQuant^{39,40} and Proteome Discoverer⁴¹, which could be used for this step of the workflow; however, we cannot report on the performance of these packages with regard to ratio compression.

In the pre-Mascot processing step, the software transfers all data from the MS1 and MS/MS scans including reporter ion

abundances, as well as information about the instrument setup from the raw file to an HDF5-format file⁴², and it calculates the S2I and P2T values for all MS/MS events in the experiment. Reporter ion abundances are corrected for isotope impurities¹⁰. Ion chromatograms are extracted for all precursor ions and used to recalculate precursor mass over charge (m/z) ratios (**Supplementary Manual**). Subsequently, a Mascot generic format (.mgf) file that contains all deisotoped and deconvoluted^{43,44} MS/MS spectra with removed reporter ions⁴³ is created that the user then submits to the Mascot search engine¹⁵. After Mascot has matched the experimental MS/MS data to *in silico*-generated peptide sequences, it generates an output file (.dat file), which contains all the identified peptide and protein information.

In the post-Mascot processing step, the information from the Mascot-generated dat file is appended to the HDF5 file, which links the identified peptide and associated protein information to the calculated S2I and P2T values, reporter ion abundances and all other spectral information. To enable protein quantification, a protein inference calculation is then performed using the identified peptides that passed the specified false-discovery rate (FDR) threshold, as determined using a decoy database⁴⁵.

Peptide fold changes are calculated using the S2I-corrected reporter ion abundances. Protein fold changes are calculated with a sum-based bootstrap algorithm³⁷ using the S2I-corrected fold changes from peptides that passed the S2I and P2T filters. The identified and quantified peptide and protein information is written to two separate txt files with an additional summary .txt file that provides experimental details such as FDR thresholds and numbers of peptides and proteins identified (**Fig. 2**).

The software package works with single or multiple raw files, with the option to combine the peptide and protein quantification information from several mass spectrometry experiments into a unified output consisting of one peptide and one protein data file. This option is necessary when samples are fractionated before mass spectrometry analysis in order to obtain more extensive proteome coverage.

All steps necessary to use the isobarQuant package for deriving protein quantification from TMT10-labeled samples analyzed by mass spectrometry are described in the PROCEDURE section of the protocol. An advanced user guide for nonstandard

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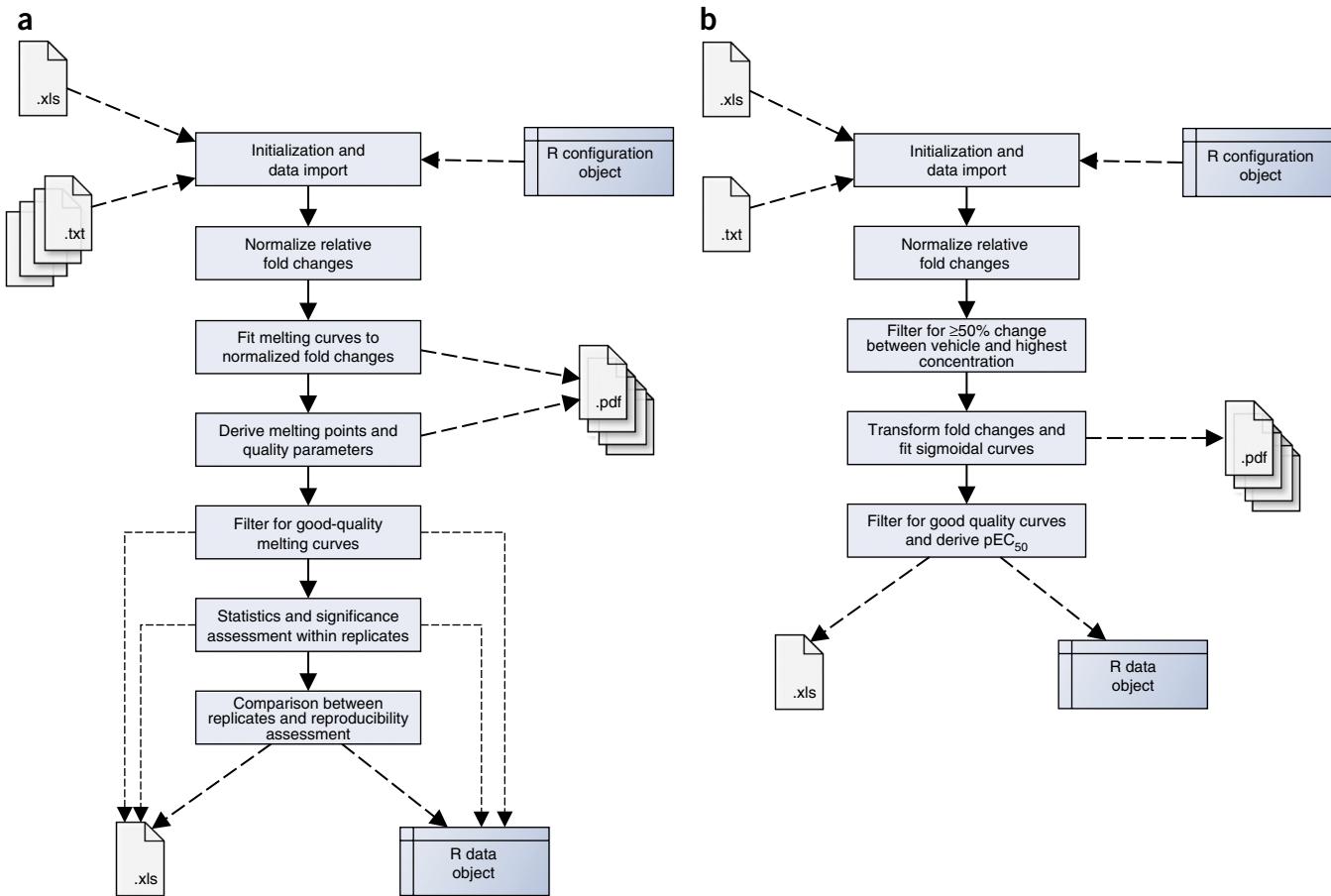


Figure 3 | Operational workflow breakdown. (a,b) Shown are the TR (a) and CCR (b) workflows of the TPP R package. Dashed lines indicate data flow to or from external files or data objects, and continuous lines indicate program flow. Initialization information and input data for each workflow can be provided either in tabular form (e.g., Excel or .txt files) or programmatically via R data objects. Both workflows return their results in R data objects, as well as in Excel files, and optionally generate .pdf files with plots that illustrate the corresponding curve fits for each protein. The two lower stages of the TR procedure are only executed when analyzing one or more control versus treatment experiments.

applications—e.g., using different isobaric mass tag labeling strategies—describes all the processing steps in detail, and it contains a glossary of the parameter names used and is available as a **Supplementary Manual**.

Data analysis of TPP-TR experiments. To analyze quantified protein data from a TPP-TR experiment, the TR workflow in the R package TPP is used. The package will automatically process the protein output files from the isobarQuant package. If other software was used to perform protein quantification, simple-to-follow instructions are available on how to format input data. The package is designed to process data from one or several experiments, to perform normalization, to fit melting curves, to determine melting points and to identify proteins that have a significant shift in thermal stability compared with controls (Fig. 3a). We strongly recommend performing at least two biological replicate TPP-TR experiments. In addition, we recommend critically examining the visualizations provided for the proteins for which a significant melting point shift is reported, as well as for those just below the applied significance threshold. The package generates a number of quality control plots to assess the reproducibility of the experiments and the effectiveness of the normalization procedure. Its output consists

of an R object and a spreadsheet (Excel) file, both of which report all relevant protein information, melting curve parameters and T_m shifts with corresponding significance estimation (Box 2). The R object can be exported from R to various file formats—e.g., CSV. The spreadsheet also contains for each protein a link to a visualization of its melting curves in a companion PDF file for all analyzed replicates and conditions. The naming conventions used in the output are explained in Table 1. The PROCEDURE section contains step-by-step instructions on how to use the TR workflow of the TPP package.

Data analysis of TPP-CCR experiments. For analysis of quantified protein data from a TPP-CCR experiment, the CCR workflow in the TPP package is used. The package will automatically process the protein output file from the isobarQuant package. For protein quantification data generated by other software, we provide simple-to-follow instructions for formatting an input file that will be processed by the TPP package. The CCR workflow of the TPP package is designed to process data from one TPP-CCR experiment, to perform normalization, to fit dose-response curves, to identify proteins that show significant dose-dependent changes in thermal stability in response to drug treatment and to determine pEC_{50} values for these proteins (Fig. 3b). The extent of

TABLE 1 | Results and parameters given in the results table of the TR workflow in the TPP R package.

Column name	Description
Always reported	
Protein_ID	Protein identifier (as given in the input file(s)), used for matching proteins across multiple input files
norm_rel_FC_label_condition	Normalized relative protein-fold change for a given condition at the corresponding isobaric label
meltPoint_condition	Melting point of the protein under the given condition, derived from fitted melting curve
slope_condition	Slope of the fitted melting curve at the inflection point under the given condition
plateau_condition	Plateau (lower horizontal asymptote) of the fitted melting curve under the given condition
R_sq_condition	Coefficient of determination for the melting curve under the given condition; it indicates how well the fold changes fit the melting curve
Plot	Link to accompanying .pdf file showing a plot of this protein's melting curves for all treatments
Only reported when analyzing one or more control versus treatment experiments	
diff_meltP_treatmentX_vs_controlX	Difference between this protein's melting points in control experiment X and treatment experiment X
min_slope_treatmentX_vs_controlX	Minimum slope of this protein's fitted melting curves in control experiment X and treatment experiment X
pVal_adj_treatmentX_vs_controlX	Benjamini-Hochberg-adjusted <i>P</i> value of this protein's melting point difference between control experiment X and treatment experiment X
passed_filter_treatmentX_vs_controlX	Did this protein meet the quality criteria ($R^2 > 0.8$, plateau <0.3) in control experiment X and treatment experiment X?
Only given when analyzing a two-replicate experiment	
min_pVals_less_0.05_and_max_pVals_less_0.1	Is one of the <i>P</i> values for the two replicate experiments <0.05 and the other one <0.1 ?
meltP_diffs_have_same_sign	Do the melting point shifts in the two control vs treatment experiments have the same sign (i.e., protein was either stabilized or destabilized in both cases)?
meltP_diffs_T_vs_V_greater_V1_vs_V2	Are both the melting point differences in the control vs treatment experiments greater than the melting point difference between the two untreated controls?
min_slopes_less_than_0.06	Is the minimum slope in each of the control vs. treatment experiments <-0.06 ?
fullfills_all_4_requirements	Does this protein fulfill all of the four requirements above?
Technical information regarding the fitting of the melting curve	
model_converged_condition	The fitting of the melting-curve fit succeeded for the given condition
Sufficient_data_for_fit_condition	There is a sufficient number (>2) of valid (non-NA) fold changes given in the input data for this condition to fit the melting curve

In the output table, label is replaced by the corresponding isobaric label (e.g., '127L'), condition is replaced by the given experiment condition (e.g., 'Vehicle_1') and treatmentX_vs_controlX is replaced by the given experiment conditions that this comparison corresponds to (e.g., 'Panobinostat_1_vs_Vehicle_1').

a protein's dose-dependent thermal stability change upon drug treatment will depend on the temperature used in the TPP-CCR experiment. If for a particular protein the temperature is outside of the TR in which its thermal stability depends on the presence of the drug, its fold changes will show no dose-dependent response. Otherwise, the range of its dose-dependent response will depend on how much a protein was stabilized or destabilized

by the drug at this temperature (**Box 3**). For instance, if a protein at 45 °C is 50% more thermostable with a high concentration of drug treatment than without drug treatment, then in a TPP-CCR experiment performed at 45 °C the fold changes of this protein, using the vehicle condition as a reference, will range from 1 in the vehicle condition to 1.5 in the highest compound concentration condition (**Box 3**). If the stabilizing (or destabilizing) effect of a

TABLE 2 | Results and parameters given in the results table of the CCR workflow in the TPP R package.

