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High-throughput generation of selected reaction-monitoring assays for proteins and proteomes

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Selected reaction monitoring (SRM) uses sensitive and specific mass spectrometric assays to measure target analytes across multiple samples, but it has not been broadly applied in proteomics owing to the tedious assay development process for each protein. We describe a method based on crude synthetic peptide libraries for the high-throughput development of SRM assays. We illustrate the power of the approach by generating and applying validated SRM assays for all Saccharomyces cerevisiae kinases and phosphatases.

Selected reaction monitoring (SRM; plural, multiple reaction monitoring)¹,² has recently emerged as a targeted proteomic technology for the consistent detection and accurate quantification of specific, predetermined sets of proteins in a complex background and in multiple samples. It exploits the capability of triple quadrupole (QQQ) mass spectrometers to selectively isolate precursor ions corresponding to the mass of the targeted peptides and to selectively monitor peptide-specific fragment ion(s). Suitable sets of precursor and fragment ion masses for a given peptide, called SRM transitions, constitute definitive mass spectrometry (MS) assays that identify a peptide and, by inference, the corresponding protein in proteome digests³. SRM has high sensitivity (low-attomolar) and a broad dynamic range (up to five orders of magnitude), and it is quantitative⁴,⁵. Once SRM assays have been established for a set of peptides, they can be used in a highly multiplexed manner (>1,000 SRM transitions per hour)⁴ and with great reproducibility, even if the measurements are carried out in different laboratories⁶. The consistency, sensitivity and completeness of datasets generated by SRM measurements compare favorably with the data generated with shotgun proteomic methods in which precursor ions are stochastically selected for fragmentation¹,⁷.

In spite of these favorable properties, SRM has not been broadly used in proteomics, and SRM-based studies have mostly focused on small numbers of proteins⁴,⁵,⁷. The effort required to develop a high-quality SRM assay for a protein has prevented the broader application of this technology. Assay development involves first the validation of the assay to confirm that it selectively monitors the analyte of interest and, second, optimization of the assay to maximize its sensitivity⁸. Optimization is achieved by determining the most suitable SRM transitions for each target peptide, along with other associated liquid chromatography (LC)-MS parameters, and is a lengthy and iterative process. Assay validation typically relies on the acquisition of full-scan MS/MS spectra for the targeted peptide on the same MS platform that will be used to deploy the assay, that is, a QQQ instrument. Acquisition of reliable MS/MS spectra of peptides in biological samples is strongly compromised by complex backgrounds that obscure the fragmentation pattern and limit the dynamic range, thus making the validation of transitions for low-abundance peptides extremely challenging. Additionally, MS/MS spectra acquisition on QQQ instruments is slow compared to fast scanning mass spectrometers such as linear ion traps. This creates the paradoxical situation that highly sensitive SRM assays have to be developed and validated by a method that has a substantially lower sensitivity and dynamic range than the SRM assay itself, which has prevented the routine development of SRM assays for low-abundance proteins.

Here we present a method for generating validated SRM assays for sets of proteins, subproteomes or whole proteomes that overcomes this limitation. It is based on the use of low-cost libraries of crude, unpurified synthetic peptides as a reference for validating and optimizing SRM assays and on a MS method to generate the assays at a throughput exceeding 100 per hour.

The method consists of the following steps (Supplementary Fig. 1). (i) A set of proteotypic peptides⁹ is selected for each target protein based on empirical data in prior proteomic datasets, such as those contained in repositories like PeptideAtlas¹⁰ or by bioinformatic prediction¹¹. (ii) The selected peptides are synthesized by Spot synthesis¹²,¹³ on a microscale and recovered from the synthesis support in a crude, unpurified form. (iii) Pools consisting of ~100 such synthesis products are analyzed by a SRM-triggered MS/MS method, whereby the detection of any of a few anticipated transitions for each peptide triggers the acquisition of a full MS/MS spectrum for the target peptide. (iv) MS/MS spectra, optionally consensus MS/MS spectra in which multiple spectra per peptide are acquired, are used to both validate the assays and extract the most favorable SRM coordinates for each peptide, such as highest-intensity fragment ions, peptide elution time and fragment...
ion relative signal intensities. (v) Optionally, the sensitivity of the SRM assays is further increased by optimizing transition- or peptide-specific MS parameters in a second LC-SRM run, in which the top transitions are measured at different parameter steps (Supplementary Discussion and Supplementary Figs. 2–4). (vi) The optimized and validated assays can then be applied to detect and quantify the target proteins in any biological sample and optionally entered into a publicly accessible repository for SRM assays8. The elution times observed for the synthetic products can be used to schedule acquisition of the SRM traces, thus drastically increasing the number of measurements per analysis in a biological sample.