Column name	Description
Protein_ID	Protein identifier as given in the input file
rel_FC_label_transformed	Transformed relative protein fold change at the corresponding isobaric label
pEC50	pEC ₅₀ (negative logarithm of the half maximal effective concentration) derived from the dose-response curve fit
Slope	Slope estimated from the dose-response curve fit
R_sq	Coefficient of determination for the dose-response curve; it indicates how well the fold changes fit the curve
passed_filter	Indicates whether the protein passes the filter criteria ($\geq 50\%$ change between vehicle and highest compound concentration and $R^2 > 0.8$)
pEC50_outside_conc_range	Indicates whether the derived pEC ₅₀ is below the lowest (nonvehicle) compound concentration and should therefore not be considered as valid
Plot	Link to accompanying .pdf file showing a plot of this protein's dose-response curve

In the output table, 'label' is replaced by the corresponding isobaric label (e.g., '127L').

compound on a protein is very small at the temperature of the TPP-CCR experiment, then it becomes difficult to confidently assign dose-dependence. We found empirically in our previous work¹² that if the measured abundance change between the vehicle and highest drug concentration condition of a protein in both biological replicates of the TPP-CCR experiment was at least 50% and the fitted sigmoid curve had an $R^2 > 0.8$, then the calculated pEC₅₀ values showed excellent reproducibility ($R^2 = 0.93$; ref. 12). We suggest considering pEC₅₀ values that pass the above criteria and reproduce within 0.5 log₁₀ units between the two biological replicates.

The CCR workflow of the TPP package normalizes the data by dividing the protein fold changes in each condition (compound concentration or vehicle) by the median fold change of all proteins in that condition. Subsequently, all fold changes of

proteins for which we found a ratio of at least 1.5-fold between the lowest (at vehicle or highest drug concentration condition) and the highest fold change (at highest drug concentration or vehicle condition) are transformed to a range between 0 and 1, and a dose-response curve is fitted. R^2 of the curve fits and pEC₅₀ values are calculated as previously described¹². For output, an R object (exportable, e.g., to CSV) and an Excel file that contain the protein information and the R^2 and pEC₅₀ values are generated. Additional columns indicate whether the quality criteria were reached, and there is a link to an accompanying PDF file showing a plot for each protein with a fitted curve (Box 3). Table 2 lists and explains the naming conventions used in the output. The PROCEDURE section contains all necessary instructions on how to use the CCR workflow of the TPP package for analyzing TPP-CCR experiments.

MATERIALS

REAGENTS

- K562 cell line (American Type Culture Collection, cat. no. CCL-243). The protocol is applicable to other cell lines or primary cells. For each new cell type, the cell amounts used for the experiment need to be adjusted depending on the cell size! **CAUTION** By using established technologies, the cell lines used should be regularly checked to ensure that they are authentic, karyotypically normal and not infected with mycoplasma.
- Histone deacetylase (HDAC) inhibitor panobinostat (Santa Cruz Biotechnology, cat. no. SC-208148)
- RPMI-1640 medium (Life Technologies, cat. no. 21875-034)
- FCS (Life Technologies, cat. no. 10270-106)
- Dulbecco's PBS (DPBS) without calcium chloride/ magnesium chloride (Life Technologies, cat. no. 14190-094)
- DMSO water-free (Sigma-Aldrich, cat. no. 41647)
- cComplete, EDTA-free protease inhibitors (Roche, cat. no. 11873580001)
- Bradford reagent (Bio-Rad, cat. no. 500-0006)
- BSA standard (Bio-Rad, cat. no. 500-0206)
- DTT (Biomol, cat. no. 04010)
- Iodoacetamide (Sigma-Aldrich, cat. no. I1149)
- NuPAGE 4× LDS sample buffer (Life Technologies, cat. no. NP0007)
- NuPAGE 4–12% (wt/vol) Bis-Tris Midi gels (Life Technologies, cat. no. WG1401A)
- NuPAGE Midi gel adapter (Life Technologies, cat. no. WA0999)
- NuPAGE MOPS running buffer, 20× (Life Technologies, cat. no. NP0001)

- Precision Plus protein standard, unstained (Bio-Rad, cat. no. 161-0363)
- Brilliant Blue G colloidal concentrate (Sigma-Aldrich, cat. no. B2025)
- Ethanol EMSURE (Merck, cat. no. 100983)
- Acetic acid, 96% (wt/vol) (Merck, cat. no. 100062)
- Nitric acid, 70% (wt/vol) (Sigma-Aldrich, nitric acid, ACS reagent, cat. no. 438073-2.5L)
- Triethylammonium bicarbonate buffer (TEAB), 1 M (Sigma-Aldrich, cat. no. T7408)
- Water, HPLC grade (Merck, cat. no. 115333)
- Formic acid SupraPure 98–100% (Merck, cat. no. 100264)
- Acetonitrile (Merck, cat. no. 100030)
- Trypsin sequencing-grade modified (Promega, cat. no. V5111)
- Lysyl endopeptidase, MS grade (LysC; Wako Chemicals, cat. no. 125-05061)
- Hydroxylamine solution (Sigma-Aldrich, cat. no. 438227)—dilute to 2.5% (vol/vol) with HPLC-grade water
- Trifluoroacetic acid (Merck, cat. no. 08262)
- Ammonia solution, 25% (wt/vol) (Merck, cat. no. 105432)
- DMSO (Fluka, cat. no. 01934-1L)
- Tryptic digest BSA (Bischoff Analysetechnik, cat. no. PTD/00006/15)

TMT reagents

- TMT126, 5 mg (Perbio Science, cat. no. 1862201B)
- TMT127, 5 mg (Perbio Science, cat. no. 1862202B)
- TMT128, 5 mg (Perbio Science, cat. no. 1862203B)
- TMT129, 5 mg (Perbio Science, cat. no. 1862204B)
- TMT130, 5 mg (Perbio Science, cat. no. 1862205B)

- TMT131, 5 mg (Perbio Science, cat. no. 1862206B)
- TMT127C, 5 mg (Perbio Science, cat. no. 1862870B)
- TMT128N, 5 mg (Perbio Science, cat. no. 1862871B)
- TMT129C, 5 mg (Perbio Science, cat. no. 1862872B)
- TMT130N, 5 mg (Perbio Science, cat. no. 1862873B)

EQUIPMENT

Sample preparation

- Water bath TW20 (Julabo, cat. no. 9550120)
- Forma Steri-Cult CO₂ incubator (Thermo Scientific, cat. no. 3311)
- CASY cell counter TT, 150 µM (Roche Innovatis, cat. no. 05651697001)
- PCR tubes (Brand, cat. no. 781305)
- Heraeus Multifuge 3SR (Thermo Scientific, cat. no. 75004371)
- Herasafe type A2 biological safety cabinets
- Peltier thermal cycler (MJ Research/Bio-Rad, cat. no. PTC-200)
- Tabletop centrifuge 5415D (Eppendorf, cat. no. 022621425)
- Ultracentrifuge tubes, 0.2 ml (Beckman Coulter, cat. no. 343775)
- Optima Max XP benchtop ultracentrifuge (Beckman Coulter, cat. no. 393315)
- pureGrade 96-well plates (Brand, cat. no. 781602)
- EnVision multilabel reader (PerkinElmer, cat. no. 2103)
- Criterion electrophoresis cell (Bio-Rad, cat. no. 165-6001)
- Plastic boxes for gel storage and staining (Licefa, cat. no. 2427744)
- Power supply power Pac200 (Bio-Rad, cat. no. 164-5050)

LC-MS analysis

- Polypropylene 96-well plates (Nunc, cat. no. 249944); with a manually drilled hole (0.4 mm) at the bottom of each well and subsequently washed. See 'Plate drilling' (Equipment Setup)
- Drill with a 0.4-mm drill bit
- Universal lids for 96-well plates (Corning, cat. no. 3098)
- Scalpels (Braun, cat. no. T998)
- Protection shield (neo-Lab, cat. no. 4-1004)
- Illumination desk, Prolite Basic 2 (Kaiser, cat. no. 2173)
- Vacuum concentrator centrifuge (UniEquip, Univapo 150ECH)
- Centrifuge 5810 (Eppendorf, cat. no. 58100000017)
- Thermomixer (Eppendorf, cat. no. 5382000015)
- Polyolefin foil for 96-well plates (HJ Bioanalytik, cat. no. 900 320)

Offline fractionation, reverse-phase pH

- UltiMate 3000 quaternary micro LC system with a biocompatible dual gradient pump (DGP-3600BM) suitable for basic pH
- Solvent rack SDR-3600
- Well-plate sampler WPS-3000TBFC used also as a fraction collector
- Column compartment TCC3000SD including a TitanHP 10-port valve
- UV detector VWD-3100
- Analytical columns used: X-Bridge C18 column, 150 mm × 1 mm, 3.5 µm (Waters, cat. no. 186003128) and X-Bridge C18 2.1 mm × 10 mm, 5 µm, Guard Cartridge (Waters, cat. no. 186003062)
- Software: Chromeleon 6.8

Online nanoLC-MS/MS

- UltiMate 3000 RSLCnano U3000 equipped with a NCS-3500RS NANO pump
- Autosampler WPS-3000TPL RS
- 50-cm C18 Reprosil aq 3-µm tip-column Nano C18
- Pep Map precolumn, 0.3 mm × 5 mm (Thermo Fisher Scientific, P/N160454)
- Nano precolumn holder, 5 mm inner diameter (i.d.) (Thermo Fisher Scientific, P/N164649)
- NanoViper connective tubing, 30 µm i.d. × 100 mm (Thermo Fisher Scientific, P/N164651)
- Nanospray Flex ion source (Eso71; Thermo Fisher Scientific)
- Column oven PRSO-V1 Sonation (PRSO-V1 and PRSO-V1-PKIT)
- Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo)
- The instruments are controlled using the following software: Foundation 3.0 SP2, Xcalibur 3.0 build 63, Dionex chromatography MS Link 2.14 build 3818, Q Exactive Orbitrap MS 2.4 build 1824, operating system Windows 7 Professional (32 bit) and Microsoft Office 2013

Software requirements for running isobarQuant

- Windows operating system (isobarQuant has been tested under Windows 7 and Windows server 2012) ▲ **CRITICAL** For isobarQuant to work, Xcalibur2.2 or Xcalibur3 (part of Thermo Foundation 3 software) must also be installed on the same computer. isobarQuant only supports files acquired on Thermo Orbitrap/Q Exactive instrumentation.
- Python 2.7, 32-bit installation (As Xcalibur is currently only available in a 32-bit version, a 32-bit Python installation is required to ensure compatibility.)

- Access to the Mascot Search Engine (Matrix Science, <http://www.matrixscience.com/>). To ensure speed to configure as required, an in-house version of Mascot is recommended. Mascot's free demo version has a limit set to 1,200 spectra, so it is not likely to be feasible to use it for data sets of this size

REAGENT SETUP

Sample buffer Dilute 4× LDS NuPAGE buffer to a 1:2 ratio and add 50 mM DTT. For a set of 20 samples (vehicle and compound), mix 320 µl of deionized water and 320 µl of 4× LDS and add 32 µl of 1 M DTT stock. The buffer should be freshly prepared.

Coomassie stain solution For each SDS Bis-Tris gel, mix 5 ml of the Brilliant Blue G stock with 20 ml of deionized water and 6.25 ml of absolute ethanol. The buffer should be freshly prepared.

Fixing solution Fixing solution is 40% (vol/vol) ethanol and 2% (vol/vol) acetic acid. The solution can be stored for 2 weeks at room temperature (23 °C).

Formic acid solution, 5% (vol/vol) Formic acid solution, 5% is 5% (vol/vol) aqueous formic acid. The solution can be stored for 2 weeks at 4 °C.

Formic acid solution, 1% (vol/vol) Formic acid solution, 1% is 1% (vol/vol) aqueous formic acid. The solution can be stored for 2 weeks at 4 °C.

Peptide extraction solution Peptide extraction solution is 60% acetonitrile and 40% (vol/vol) aqueous 1% (vol/vol) formic acid solution. The solution can be stored for 2 weeks at 4 °C.

TMT labeling solution 1 TMT labeling solution 1 is 10% acetonitrile and 90% 200 mM triethylammonium bicarbonate buffer (TEAB) (vol/vol) in water. This solution should be freshly prepared.

TMT labeling solution 2 TMT labeling solution 2 is 60% (vol/vol) 200 mM TEAB in water and 40% (vol/vol) acetonitrile. This solution should be freshly prepared.

Fractionation basic pH mobile phase A Fractionation mobile phase A is 1.25% (vol/vol) ammonia in HPLC water. Mobile phase should be freshly prepared.

Fractionation basic pH mobile phase B Fractionation mobile phase B is acetonitrile (70% (vol/vol)) and 1.25% (vol/vol) ammonia in HPLC water. Mobile phase should be freshly prepared.

Fractionation basic pH loading pump mobile phase Fractionation loading pump mobile phase is 1.25% (vol/vol) ammonia in HPLC water. Mobile phase should be freshly prepared.

NanoLC pump mobile phase A NanoLC pump mobile phase A is 3.5% DMSO (vol/vol) and 0.1% formic acid (vol/vol) in HPLC water. Mobile phase should be freshly prepared.

NanoLC pump mobile phase B NanoLC pump mobile phase B is 3.5% DMSO (vol/vol) and 0.1% formic acid (vol/vol) in acetonitrile. Mobile phase should be freshly prepared.

Loading pump mobile phase Loading pump mobile phase is 0.05% trifluoroacetic acid (vol/vol) in HPLC water. Mobile phase should be freshly prepared.

EQUIPMENT SETUP

Plate drilling 96-well polypropylene plates are drilled using a 0.4-mm drill bit. Remove drilling debris by shaking the plates upside down. Stack three drilled plates over one nondrilled plate and pipette 100 µl of 100% ethanol into the top plate. Spin down ethanol using the Eppendorf centrifuge (type 5810) at 1,000 r.p.m. for 1 min at room temperature. Repeat this with 1% (vol/vol) formic acid. Inspect the plate for poorly drilled wells, and repeat drilling and cleaning if necessary.

Off-line fractionation at basic pH Note that all connections should be made with peak tubing, as fused silica is not stable in the long term at high pH. The sample is loaded with a flow of 30 µl/min to the trap column, and it is separated on the analytical column using a gradient starting from 3% up to 60% fractionation basic pH mobile phase B over 85 min with a flow of 40 µl/min. With the start of the elution, fractions are collected for 1 min each into a 96-well plate. After 24 fractions, the 25th fraction is collected in the same well as the first, the 26th fraction is collected in the same well as the second and so on. Thus, three or four fractions are collected into each well, which ensures the optimal use of instrument time in the subsequent LC-MS/MS.

On-line nanoLC-MS/MS The sample is loaded with a flow of 6 µl/min to the trap column (60 °C) and eluted over the analytical column (we typically use in-house-manufactured columns, but any commercial analytical column such as the 75-µm ID × 50 cm C18 column (Acclaim PepMap RSLC C18, 50 cm × 75 µm × 2 µm, 100 Å) used in Kocher *et al.*⁴⁶ will work; 55 °C) with a gradient starting from 3.5% up to 35% NanoLC pump mobile phase B over 103 min with a flow of 350 nl/min.

PROTOCOL

Instrument tune settings The instrument tune settings are as follows: spray voltage 1.9 kV and 2.8 kV (depending on the column), capillary temperature of 250 °C and S-lens RF level of 60.0.

Instrument method settings A crucial parameter in this method is the high-resolution (35,000) MS/MS scan setting, which is necessary to resolve the neutron-encoded reporter ions and to enable quantification. The isolation window of 1 Th should be used to minimize cofragmentation³⁷, and the MS/MS automatic gain control (AGC) target of 2×10^5 should be used to avoid cloud coalescence¹¹.

Parameter	Setting
Global settings	
User role	Advanced
Use lock masses	Off
Lock mass injection	–
Chrom. peak width (FWHM)	9 s
Time	
Method duration	Gradient time
Customized tolerance (+/–)	
Lock masses	–
Inclusion	–
Exclusion	–
Neutral loss	–
Mass tags	–
Dynamic exclusion	–
General	
Runtime	10 min after injection until set gradient time
Polarity	Positive
In-source collision-induced dissociation (CID)	0.0 eV
Default charge state	2
Inclusion	–
Exclusion	–
Tags	
Full MS	
Microscans	1
Resolution	70,000
AGC target	3.00E+06
Maximum IT	250 ms
Number of scan ranges	1
Scan range	375–1200 <i>m/z</i>
Spectrum data type	Profile
DD-MS2/dd-SIM	
Microscans	1
Resolution	35,000
AGC target	2.00E+05
Maximum IT	120 ms
Loop count	10
MSX count	1
TopN	10
Isolation window	1.0 <i>m/z</i>
Isolation offset	0.0 <i>m/z</i>
Fixed first mass	100.0 <i>m/z</i>
NCE/stepped NCE	33
Spectrum data type	Profile

IT, injection time; NCE, normalized collision energy.

Parameter	Setting
dd Settings	
Underfill ratio	0.10%
Intensity threshold	1.70E+03
Apex trigger	–
Charge exclusion	Unassigned, 1, 7, 8, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	40.0 s

Installation of isobarQuant Download and unzip the package containing isobarQuant (<https://github.com/protcode/isob/archive/1.0.0.zip>). Ensure that all required Python libraries (as listed in README within the .zip file) are installed and that they are accessible from the location at which the Python scripts will run. Before running the software, make sure that you agree to the terms of the free license. See **Supplementary Manual** for details.

Mascot setup Set the parameter ‘ProteinsInResultsFile’ in Mascot’s configuration file to 3. This will guarantee that all protein entries in the generated Mascot .dat files will contain protein description information from the .fasta file used for searching. Information on how to do this can be found in Chapter 6, ‘Configuration & Log Files’ of the Setup & Installation Manual included with Mascot. Use a single .fasta file containing both forward and reverse (decoy) hits for searching. Details of how to create this may be found on the Matrix Science web site http://www.matrixscience.com/help/decoy_help.html. The TPP-TR and TPP-CCR experiments were searched against the October 2014 release of the Uniprot human database (<http://www.uniprot.org/proteomes/UP000005640/>) combined with a sequence-reversed (decoy) version of all proteins.

Installation for running the TPP R package To install R, download the latest release version of R from <http://cran.r-project.org/> and install it. For details refer to the R Installation and Administration manual. (A useful quick reference for R commands can be found at <http://cran.r-project.org/doc/contrib/Short-refcard.pdf>.)

To install the TPP package from Bioconductor, you need to start the R program. On Windows and OS X, this will usually mean double-clicking on the R application; on UNIX-like systems, type

\$ R

at a shell prompt. Then run the following commands (note that the first line is only required under Windows):

```
> setInternet2(TRUE)
> source("http://bioconductor.org/biocLite.R")
> biocLite("TPP")
```

Now you can load the package by running the following command:

```
> library("TPP")
```

Note that during the loading procedure, the package checks whether you have a zip application available to R, which is required to generate valid Excel output files. If the package could not locate a zip application in your system PATH, it displays a corresponding message after loading. In this case, please install Rtools from <http://cran.r-project.org/bin/windows/Rtools/>. During the Rtools installation, you will be asked whether the system PATH shall be edited. Make sure to check the corresponding box. After the Rtools installation has been completed, restart your R session for the changes to take effect, and load the TPP package again:

```
> library("TPP")
```

All steps for running the TPP package are described in the protocol. Detailed explanations, regarding the functionality and use of the TPP package, can also be found in the accompanying vignette. To view the vignette, type the following into the R command line:

```
> browseVignettes("TPP")
```

PROCEDURE

Part 1, performing the cell handling and compound treatment part of the TPP-TR and TPP-CCR experiments • TIMING 7 h

▲ **CRITICAL** Part 1 of the PROCEDURE (Steps 1 and 2) connects our protocol with the protocol of Jafari *et al.*³. We describe which steps from the Jafari *et al.*³ protocol should be followed in order to perform the cell handling and compound treatment steps of TPP-TR and TPP-CCR experiments on intact K562 cells using panobinostat. For a full version of this part of the PROCEDURE, please see **Supplementary Methods**.

1| Perform two biological replicates of a TPP-TR experiment: two pairs of vehicle (DMSO)-treated and compound (1 μ M panobinostat)-treated experiments. Heat the samples to the following ten temperatures for each experiment: 37, 41, 44, 47, 50, 53, 56, 59, 63 and 67 °C. Except for the cell line used (K562 instead of HL-60), the duration of incubation of cells with compound (5 h instead of 30 min) and compound concentration (1 μ M instead of 20 μ M), the TPP-TR experimental steps are exactly as described in Steps 1–15 of the protocol by Jafari *et al.*³.

! **CAUTION** Instead of applying 20,000g for centrifugation as in the Jafari *et al.*³ protocol at Step 15, we transferred the cell extracts to polycarbonate thick-wall tubes and performed an ultracentrifugation step at 100,000g at 4 °C for 20 min.

▲ **CRITICAL STEP** Refer to Steps 1–16 of the **Supplementary Methods**.

2| Perform two biological replicates of a TPP-CCR experiment. Treat the cells with nine different concentrations of panobinostat (10, 2.5, 0.625, 0.15625, 0.03906, 0.00977, 0.00244, 0.00061 and 0.00015 μ M), including one vehicle control. Heat the samples to 55 °C. Except for the cell line used (K562 instead of HL-60) and duration of incubation of cells with compound (5 h instead of 30 min), the TPP-CCR experimental steps are exactly as described in Steps 18–30A(iv) of the protocol by Jafari *et al.*³.