To test the validity of using crude, unpurified synthetic peptides for generating SRM transitions, we evaluated a set of 20 such unpurified peptides. We analyzed each sample individually by LC-MS/MS using a high-resolution Orbitrap instrument (Fig. 1a,b and Supplementary Fig. 5). Synthesis byproducts and other contaminants were present in each sample. However, for all 20 peptides the extracted ion chromatogram of the target peptide represented the highest peak in the corresponding base peak chromatogram, indicating that the peptide of interest was the predominant component or was one of the most abundant components.

We also selected ten peptide sequences from medium- to high-abundance yeast proteins and analyzed the similarity between the QQQ MS/MS spectra for the same peptide, derived from a yeast digest or from chemical synthesis products, respectively. In all cases the spectra were indistinguishable in terms of distribution and relative intensities of assigned fragment ions (data not shown). This supports the use of MS/MS spectra acquired from unpurified synthetic peptide mixtures to predict the fragmenta-
tion pattern of naturally occurring peptides.

To test the similarity of relative fragment ion intensities in full MS/MS spectra and SRM traces of the same peptides, we analyzed 50 yeast peptides from medium- to high-abundance proteins on a QQQ instrument in both MS/MS and SRM mode acquired at the same nominal collision energy. We monitored seven to ten SRM transitions per peptide that matched the most intense peptide fragment ions observed in the QQQ full MS/MS spectrum and correlated their signal intensities. The average correlation coefficient was 0.88, indicating a high degree of similarity between SRM and MS/MS

Figure 1 | Synthetic peptide libraries are used to generate SRM assays. (a, b) Unpurified preparations of the indicated peptides, analyzed by LC-MS/MS on an Orbitrap mass spectrometer. For each peptide injected, the base peak chromatogram (BPC) and the extracted ion chromatogram (XIC) of the mass to charge ratio (m/z) of the peptide in its predominant charge state (tolerance 0.1 Da) are shown. For clarity of presentation, XIC and BPC traces are shown with an artificial offset of 1 min. (c) Comparison of full fragment ion spectra with SRM transition intensities for 50 peptides. We selected 7–10 peaks assigned to b or y ions from QQQ MS/MS spectra and measured in SRM mode. Fragment ion intensity distribution of identified peaks was very similar (r > 0.8) to the SRM transitions. (d) Example of SR transitions displayed as chromatographic traces (inset) or as a spectrum imitation (SRM intensities) compared to the corresponding MS/MS spectrum. Red peaks in the MS/MS spectrum indicate peaks matched to b and y ions.

Figure 2 | Development of SRM assays for ~100 synthetic peptides. (a–c) Peptides were analyzed in SRM-triggered MS/MS mode, using as triggering transitions those associated with the first fragment ion of the y ion series with m/z greater than the m/z of the precursor for each of the two main charge states of the peptides (doubly and triply charged). SRM-XIC chromatogram for the whole set of peptides (a), XIC of the SRM traces recorded for the two charge states of peptide EAENANLELESK (b) and associated MS/MS spectrum recorded during the analysis (c).
traces (Fig. 1c,d). Moreover, when we considered the most intense fragment ions, for 96% of the peptides the three most intense fragment ions extracted from a full-scan MS/MS spectrum included the most intense SRM transition (Supplementary Figs. 6,7). This value increased to 100% when we considered the top four fragment ions. Thus it is legitimate to extract the most intense fragment ion signals for SRM analysis from assigned MS/MS spectra acquired on a QQQ instrument, allowing the validation and optimization of the SRM assays in a single operation, provided that MS/MS spectra are generated for each peptide of interest.

To test whether our method for generating validated SRM transitions using crude synthetic peptide preparations could be applied at high throughput, we randomly chose 125 S. cerevisiae proteins, including 15 proteins that had not been observed in publicly available proteomic datasets or quantified by affinity-based techniques. We synthesized 480 proteotypic peptide sequences (five per protein; 71 peptides had been previously observed, 409 were predicted) with the SPOT synthesis technology on a small scale. We used these proteins to test our approach and optimize its steps (for example, in terms of optimal number of peptides injected, peptide amount and mass spectrometer settings to maximize the success rate; Supplementary Tables 1,2 and Online Methods). We combined peptide aliquots to produce five samples, each containing 96–140 peptides (Supplementary Table 1) and analyzed them on a QQQ instrument using predicted SRM transitions to trigger acquisition of full MS/MS spectra for the doubly and triply charged form of each peptide (Fig. 2). We developed SRM assays for 432 of 480 peptides in <6 h of instrument time. The average success rate of peptide identification was 89%, (Supplementary Tables 1 and 2) with a 1% false positive error rate, based on a decoy search strategy. The success rate did not substantially decrease (83%) when we saturated the capabilities of the acquisition software by targeting 150 peptides per run. Proteotypic peptides selected based on empirical evidence or by bioinformatic prediction resulted in comparable success rates (86% and 91%, respectively). Overall, we developed SRM assays with a minimum of one proteotypic peptide per protein for 124 of 125 target proteins (99.2%).

We then attempted to generate SRM assays for all yeast kinases and phosphatases, proteins that are of high biological interest, but which are generally found at low abundance. A shotgun proteomic analysis of a total yeast lysate with a high-performance LC-MS/MS system only identified four kinases and three phosphatases (Supplementary Table 3). Our protein set included known kinases and phosphatases and hypothetical proteins with putative kinase or phosphatase activity, a total of 156 proteins, of which 120 and 36 were annotated with kinase and phosphatase activity, respectively (Supplementary Table 4). We selected and synthesized 816 peptides (three to seven per protein, 51% of which we chose by bioinformatic prediction; Supplementary Discussion). We developed SRM assays for 698 peptides (86%); the assays included at least one proteotypic peptide for each protein (100% protein coverage; Supplementary Table 5) and are publicly available for community use in MRMAtlas.

To compare the efficiency of generating validated SRM assays via the use of synthetic peptide libraries with that achieved by SRM-triggered MS/MS measurements on natural protein samples, we used our LC-SRM-MS/MS method to analyze a total yeast lysate on the same QQQ instrument. We identified only one phosphatase-derived peptide (GSKPGQQVQDEEER from GLC7; Supplementary Table 6), and therefore developed an SRM assay for only one of 156 proteins.

We used the SRM assays generated with the synthetic peptides to detect kinases and phosphatases in an unfractionated yeast proteome tryptic digest via time-scheduled SRM. Overall, we unambiguously detected 84 kinases and 26 phosphatases. This corresponds to 71% of the targeted protein set and includes proteins with abundances of ~20,000 to 112 copies.

Figure 3 | Analysis of yeast protein kinases and phosphatases. (a) Detection of the kinase Snf1 (590 copies per cell) in a total yeast digest, using an SRM assay for the Snf1-derived proteotypic peptide: SSSLADGAHIGNYQIK. The signal was acquired via time-scheduled SRM using a 4000QTrap. Three SRM transition (labeled as precursor to fragment; thomson) traces for each of the two main peptide charge states are shown. (b) Distribution of cellular abundances of all protein kinases and phosphatases in S. cerevisiae: enzymes detected by SRM in an unfractionated yeast sample; enzymes detected via SRM when adding one step of off-gel electrophoresis fractionation; enzymes detected via in-depth fractionation and shotgun LC-MS/MS (dataset in ref. 15); and enzymes detected via shotgun proteomic analysis of an unfractionated yeast digest (pooled results from five LC-MS/MS runs). Protein abundances were derived from a tandem affinity purification (TAP)-tag dataset14 and the dataset in ref. 15, was analyzed as described in Supplementary Discussion. ‘No TAP tag’ indicates proteins that could not be measured in the TAP-tag study, and ‘No fractionation and no TAP tag’ indicates proteins that could neither be measured in the TAP-tag study, nor via in-depth fractionation coupled to shotgun proteomics. (c) Example of resolved ambiguity in the identification of peptide TLSGNDYINASYVK from kinase Ptp1. Relative intensities of fragment ions in the QQQ MS/MS spectrum (23.5 min) of the synthetic peptide were used to distinguish the correct protein peak group from that of a different analyte, which could constitute a false positive identification in the biological sample.
per cell\textsuperscript{14} and some previously undetected proteins (Fig. 3a, b). We observed no bias to medium-high abundance proteins (Fig. 3a, b), and the congruence of fragmentation patterns obtained from native and synthetic peptides allowed us to increase the confidence of detecting the targeted peptides in case of small or ambiguous signals (Fig. 3c). We detected six additional kinases and two phosphatases (final coverage, 76%; Fig. 3b and Supplementary Table 7) if we analyzed off-gel electrophoresis fractions of the sample. The kinase and phosphatase coverage achieved by SRM using an unfraccionated yeast total proteome digest was almost as high as that obtained in a recent yeast proteomic analysis based on a large-scale sample fractionation\textsuperscript{15} (Fig. 3b).

There are several possible reasons why not all the targeted proteins were detected. They include, (i) absence of the protein in the sample, that is, no expression under the cell conditions used, (ii) low abundance and selection of proteotypic peptides with suboptimal MS signal response or (iii) modification of the targeted protein in the selected proteotypic peptide and thus absence of the targeted peptide in the sample.