! **CAUTION** Instead of applying 20,000g for centrifugation as in the Jafari *et al.*³ protocol at Step 30A(iv), we transferred the cell extracts to polycarbonate thick-wall tubes and performed an ultracentrifugation step at 100,000g at 4 °C for 20 min.

▲ **CRITICAL STEP** Refer to Steps 17–33 of the **Supplementary Methods**.

Part 2, preparing samples, TMT10 labeling, offline fractionation and analysis by high-resolution mass spectrometry • TIMING ~8 d

▲ **CRITICAL** Part 2 of the PROCEDURE (Steps 3–46) describes all steps for obtaining raw quantitative TMT10-plex mass spectrometry data for each experimental condition (an experimental condition is defined as being one TPP-TR vehicle-treated experiment, one TPP-TR compound-treated experiment or one TPP-CCR experiment)

Determining protein concentration and alkylation • TIMING 2 h

3| Take out ultracentrifuge tubes with the help of tweezers and place them into a precooled cooling rack. Carefully take off 30 μ l of the supernatant containing the soluble protein fraction and transfer it to 1.5-ml Eppendorf tubes and keep them on ice.

▲ **CRITICAL STEP** Be sure not to disturb or touch the pellet, and also avoid touching the tube wall, especially if the pellet is not clearly visible.

■ **PAUSE POINT** Lysate samples can be snap-frozen in liquid nitrogen and kept at –80 °C for several days at this point.

4| For protein concentration measurement, take 3 μ l of the two lowest-temperature samples (if TPP-TR samples are used) or the two lowest compound concentration samples (if TPP-CCR samples are used) of each set, or use residual supernatant of these samples if it is possible to take off safely without aspirating the pellet.

5| Add a volume of sample buffer that is equal to the volume of the protein sample to each tube and incubate it for 30 min in an Eppendorf shaker (700 r.p.m. at 50 °C).

■ **PAUSE POINT** Samples can be stored frozen at –20 °C after incubation for several weeks.

6| In the meantime, prepare the Bradford assay and measure protein concentrations of the respective temperature or compound concentration samples (see above) of each set.

7| To alkylate cysteine residues, add 100 mM iodoacetamide to each sample, mix it well and incubate the mixture in the dark at room temperature for 30 min.

▲ **CRITICAL STEP** Use either freshly dissolved iodoacetamide solution or heat up frozen 1 M iodoacetamide stock at 50 °C and mix it vigorously before one-time use. Do not reuse any stocks, and make sure to keep exposure to light at a minimum (iodoacetamide is light-sensitive and unstable).

SDS-PAGE • TIMING 1.5 h

8| Take the average of the determined protein concentration for the two samples as a guide value to calculate the volume corresponding to 25 μ g of protein, and load this volume of each of the samples, starting with the lowest temperature (for TPP-TR experiments) or highest compound concentration (for TPP-CCR experiments).

PROTOCOL

9| Assemble the electrophoresis chamber according to the manufacturer's instructions.

10| Fill the upper chamber with freshly prepared MOPS buffer (diluted 20× stock) and the lower compartment with the same buffer. For a normal chamber (stocked with two gels), ~0.5 liters of buffer is needed in total.

11| To avoid edge effects, the ten samples of one treatment set must be split onto two 12+2-well SDS gels, and every empty lane needs to be filled with the same volume of 1× LDS buffer.

▲ **CRITICAL STEP** As subsequent band cutting requires sufficient space between the individual sample bands, it is crucial not to load the samples in adjacent wells but to always leave one lane free in between.

12| Run the gels for only 15 min at a constant voltage of 80 V. Prevent the samples from moving too far into the gel: 1–2 cm is sufficient.

After the run, remove the gels from the chamber and open the cassettes according to the supplier's protocol. Have clean boxes with fixing solution ready. For 1 gel, ~30 ml of fixing solution is needed.

13| Prepare Coomassie stain solution, and after 30–60 min replace the fixation solution with ~30 ml of Coomassie solution. Incubate the gels for up to 3 min until the bands are clearly visible but not too intensely stained.

! **CAUTION** If the protein bands are stained for longer than 5 min, the process of destaining after cutting will take substantially longer, and it might lead to further problems if the dye cannot be removed completely.

14| Replace Coomassie solution with the destain solution made of 25% ethanol and 5% acetic acid (vol/vol). Change the buffer until the gel background has been adequately destained, and wash the gels again in deionized water (repeat washing steps until water stays colorless and bands are clearly visible without remaining background). Be aware that the intensity of the bands will increase in water, so even if the bands are rather pale directly after destaining they might be in order after the gel is transferred to water.

15| Check the gels for obvious outliers or a generally unexpected band intensity trend.

? **TROUBLESHOOTING**

■ **PAUSE POINT** Stained gels can be stored in sterile deionized water up to 1 week at 4 °C without obvious loss of quality.

Gel cutting and digestion ● **TIMING** ~16 h

▲ **CRITICAL** All steps need to be carried out under keratin-free conditions to avoid sample contamination. Surfaces and tools need to be cleaned beforehand, and all work should be done behind a protection shield.

16| Put a drilled and washed 96-well plate on top of a standard 96-well plate, and add 100 µl of 40% (vol/vol) ethanol in 5 mM TEAB to all wells. Discard the water in which the Coomassie-stained gels have been stored, and use a clean scalpel and an illumination desk to cut each band into three different gel pieces, which are then transferred into individual wells of the drilled plate. Use tweezers to shred the gel pieces inside each well.

17| Destain the gel pieces by incubation for 1 h at 55 °C. If they are still blue afterward, replace the destaining solution (centrifugation step: 1 min at 200g at 4 °C) and repeat the procedure for 1 h.

18| Add 100 µl of 100% ethanol to each well, and wait until the gel pieces are shrunken and white. Centrifuge for 1 min at 200g at room temperature to remove ethanol, and repeat this step once.

19| Dissolve 20 µg of LysC (1 vial) in 2 ml of 5 mM TEAB and add 15 µl to each well and gel piece. Incubate the samples at 4 °C until they are colorless and completely swollen back to their normal size (~5 min).

20| Spin the plate for 1 min at 200g at room temperature and discard the liquid. Now add 20 µl (or if necessary more) of 5 mM TEAB to each well so that the gel pieces are completely covered. Put the drilled plate onto a new plate and both of them in a plastic bag to incubate the gel pieces for 4 h at 37 °C.

! **CAUTION** From this step on, all flow-through fractions (extracts) need to be collected in one plate; fractions of 1 gel piece will be pooled in one well.

! **CAUTION** Sometimes the gel pieces can hinder the drainage of extraction buffer after the single extraction steps, and if this occurs the liquid may need to be transferred by hand to the corresponding well in the pooling plate.

21| Dissolve 20 µg of trypsin in 2 ml of 5 mM TEAB (0.01 mg/ml LysC solution) and add 15 µl of trypsin working solution to each well. If necessary, add an additional volume of 5 mM TEAB to each well to make sure that the gel pieces are sufficiently covered. Put the plate (on top of the pooling plate) into a plastic bag and incubate it overnight at 37 °C.

Protein extraction ● **TIMING** ~5 h

22| Stop digestion with 5 µl of 5% (vol/vol) formic acid, and incubate the samples for 30 min at room temperature with 20 µl of additionally added 1% (vol/vol) formic acid solution. Centrifuge the plate for 1 min at 200g at room temperature and collect the flow-through in a washed 96-well plate.

23| Add an additional 20 µl of 1% (vol/vol) formic acid solution to the gel pieces and incubate it again for 30 min at room temperature. Centrifuge the plate for 1 min at 200g at room temperature and collect the fraction in the previously used plate.

24| Add 20 µl of peptide extraction solution to the gel pieces and incubate them for 30 min. Centrifuge the plate for 1 min at 200g at room temperature and collect the flow-through samples.

25| Add 30 µl of 100% acetonitrile to the gel pieces and incubate them for 15 min. Centrifuge the plate for 1 min at 200g at room temperature and collect the fractions; repeat the addition of acetonitrile and centrifugation once.

26| Lyophilize the pooled extraction samples (~125 µl/gel piece) for ~3 h depending on how fast the drying process progresses.

! CAUTION Make sure that the samples always remain frozen.

■ **PAUSE POINT** Samples can be stored frozen at -20 °C after lyophilization for several weeks.

TMT labeling ● **TIMING** 5 h

27| Equilibrate TMT reagent to room temperature, and add 580 µl of acetonitrile to each vial (final concentration 24 mM), vortex the solution and centrifuge the vial briefly (500g at room temperature for 30 s). The remaining TMT reagent can be stored at -20 °C, but it should be used within 1–2 weeks.

! CAUTION Before each incubation step, the plates always need to be covered and sealed with polyolefin foil.

28| Dissolve lyophilized samples in 10 µl of TMT labeling solution 1 and shake them on an Eppendorf shaker for 15 min (400 r.p.m. at 20 °C).

29| Add 10 µl of TMT reagent (24 mM) to each sample and incubate the samples on an Eppendorf shaker for 1 h (400 r.p.m. at 20 °C).

30| Add 5 µl of 2.5% (vol/vol) hydroxylamine solution to each well and shake it on an Eppendorf shaker for 15 min (400 r.p.m. at 20 °C). Afterward, combine all TMT-labeled samples of one experiment in a single 1.5-ml reaction tube. Wash each well again with 5 µl of TMT labeling solution 2, and add this solution to pooled samples.

31| Freeze the plate carefully in liquid nitrogen and lyophilize the samples.

! CAUTION Make sure that the samples always remain frozen.

32| Dissolve the sample in 40 µl of 8% formic acid/20% acetonitrile solution (vol/vol), shake it on an Eppendorf shaker for 15 min (850 r.p.m. at 20 °C), and if a pellet is still visible, vortex the sample before proceeding.

33| Transfer the sample to the corresponding well on the washed 96-well plate; wash each well with 20 µl of wash solution 2 and add it to the respective well on the plate.

34| Freeze the sample in liquid nitrogen and lyophilize it.

■ **PAUSE POINT** Samples can be stored frozen at -20 °C after incubation for several weeks.

Offline prefractionation reverse-phase high basic pH ● **TIMING** 2.5 h (per experimental condition), 15 h in total

35| Add 15 µl of basic pH loading pump mobile phase solvent to each sample.

36| Seal the plate with polyolefin foil and place it for 15 min on an Eppendorf shaker (500 r.p.m. at room temperature).

37| Place it into the autosampler of the UPLC system used for offline sample fractionation at pH 12.

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38| Place a new 96-well plate in the sampler to collect the fractions.

39| Start the fractionation (the sample is completely injected).

! CAUTION The plate from which the sample was picked up cannot be removed from the well-plate sampler while the gradient is running.

? TROUBLESHOOTING

40| Freeze the plate and lyophilize it.

41| Store the dry plate at -20 °C.

■ **PAUSE POINT** Samples can be stored frozen at -20 °C after lyophilization for several weeks.

Online nanoLC-MS/MS ● TIMING 24 h per experimental condition, 6 d in total

42| Add 10 µl of 0.05% (vol/vol) trifluoroacetic acid in HPLC water to each of the samples (wells).

43| Seal the plate and place it on an Eppendorf shaker for 15 min (500 r.p.m. at 20 °C).

44| Place the plate on the sampler of the online nanoLC-MS/MS system, and start the analysis. 5 µl (50% of the sample) is injected.

▲ **CRITICAL STEP** We recommend measuring only one of the 24 fractions first in order to check the technical quality of the experiment before running the remaining fractions.

? TROUBLESHOOTING

45| The number of fractions analyzed per experiment can be adjusted depending on the analytical depth required. Here we analyzed nine fractions per experimental condition.

46| Run blanks after analyzing the fractions of each experimental condition to avoid carry over.

Part 3, identifying and quantifying proteins ● TIMING 13 h per experimental condition, ~3.5 d for all experiments

▲ **CRITICAL** Part 3 of the PROCEDURE (Steps 47–53) describes all steps for analyzing raw quantitative TMT10-plex mass spectrometry data and obtaining quantitative protein data for each experimental condition (one experimental condition = one TPP-TR vehicle-treated, one TPP-TR compound-treated or one TPP-CCR experiment).

47| Create one folder per experimental condition (e.g., 'C:\vehicle_1'). The name is important, as it will later form the basis of the output file names created by the software. The folder should be accessible from the location where isobarQuant will run.

48| Copy all Xcalibur .raw files from each of the experimental conditions to the corresponding folders created in the previous step (e.g., nine .raw files from the first vehicle TPP-TR experiment to 'C:\vehicle_1').

49| On the workstation on which isobarQuant has been installed, open a command prompt (this may be done by typing 'cmd' at the bottom of the Start Menu) and change to the directory where isobarQuant has been unzipped. The pre-Mascot workflow has two configuration files (preMascot.cfg and QuantMethods.cfg) associated with it. The settings of these typically do not need to be changed, except for exploratory purposes. They are described in detail in the **Supplementary Manual**. To start the pre-Mascot workflow, run the command as shown below: The first argument '--datadir' is the location of the folder from Step 47 and the second '--quant' is the type of isobaric quantification, here TMT10.

```
C:\isobarQuant>python preMascot.py --datadir c:\vehicle_1 --quant TMT10
```

The time taken to process each .raw file depends on its size. On a computer with 4 GB RAM and a dual-core 2,300 MHz processor running Windows server 2012, it took ~50 min to process each .raw file containing an average of 35,000 spectra, totaling 9 h for all nine fractions in one of the conditions. For each .raw file, two new files are created in this step: a .hdf5 file (see **Supplementary Manual** for details) and a .mgf file for the Mascot search. The configurable parameters for this part of the workflow default to those used for processing the panobinostat TPP-TR and TPP-CCR data, and they may be changed if required. The default parameters are applicable for analyzing .raw files, which have been acquired following similar protocol steps as outlined above. Consult the **Supplementary Manual** for details.

50 Start Mascot searches using the .mgf files created in the previous step. For the panobinostat TPP-TR and TPP-CCR experiments, the following Mascot search settings are used: 10 p.p.m. mass accuracy (monoisotopic mass) and 0.02-Da fragment ion mass accuracy. Variable modifications selected were acetyl (protein N terminal), oxidation (M) and TMT6plex (N terminal), and fixed modifications selected were carbamidomethyl (C) and TMT6plex (K). The maximum number of missed cleavages was set to 3, the enzyme was set to trypsin/P, quantification was left as 'none' and peptide charge was set to 2+ and 3+. The 'instrument' chosen was a modified ESI-TRAP, which was set to include immonium ion fragments and to allow for '2+ fragments if precursor 3+ or higher', instead of '2+ fragments if precursor 2+ or higher'. Once this has been completed, the Mascot results files (in .dat format) may be exported to 'C:\vehicle_1' via the browser interface by clicking 'Export' on the main search results page and then selecting 'Mascot DAT file'. Clicking 'Export search results' on the following page triggers the download of the .dat file. If you have access to the file system of the Mascot server itself, the .dat file may be retrieved directly from there. Searching nine .mgf files will take ~3 h, but this can vary depending on the size of the database selected and the number of processors in the Mascot server.

! CAUTION Make sure that no other .dat files from previous Mascot searches are in the 'C:\vehicle_1' folder.

51 Before starting the post-Mascot workflow, ensure that the settings in the configuration files outputResults.cfg and postMascot.cfg (found within the downloaded isobarQuant package) are as required. The values used to process the panobinostat TPP-TR and TPP-CCR data described in this protocol are set to be the default in the software release. We have found these values to be suitable for processing the majority of Orbitrap shotgun proteomics data.

These settings state that proteins are identified by at least one peptide that has a length of >6, that passes the FDR cutoff of 1% and whose Mascot score difference to the next highest score is at least 10. A protein FDR will be calculated and displayed for each protein in the final output. For protein quantification, only reporter signals from unique peptides of a minimum length of 6 whose Mascot scores pass the 1% FDR threshold will be used. Additionally, the calculated S2I must be >0.5, the P2T must be >4 and the delta of the peptide's Mascot score to the next-best-scoring Mascot-suggested peptide must also be >5. By default, the reference condition for calculation of relative fold changes was set to be the highest mass isobaric label (131L). To change this setting, the user can provide the parameter '--quantification.reference' followed by the required isobaric label when starting the post-Mascot workflow (see below). The fold changes are calculated by performing a bootstrap sum ratio³⁷ when a minimum of four quantifiable peptides are available; otherwise, a simple sum ratio is used. Further details of these parameters and information on how to alter them may be found in the **Supplementary Manual**.

! CAUTION Make sure that the reference condition for fold-change calculation corresponds to the lowest temperature for TPP-TR experiments and the lowest (vehicle) compound concentration for TPP-CCR experiments.

52 The post-Mascot workflow is started in a similar way to the pre-Mascot workflow. From the command prompt, navigate to the directory in which isobarQuant is installed, and start the workflow as below, indicating via the parameter '--mergeresults' whether to merge the search results of the multiple mass spectrometry experiments or not. The directory that contains the .hdf5 files and Mascot results (.dat) files is provided via the '--datadir' argument, here 'C:\vehicle_1'.

```
C:\isobarQuant>python postMascot.py --datadir c:\vehicle_1 --mergeresults yes
```

This will initiate protein inference, protein quantification and output generation, during which peptides are filtered according to the criteria listed above and reporter ion intensities are corrected for ratio compression using the S2I values, as described³⁵. Detailed information on each step can be found in the **Supplementary Manual**. At the end of this process, four new files are created in the 'C:\vehicle_1' directory. Three of these are .txt files and one is a .hdf5 file. They are named according to the scheme:

vehicle_1_merged_results_rundate_runtim suffix

The suffix describes the content as follows:

Protein output (_proteins.txt)	Contains the calculated protein fold changes and may be used with the R package described in this protocol. Further description of the columns in this file is given in the Supplementary Manual
Peptide output (_peptides.txt)	Contains information about the individual peptides associated with each protein. Further description of the columns in this file is given in the Supplementary Manual
Summary output (_summary.txt)	Contains some statistical information about the runs performed

The .hdf5 file itself is effectively a database of all the outputs from the previous step. For standard-use cases, no access to this file is needed. This file and its format are described in detail in the **Supplementary Manual**.

The post-Mascot processing takes ~20 min for each .raw file.

?

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53 | Repeat Steps 47–52 for all experimental conditions. Suggested folder names for the result files are as follows: C:\vehicle_1, C:\vehicle_2, C:\panobinostat_1, C:\panobinostat_2 for TPP-TR and C:\TPP_CCR_1, C:\TPP_CCR_2, for TPP-CCR.

Part 4, determining melting curves and significant T_m shifts from the quantitative protein data of a TPP-TR experiment

● **TIMING** 1 h

▲ **CRITICAL** Part 4 of the PROCEDURE (Steps 54–56) describes all steps for analyzing quantitative protein information from a TPP-TR experiment. The TPP R package analyzes both biological replicates of the TPP-TR experiment (two pairs of vehicle- and compound-treated experiments) at once and compares these with each other.

54 | Analyzing your TPP-TR data with the TPP package. The default parameters in the TPP package enable processing of protein quantification data from files that were generated by the isobarQuant Python package, but it is also compatible with any other protein quantification files as long as they are in a tab-separated text format and contain at least one column with protein identifiers and columns with protein fold changes for each used isobaric label (in the case of TPP-TR corresponding to different temperatures).

The inputs to the TPP-TR analysis are specified via a configuration table. An example for such a configuration table is included in the TPP package. To obtain the example table, load the TPP package by typing

```
> library("TPP")
```

in an R command window. Now you can type

```
> system.file('example_data', package = 'TPP')
```

to obtain the path to where the example data were stored during the installation of the TPP package (e.g., 'C:/Users/myName/Documents/R/win-library/3.0/TPP/example_data'). If you navigate to this location, you will find the folders 'CCR_example_data' and 'TR_example_data'. Copy the Excel sheet 'Panobinostat TPP-TR_config.xlsx' from 'TR_example_data' to a location of your choice—e.g., 'C:\TPP-TR_data'; in the following, we refer to this as 'TPP-TR_data_location'.

If you open 'Panobinostat TPP-TR_config.xlsx' in a spreadsheet program, you will find a configuration table containing the following columns (**Fig. 4a**):

Column	Content
Experiment	Identifiers for the experiments to be analyzed together. The identifiers can be chosen according to your preferences, but they should not contain blanks or special characters except for underscores, and they should begin with a letter, not a digit
Condition	Specifies for each experiment whether it is a 'Treatment' or a 'Vehicle' experiment (these designators must be provided with this exact spelling). If you are not dealing with control vs. treatment experiments, set the values in this column to 'NULL'
Replicate	Specifies for each experiment what replicate it belongs to (indicate replicates by integers). If you are not dealing with replicate experiments, set the values in this column to 'NULL'
Labels	The temperatures that the isobaric labels correspond to. These can be identical for all experiments (recommended) or differ between the experiments
Path	Absolute path to the corresponding input file for each experiment

Whereas the headers of the columns that are shaded orange in **Figure 4a** must match those of the fold-change columns in the input files, the names of the 'Experiment', 'Condition', 'Replicate' and 'Path' columns must not be changed.

55 | If you want to analyze files generated by isobarQuant, follow the steps in option A; otherwise, follow those in option B.

(A) Analyzing files generated by isobarQuant

- Copy the four proteins .txt files generated by isobarQuant from the four folders: C:\vehicle_1, C:\vehicle_2, C:\panobinostat_1, C:\panobinostat_2 to 'TPP-TR_data_location' (see above).
- In the 'Panobinostat TPP-TR_config.xlsx', replace the placeholder <copy path here> by the absolute paths to the corresponding files in your 'TPP-TR_data_location'. The rest of the configuration suits the experiment described in this protocol (**Fig. 4b**).

N														
1	Experiment	Condition	Replicate	126	127L	127H	128L	128H	129L	129H	130L	130H	131L	Path
2	Vehicle_1	Vehicle		1	67	63	59	56	53	50	47	44	41	37 <copy path here>
3	Vehicle_2	Vehicle		2	67	63	59	56	53	50	47	44	41	37 <copy path here>
4	Panobinostat_1	Treatment		1	67	63	59	56	53	50	47	44	41	37 <copy path here>
5	Panobinostat_2	Treatment		2	67	63	59	56	53	50	47	44	41	37 <copy path here>

N														
1	Experiment	Condition	Replicate	126	127L	127H	128L	128H	129L	129H	130L	130H	131L	Path
2	Vehicle_1	Vehicle		1	67	63	59	56	53	50	47	44	41	C:\TPP-TR_data\vehicle_1_merged_results_20150227_0908_proteins.txt
3	Vehicle_2	Vehicle		2	67	63	59	56	53	50	47	44	41	C:\TPP-TR_data\vehicle_2_merged_results_20150227_0847_proteins.txt
4	Panobinostat_1	Treatment		1	67	63	59	56	53	50	47	44	41	C:\TPP-TR_data\panobinostat_1_merged_results_20150226_1013_proteins.txt
5	Panobinostat_2	Treatment		2	67	63	59	56	53	50	47	44	41	C:\TPP-TR_data\panobinostat_2_merged_results_20150226_1139_proteins.txt

N														
1	Prot_ID	numSpec	FC_TMT126	FC_TMT127L	FC_TMT127H	FC_TMT128L	FC_TMT128H	FC_TMT129L	FC_TMT129H	FC_TMT130L	FC_TMT130H	FC_TMT131L	other columns ...	
2	P45974	11	0.0121145	0.0247455	0.051806	0.133083	0.370148	0.626196	0.934595	0.924412	0.972442	1	...	
3	O15143	4	0.036972	0.120039	0.342576	0.457391	0.668562	0.840216	1.03664	1.03774	1.00186	1	...	
4	O15144	8	0.0239136	0.196391	0.43366	0.583243	0.921825	1.11436	1.08541	0.901592	0.888808	1	...	
5	O15145	6	0.0108314	0.0638404	0.271248	0.429889	0.742479	0.993796	0.916678	0.868269	0.876317	1	...	
6	O15294	4	0.0045511	0.0302714	0.0491007	0.0436317	0.101716	0.477043	0.809563	0.934916	0.98174	1	...	
7	O007704	6	0.2600412	0.2800620	0.28524	0.281207	0.280565	0.280582	0.282411	0.282558	0.282607	1	...	

N														
1	Experiment	Condition	Replicate	TMT126	TMT127L	TMT127H	TMT128L	TMT128H	TMT129L	TMT129H	TMT130L	TMT130H	TMT131L	Path
2	Vehicle_1	Vehicle	1	67	63	59	56	53	50	47	44	41	37	C:\myTPP-TRdata\myInputFile_Vehicle1.txt
3	Vehicle_2	Vehicle	2	67	63	59	56	53	50	47	44	41	37	C:\myTPP-TRdata\myInputFile_Vehicle2.txt
4	Panobinostat_1	Treatment	1	67	63	59	56	53	50	47	44	41	37	C:\myTPP-TRdata\myInputFile_Panobinostat1.txt
5	Panobinostat_2	Treatment	2	67	63	59	56	53	50	47	44	41	37	C:\myTPP-TRdata\myInputFile_Panobinostat2.txt

Figure 4 | Example tables required to analyze TPP-TR data using the TPP package. (a) Panobinostat TPP-TR experiment_config.xlsx as it is provided as part of the TPP package. (b) Panobinostat TPP-TR experiment_config.xlsx exemplarily modified to analyze input files generated by isobarQuant. (c) Example input file for the TPP package containing TPP-TR data not generated by isobarQuant. (d) Panobinostat TPP-TR experiment_config.xlsx exemplarily modified to analyze input files not generated by isobarQuant.

- (iii) Save the modified file.
- (iv) On the basis of the information in the Excel file, the function analyzeTPPTR() controls the workflow illustrated in **Figure 3a** and described in detail in Savitski *et al.*¹². To start the analysis with the default parameters and to write the resulting data frame to a variable called 'TPPTR_result', run the following command (Replace 'TPP-TR _data _location' by the path to your modified 'Panobinostat TPP-TR_config.xlsx' file. Note that R does not accept backslashes, but it rather uses forward-slashes for paths (as on OS-X or Unix) also in Windows.):

```
> TPPTR_result <- analyzeTPPTR("TPP-TR_data_location" /Panobinostat TPP-TR_config.xlsx")
```

Optionally, use the 'resultPath' parameter when calling analyzeTPPTR() to control the output location. By default, the output will be written to the folder in which the first input file listed in the configuration table is located.

(B) Analyzing input files not generated by isobarQuant

- (i) Prepare one input file per experiment that is supposed to be part of the analysis, and store them in one folder—e.g., 'C:\my_TPP-TR_data'. Make sure that all your input files are in a tab-separated text format and that they contain at least the following columns (**Fig. 4c**): a unique protein identifier that will be used for matching proteins across multiple input files (blue shading in **Fig. 4c**) and one column per isobaric label (in the case of TPP-TR corresponding to the different temperatures), which contains the relative fold changes between the label indicated in the column name and the label representing the lowest temperature (orange shading in **Fig. 4c**). If you are using a different number of temperatures (i.e., isobaric labels), you may have to adjust the filter criteria for normalization. See the package vignette for details.

The green shading in **Figure 4c** shows an optional column where in each row the value of that column indicates the quality of the data in the row (the higher the better). If there are multiple rows with the same protein identifier in an input file (not recommended, see above), the one with the highest quality value will be used for further analysis (green shading in **Fig. 4c**). The order of the columns is not important, and any other column in the input files will not be used and just be transferred to the output table of the TPP package.

! CAUTION Make sure that the fold changes in your input files are calculated relative to the lowest temperature.

- (ii) Modify the table 'Panobinostat TPP-TR_config.xlsx' (**Fig. 4d**): to do this, first adapt the information in the 'Experiment', 'Condition' and 'Replicate' columns to suit your experimental setting, and then replace the orange

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shaded columns in **Figure 4d** so that, together with a common prefix, their column names form those of the fold-change columns in the input files. In the given example, the common prefix is 'FC_', so that, e.g., 'FC_ + 'TMT126' = 'FC_TMT126' (see orange shaded column headers in **Fig. 4c**). Adjust the temperature values in these columns to match your experimental setting. In the 'Path' column, specify the absolute path to the corresponding input file for each experiment (**Fig. 4d**).

- (iii) Store this configuration table in the folder with your input files from Step 55B(i), e.g., as 'C:\my TPP-TR_data\myConfigTable.xlsx'.
- (iv) On the basis of the information in the Excel file, the function `analyzeTPPTR()` controls the workflow illustrated in **Figure 3a** and described in detail in Savitski *et al.*¹². To start the analysis and to write the resulting data frame to a variable called 'TPPTR_result', type the following (Replace 'C:/my TPP-TR_data/myConfigTable.xlsx' by the path to your configuration table and note that R does not accept backslashes in paths!):

```
> TPPTR_result <- analyzeTPPTR ("C:/my TPP-TR_data/myConfigTable.xlsx",
  idVar= "Prot_ID", qualColName= "numSpec", fcStr= "FC_")
```

The parameter 'idVar' specifies the name of the column that contains the unique protein identifiers (see Step 55B(i)), 'qualColName' specifies the quality column (see Step 55B(i)) and 'fcStr' specifies the prefix for the fold-change columns (see Step 55B(ii)).

Optionally, use the 'resultPath' parameter when you are calling `analyzeTPPTR()` to control the output location. By default, the output will be written to the folder in which the first input file listed in the configuration table is located.

TROUBLESHOOTING

56 | After the analysis has finished (by using all cores on a 2.5-GHz Windows7 PC with 4 cores and 4 GB RAM, the analysis took ~35 min on four input files containing just over 6,000 unique proteins in total), navigate to the output location to inspect your results. A new folder called 'TPP_results' is created at that location, which contains the following objects:

Object	Content
results TPP TR.xlsx	Excel spreadsheet containing all results and links to the melting-curve plots
dataObj	Folder containing R data objects that hold the contents of 'results TPP TR.xlsx' in an R data frame ('results TPP TR.Rdata'), a list of data objects with intermediate results after melting curve fitting and parameter determination ('fittedData.Rdata') and a list of data objects with intermediate results after normalization ('normalizedData.Rdata')
Melting_Curves	Folder containing one pdf file per protein showing a plot of the melting curve fits and the corresponding parameters
QCplots.pdf	Pdf file containing QC plots to visualize the median curve fits used in the normalization step, the normalization effects on the fold changes and the distribution of melting point differences with respect to the melting curve slopes used for significance assessment

The links to the pdf files in 'results TPP TR.xlsx' are relative links, so that if the result folder is moved they remain functional as long as the location of the 'Melting_Curves' folder relative to the 'results TPP TR.xlsx' spreadsheet is maintained.

TROUBLESHOOTING

Part 5, determining compound potency on proteins from the quantitative protein data of a TPP-CCR experiment

● **TIMING** 0.5 h

▲ **CRITICAL** Part 5 of the PROCEDURE (Steps 57–59) describes all steps for analyzing quantitative protein information from a TPP-CCR experiment. The TPP R package analyzes the two biological replicates of the TPP-CCR experiment one by one, and it determines pEC₅₀ values for proteins that pass the necessary requirements.

57 | Analogous to the TPP-TR analysis, the TPP-CCR analysis is configured via a configuration table. An example for such a configuration table is included in the TPP package. To obtain the example table, load the TPP package by typing

```
> library ("TPP")
```

and then type the following to obtain the path to the included example data. From the folder 'CCR_example_data' at the returned location, copy the Excel sheet 'Panobinostat TPP-CCR_config.xlsx' to 'TPP-CCR_data_location' (a location of your choice; e.g., 'C:\TPP-CCR_data\'):

```
> system.file('example_data', package = 'TPP')
```

If you open 'Panobinostat TPP-CCR_config.xlsx' you will find a configuration table containing the following columns (**Fig. 5a**):

Column	Content
Experiment	Identifier for the experiment to be analyzed
Columns that are named by the identifiers of the isobaric labels as they appear in the headers of the fold change columns in the input files (see Step 58B(ii) below)	The compound concentrations in μM that the isobaric labels correspond to
Path	Absolute path to your input file

Whereas the headers of the columns that are shaded orange in **Figure 5a** must match those of the fold-change columns in the input files, the names of the 'Experiment' and 'Path' columns must not be changed.

58 If you want to analyze the files generated by isobarQuant, follow the steps in option A; otherwise, follow those in option B.

(A) Analyzing files generated by isobarQuant

- Copy the proteins .txt file generated by IsobarQuant to 'TPP-CCR_data_location' (see above).
- In the 'Panobinostat TPP-CCR_config.xlsx', replace the placeholder <copy path here> by the path to the corresponding file in your 'TPP-CCR_data_location'. The rest of the configuration suits the experiment described in this protocol (**Fig. 5b**).
- Save the modified file.
- On the basis of the information in the Excel file, the function `analyzeTPPCCR()` controls the workflow illustrated in **Figure 3b** and described in detail in **Box 3**. To start the analysis with the default parameters and to write the resulting data frame to a variable called 'TPPCCR_result', run the following command (Replace 'TPP-CCR_data_location' by the path to your modified 'Panobinostat TPP-CCR_config.xlsx' file and note that R does not accept backslashes in paths!):

```
> TPPCCR_result <- analyzeTPPCCR(
  " 'TPP-CCR_data_location' /Panobinostat TPP-CCR_config.xlsx" )
```

Optionally, use the 'resultPath' parameter when calling `analyzeTPPCCR()` to control the output location. By default, the output will be written to the folder in which the input file given in the configuration table is located.

a													
	A	B	C	D	E	F	G	H	I	J	K	L	
1	Experiment	126	127L	127H	128L	128H	129L	129H	130L	130H	131L	Path	
2	Panobinostat_1	10	2.5	0.625	0.15625	0.03906	0.00977	0.00244	0.00061	0.00015	0	<copy path here>	

b													
	A	B	C	D	E	F	G	H	I	J	K	L	
1	Experiment	126	127L	127H	128L	128H	129L	129H	130L	130H	131L	Path	
2	Panobinostat_1	10	2.5	0.625	0.15625	0.03906	0.00977	0.00244	0.00061	0.00015	0	C:\TPP-CCR_data\panobinostat_1_merged_results_20150227_1017_proteins.txt	

c														
	A	B	C	D	E	F	G	H	I	J	K	L	M	
1	Prot_ID	numSpec	FC_TMT126	FC_TMT127L	FC_TMT127H	FC_TMT127H	FC_TMT128L	FC_TMT128H	FC_TMT129L	FC_TMT129H	FC_TMT130L	FC_TMT130H	FC_TMT131L	other columns ...
2	P30613	17	1.44376	1.64708	1.37119	1.22776	1.19985	1.26227	0.813251	1.33376	1.05782	1	...	
3	P23919	8	1.69997	1.86556	1.39599	1.28556	1.3479	1.4173	0.915872	1.46516	1.13496	1	...	
4	Q07960	5	1.14255	1.07415	1.12082	0.921415	0.71106	0.802999	0.681458	1.50051	0.940735	1	...	
5	Q9NXR7	5	1.71619	2.00844	1.5165	1.40825	1.28814	1.38825	0.873972	1.58082	1.11446	1	...	
6	P40429	9	0.226722	0.375417	1.01621	1.05184	0.394098	0.36521	1.08728	1.44252	1.43675	1	...	
7	P0E38C	1	0.42208	0.545032	0.862026	1.00102	0.476247	0.534734	0.915541	1.05553	1.08742	1	...	

d													
	A	B	C	D	E	F	G	H	I	J	K	L	
1	Experiment	TMT126	TMT127L	TMT127H	TMT128L	TMT128H	TMT129L	TMT129H	TMT130L	TMT130H	TMT131L	Path	
2	Panobinostat_1	10	2.5	0.625	0.15625	0.03906	0.00977	0.00244	0.00061	0.00015	0	C:\my_TPP-CCR_data\myInputFile_Panobinostat_1.txt	

Figure 5 | Example tables required to analyze TPP-CCR data using the TPP package. (a) Panobinostat TPP-CCR experiment_config.xlsx as it is provided as part of the TPP package. (b) Panobinostat TPP-CCR experiment_config.xlsx exemplarily modified to analyze an input file generated by isobarQuant. (c) Example input file for the TPP package containing TPP-CCR data not generated by isobarQuant. (d) Panobinostat TPP-TR_config.xlsx exemplarily modified to analyze an input file not generated by isobarQuant.

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(B) Analyzing input files not generated by isobarQuant

(i) Prepare an input file that is supposed to be analyzed, and store it in a folder; e.g., 'C:\my_TPP-CCR_data\'.

Make sure that your input file is in a tab-separated text format and that it contains at least the following columns (**Fig. 5c**): a unique protein identifier (blue shading in **Fig. 5c**) and one column per isobaric label (in the case of TPP-CCR corresponding to the different compound concentrations), which contains the relative fold changes between the label indicated in the column name, and the label representing the lowest (vehicle) compound concentration (orange shading in **Fig. 5c**). The green shading in **Figure 5c** marks a column whose values indicate the quality of the data in the corresponding row (the higher the better). This column is optional. If there are multiple rows with the same protein identifier in an input file (not recommended, see above), the one with the highest-quality value will be used for further analysis.

! CAUTION Make sure that the fold changes in your input files are calculated relative to the lowest (vehicle) compound concentration.

(ii) Modify the table 'Panobinostat TPP-CCR_config.xlsx' (**Fig. 5d**). To do this, first adapt the information in the 'Experiment' column to suit your experimental setting, and then replace the orange shaded columns so that, together with a common prefix, their column names form those of the fold-change columns in the input file. In the given example, the common prefix is 'FC_', so that, e.g., 'FC_ + 'TMT126' = 'FC_TMT126' (see orange shaded column headers in **Fig. 5c**). Adjust the compound concentrations in μM in these columns to match your experimental setting.

(iii) In the 'Path' column, specify the absolute path to your input file.

(iv) Store this configuration table in the folder with your input files from Step 58B(i), e.g., as 'C:\my_TPP-CCR_data\myConfigTable.xlsx'.

(v) On the basis of the information in the Excel file, the function `analyzeTPPCCR()` controls the workflow illustrated in **Figure 3b** and described in detail in **Box 3**. To start the analysis and to write the resulting data frame to a variable called 'TPPCCR_result', type the following command in R (Replace 'C:/my_TPP-CCR_data/myConfigTable.xlsx' by the path to your configuration table and note that R does not accept backslashes in paths!):

```
> TPPCCR_result <- analyzeTPPCCR(  
  "C:/my_TPP-CCR_data/myConfigTable.xlsx",  
  idVar= "Prot_ID", qualColName= "numSpec", fcStr= "FC_")
```

The parameter 'idVar' specifies the name of the column that contains the unique protein identifiers (see Step 58B(i)), 'qualColName' specifies the quality column (see Step 58B(i)) and 'fcStr' specifies the prefix for the fold-change column (see Step 58B(ii)).

Optionally, use the 'resultPath' parameter when you are calling `analyzeTPPCCR()` to control the output location.

By default, the output will be written to the folder in which the input file given in the configuration table is located.

? TROUBLESHOOTING

59 After the analysis has finished (on a 2.5 GHz Windows7 PC with 4 GB RAM, the analysis of a file with ~3,600 proteins takes ~3 min), navigate to the output location to inspect your results. A new folder called 'TPP_results' is created at that location, which contains the following objects:

Object	Content
results_TPP-CCR.xlsx	Excel spreadsheet containing all results and links to the dose-response curve plots
DoseResponse_Curves	Folder containing one pdf file per protein showing a plot of the dose-response curve fit and the corresponding parameters
results_TPP_CCR.Rdata	R data object that holds the contents of 'results_TPP-CCR.xlsx' in an R data frame

The links to the pdf files in 'results_TPP_CCR.xlsx' are relative links, so that if the result folder is moved they remain functional as long as the location of the 'DoseResponse_Curves' folder relative to the 'results_TPP_CCR.xlsx' spreadsheet is maintained.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
15	Gel QC reveals a band outlier (one band has a stronger staining compared with other bands at the same temperature)	Part of the pellet (aggregated protein fraction) accidentally aspirated into the soluble fraction	Discard the sample and repeat the experiment
39	No UV and pressure trace after the fractionation	Sample not picked up by the autosampler Leakage	Check the autosampler, and repeat fractionation Check the HPLC and repeat the experiment
44	No peaks are eluting	Sample not picked up Leakage	Check the autosampler, and repeat the step Check the nanoLC tubings and repeat the step
	Weak low-quality MS1 and MS/MS spectra	Bad ionization efficiency HCD energy settings not optimal	Check the electrospray performance, adjust the voltage settings if necessary and repeat the step
	Good-quality MS1 and bad-quality MS/MS spectra	Instrument is poorly calibrated	Check higher-energy collisional dissociation (HCD) energy settings, adjust if necessary, and repeat the step
	Poor protein identification after searching with Mascot		Recalibrate the instrument and repeat Step 44
52	All FDR values for protein and peptide show 0	No decoy data (reverse hits) present in the .fasta file used for searching	Add decoy hits to .fasta file on Mascot server (see http://www.matrixscience.com/help/decoy_help.html)
	postMascot.py exits saying 'No *.dat files found for processing'	Mascot .dat files were not exported to the corresponding data directory; e.g., C:\vehicle_1, or the wrong datadir name was entered	Check that the value given as argument for -datadir exists and contains .dat files
	Failed digestion; either no or very few peptides were detected	Wrong trypsin or LysC concentration (at Step 21)	Repeat the experiment
	Mascot search results show an absence of peaks corresponding to TMT10 peptide labeling, indicating that TMT10 labeling failed	Reagents degraded	Check the expiration date of TMT10 reagents; if necessary, purchase new reagents and repeat the experiment
		Samples and buffers contaminated with amine-containing component	Repeat the experiment
55,58	Functions analyzeTPPTR() or analyzeTPPCR() exit while importing data	Column headers in config table and input file(s) do not match	Check that the column names in your input file(s) and your config table match, as explained in Steps 55B(ii) and 58B(ii)
55	Function analyzeTPPTR() exits with an error message: 'Importing TR dataset: NA Error in file(file, "*") : cannot open the connection'	Config table contains non-empty rows that do not specify an experiment	Delete all rows from your config table that do not specify an experiment to be analyzed
	Function analyzeTPPTR() exits with an error message: 'Error in modelList[[r2Best]] : attempt to select less than one element'	Wrong reference label. Possibly the highest temperature instead of the lowest was chosen as reference label during fold-change calculation	Rerun the fold-change calculation with the correct reference label. For isobarQuant, see Step 51
	A substantial population of identified proteins are outliers in one temperature condition of the TPP-TR experiment	Partial resolubilization of the pellet when aspirating	Repeat the TR workflow of the TPP package without using the affected temperature condition

(continued)

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TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
56,59	When you try to open the result Excel file, Excel complains that it 'found unreadable content' in the file and cannot open it	There is no zip application in your system PATH. This is required by the openxlsx package to generate valid Excel files	Install Rtools and make sure to check the corresponding box when you are asked whether the system PATH shall be edited during the installation. See 'Installation for running the TPP R package' in Equipment Setup
58	Protein with significant T_m shift in TPP-TR did not show a dose response in TPP-CCR	False positive in TPP-TR Wrong TPP-CCR temperature for this protein	Protein should not be considered as a potential compound target Select a better temperature for the TPP-CCR experiment

● TIMING

Part 1

Steps 1 and 2, performing the cell handling and compound treatment part of the TPP-TR and TPP-CCR experiments: 7 h

Part 2

Preparing samples, TMT10 labeling, offline fractionation and analysis by high-resolution mass spectrometry of TPP-TR and TPP-CCR experiments: ~8 d

Steps 3–7, determining protein concentration and alkylation: 2 h

Steps 8–15, SDS-PAGE: 1.5 h

Steps 16–21, gel cutting and digestion: ~16 h

Steps 22–26, protein extraction (all steps are done at room temperature): ~5 h

Steps 27–34, TMT labeling: 5 h

Steps 35–41, offline prefractionation reverse-phase high basic pH: 2.5 h (per experimental condition = one TPP-TR vehicle-treated experiment, one TPP-TR compound-treated experiment or one TPP-CCR experiment), 15 h in total

Steps 42–46, online nanoLC-MS/MS: 24 h per experimental condition, 6 d in total

Part 3

Steps 47–53, identifying and quantifying proteins: 13 h per experimental condition, ~3.5 d for all experiments

Part 4

Steps 54–56, determining melting curves and significant T_m shifts from the quantitative protein data of a TPP-TR experiment: 1 h

Part 5

Steps 57–59, determining compound potency on proteins from the quantitative protein data of a TPP-CCR experiment: 0.5 h

ANTICIPATED RESULTS

TPP of cells treated with panobinostat

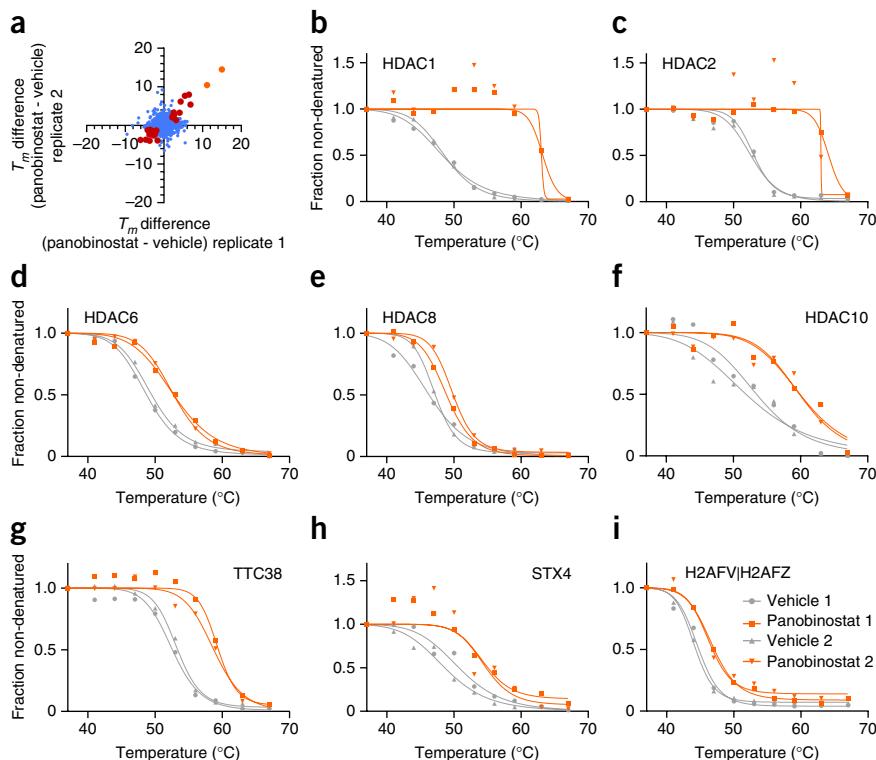
Throughout the protocol, we follow TPP-TR and TPP-CCR experiments performed on K562 cells using the clinical HDAC inhibitor panobinostat^{6,17}. Two biological replicates of TPP-TR were performed after treatment of cells with panobinostat, and in total 6,004 proteins were identified. HDACs 1, 2, 4, 6, 7, 8 and 10 were identified and quantified in both vehicle- and compound-treated conditions. Pronounced changes in thermal stability were observed for the HDACs 1, 2, 6, 8 and 10, as well as for other proteins (Fig. 6); TTC38, syntaxin-4 (STX4) and zinc-finger and FYVE domain-containing 28 (ZFYVE28; Supplementary Data 1) are most prominent among them.

We recommend manually inspecting cases of T_m shifts that fall just outside the specified significance thresholds (Box 2). For instance, HDAC1 and HDAC2 (Fig. 6b,c) did not pass the significance requirements even though the melting point shift is large, because the R^2 fit of the melting curve in one of the biological replicates of the TPP-TR experiments was below 0.8 for both proteins (Box 2). We would not recommend repeating the experiment for cases like this, as the large melting point shifts reproduced in both biological replicates and the R^2 fit was just below the required value. Rather, we would suggest flagging the proteins as potential hits and validating them by performing TPP-CCR experiments.

Among targets with smaller, but significant, changes in thermal stability, an interesting one is the H2A histone family, member V or Z protein (H2AFV or H2AFZ; the two variants cannot be distinguished on the basis of identified peptides; Fig. 6i), which is a likely case of an indirect target of panobinostat (Box 1), as it is known that HDAC inhibition leads to hyperacetylation of H2AFV/H2AFZ⁶.

Figure 6 | Panobinostat TPP-TR experiment.

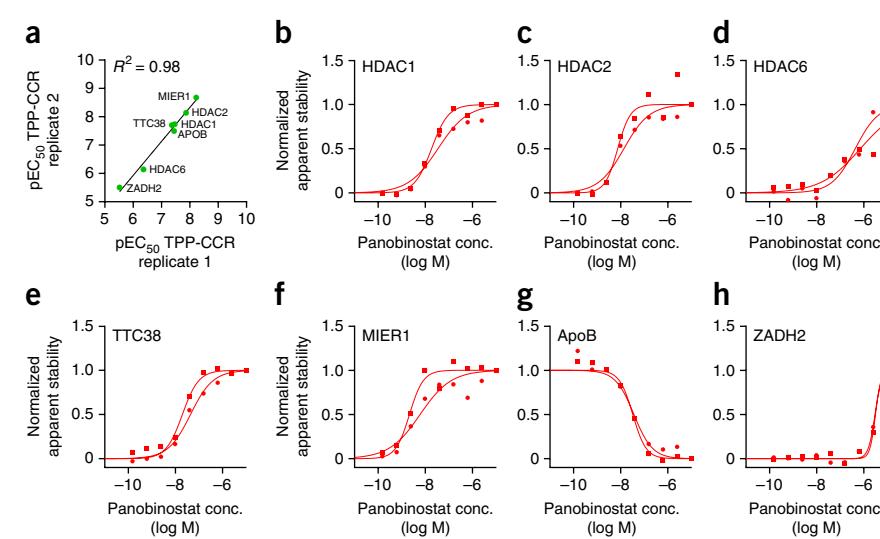
(a) Scatter plot of T_m shifts calculated from the two biological replicates of the panobinostat versus vehicle treatment experiment. Panobinostat-induced T_m shifts that passed the significance criteria are shown in red. The points corresponding to the HDAC1 and HDAC2 proteins are colored orange; both proteins passed the significance requirement in one biological replicate and were just outside the threshold for significance in the other. (b–i) Melting curves of proteins for which strong and significant or borderline significant thermal stabilization was observed when treating cells with panobinostat; HDAC 1, 2, 6, 8 and 10 (b–f); TTC38 (g); STX4 (h); and H2AFV or H2AFZ (i).



We performed two biological replicates of a TPP-CCR experiment to determine the compound potency for a subset of these targets. The TPP-CCR experiment was performed at 55 °C, which, based on the TPP-TR data, appeared ideal for the determination of pEC_{50} values (i.e., the drug concentration required to elicit a half-maximal thermal shift) for HDAC 1, 2, 6 and 10 proteins, as well as for TTC38, STX4 and ZFYVE28. Reproducible pEC_{50} values were determined for HDAC 1, 2 and 6 in both biological replicates of the TPP-CCR experiment (Fig. 7) and showed good correlation with the previously reported half-maximal inhibitory concentration (IC_{50}) values for panobinostat⁶. HDAC10 was identified by a single spectrum in both TPP-CCR experiments; a pEC_{50} could be determined in one experiment but not in the other, thus yielding inconclusive results (Supplementary Data 2). The thermal stability of STX4 did not exhibit any dose-dependent behavior in either of the experiments, strongly suggesting that the result from the TPP-TR experiment was a false positive (Supplementary Data 2). ZFYVE28 was not identified in the TPP-CCR experiments, and hence the effect of panobinostat on its thermal stability could not be quantitatively verified.

Both TPP-CCR experiments showed that panobinostat elicits a significant effect on the thermal stability of the TTC38 protein even at low concentrations (Fig. 7e). This direct or indirect effect of the drug on TTC38 is a novel finding for the mode of action of panobinostat that will need to be further investigated using orthogonal biochemical and functional assays.

In addition, a known HDAC1/HDAC2 complex member, mesoderm induction early response 1, transcriptional regulator (MIER1)⁶ is affected by panobinostat (Fig. 7f). The ligand-binding effect on thermal stability of protein complexes formed by a target protein has been observed in previous experiments for staurosporine-stabilized cycline-dependent kinase complexes¹². We did not observe a significant shift for this HDAC complex member in the TPP-TR experiments. This further strengthens

**Figure 7 |** Panobinostat TPP-CCR experiment.

(a) pEC_{50} values derived from data fulfilling the specified fold-change and curve-fit requirements (Box 3) reveal excellent correlation between biological replicates of the TPP-CCR experiment using panobinostat. All pEC_{50} values reproduced within 0.5 \log_{10} units in both experiments. (b–h) TPP-CCR profiles for panobinostat at 55 °C for HDAC1 (b), HDAC2 (c), HDAC6 (d), TTC38 (e), MIER1 (f), ApoB (g) and ZADH2 (h).

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the notion that TPP-CCR experiments can detect compound effects for a subgroup of proteins favored by the chosen temperature, with greater sensitivity compared with TPP-TR¹².

Panobinostat also had a destabilizing effect on apolipoprotein B (ApoB; **Fig. 7g**), a protein involved in cholesterol homeostasis. Treatment of cells with HDAC inhibitors is linked to lowered levels of cholesterol probably because of lower expression levels of apolipoproteins including ApoB⁴⁷. Finally, we have identified the zinc-binding alcohol dehydrogenase domain-containing protein 2 (ZADH2) as a protein potentially directly targeted by panobinostat with low potency (**Fig. 7h**). This protein is related to other alcohol dehydrogenases that have been previously reported as targets of hydroxamate-containing compounds⁶.

To differentiate between direct and indirect targets of panobinostat, the next step would now be to perform additional TPP experiments in cell extracts, as outlined in **Box 1**. The overlap of proteins with affected thermal stability as a result of panobinostat treatment in TPP experiments in living cells compared with cell extracts will delineate the direct targets of panobinostat. Proteins with affected thermal stability in TPP experiments in living cells but not in cell extracts are likely to be indirect targets.

Mass spectrometry data are available for download at ProteomicsDB (<https://www.proteomicsdb.org/#projects/4221>).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS H.F., D.C., T.M., F.B.M.R., W.H. and M.M.S. conceived the project and wrote the manuscript; M.M.S., F.B.M.R., T.W., M.B. and G.D. designed the mass spectrometry experiments; T.W. and I.T. conducted and supervised the experiments; H.F., T.M., D.C., G.M.A.S., T.W., C.D., F.B.M.R. and M.M.S. analyzed proteomics data; T.M. and G.M.A.S. developed the isobarQuant package; D.C., H.F., C.D., S.G. and W.H. developed the TPP package; T.W., I.T., G.M.A.S., M.B. and G.D. contributed to the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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