In summary, our method to rapidly generate SRM assays using crude synthetic peptides expands the application of SRM-based targeted MS for high-throughput protein detection and quantification. It eliminates a substantial bottleneck in SRM assay development, the generation of full-scan MS/MS spectra from low-abundance peptides in a QQQ mass spectrometer (Supplementary Discussion). With the Spot-synthesis technology, it is possible to generate crude peptides at dramatically lower cost ($5–15 each), compared to classical peptide synthesis of purified peptides ($\sim$500 each). This should facilitate the use of peptide libraries representing larger protein sets, subproteomes or complete proteomes. Crude peptide libraries can be generated at a very high rate of >50,000 peptides per month, which is well matched by the throughput of generating the corresponding SRM assays with our MS approach to concurrently validate and optimize the transitions. At a pace of >100 peptides per hour, SRM assays for 50,000 peptides, roughly the number of peptides required to cover the proteins of the human proteome, could be validated in ~500 h on a single LC-MS/MS system. This opens exciting possibilities in biotechnological, biomedical, pharmaceutical and biological applications, and makes the quantitative analysis of a whole proteome by SRM a concrete possibility.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

P.P. and R.A. designed the experiments and the method workflow and wrote the paper. O.R. conceived the data analysis pipeline and performed part of the data analysis. P.P., R.S. and F.D. performed the experiments and analyzed data. T.F. compared results with those of ref. 15. H.W. supervised optimization of the SPOT-synthesis technology for producing small-scale, unpurified peptides to be used in SRM or multiple reaction monitoring assays; B.D. contributed to technical aspects of the SRM or HRM approach development. R.A. supervised the project.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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ONLINE METHODS

Generation of the peptide library. For 125 proteins, proteotypic peptides (PTPs) were selected according to the following criteria. Peptides were chosen with a length of 6–20 amino acids. Only fully tryptic peptides, with no missed cleavages, that were unique for the targeted protein were considered. For proteins previously observed in shotgun proteomics experiments three proteotypic peptides most frequently observed by mass spectrometry were selected based on the information present in the S. cerevisiae build of the proteomics data repository PeptideAtlas.10 Peptide identifications in PeptideAtlas deriving from isotope-coded affinity tags (ICAT) experiments or experiments involving other labeling reagents were not considered. For proteins not observed in PeptideAtlas, a set of five peptides with good MS properties were derived by bioinformatic prediction, using the publicly available tool PeptideSieve11. Only peptides with a PeptideSieve score >0.3 were considered. For proteins with an entry in PeptideAtlas, but with less than three peptides available, up to two additional peptides, were selected with PeptideSieve. The final set of 480 peptides was synthesized on a microscale using the Spot-synthesis technology, lyophilized in a 96-well plate (~50 nmol of peptide material per well; JPT Peptide Technologies) and used in an unpurified form. Peptides were resuspended in 20% acetonitrile, 1% formic acid, vortexed for 20 min and sonicated for 15 min in the 96-well plate. Aliquots (1/500,000 of the starting material) of each peptide contained in a well were mixed (96-peptide mixes; Supplementary Table 1), evaporated on a vacuum centrifuge to dryness, resolubilized in 0.1% formic acid and immediately analyzed. Nine synthetic peptides (AAVYHHFISDGVR, HIQNDIQLHAKG, TEVVSVNHLYLDK, GQGHEFAHLLRL, TEHPFVTVEEFVLPK, TNIQGNNLLEESR, NQQNTWLTAFVLK, LVAYYTLIGASGQR, ITPNLAEFAFSLYR) with elution times immediately analyzed. Nine synthetic peptides (AAVYHHFISDGVR, HIQNDIQLHAKG, TEVVSVNHLYLDK, GQGHEFAHLLRL, TEHPFVTVEEFVLPK, TNIQGNNLLEESR, NQQNTWLTAFVLK, LVAYYTLIGASGQR, ITPNLAEFAFSLYR) with elution times spanning the whole solvent gradient were spiked into each mixtures; Supplementary Table 1), evaporated on a vacuum centrifuge to dryness, resolubilized in 0.1% formic acid and immediately analyzed. Nine synthetic peptides (AAVYHHFISDGVR, HIQNDIQLHAKG, TEVVSVNHLYLDK, GQGHEFAHLLRL, TEHPFVTVEEFVLPK, TNIQGNNLLEESR, NQQNTWLTAFVLK, LVAYYTLIGASGQR, ITPNLAEFAFSLYR) with elution times spanning the whole solvent gradient were spiked into each mixture to facilitate the correlation of relative retention times between LC-MS/MS runs. Sample mixes containing 140 peptides were also prepared (Supplementary Table 1). For the 156 yeast protein kinases and phosphatases (Supplementary Table 4), 816 peptides were selected and synthesized based on the criteria listed above, and sample mixes containing 96 peptides were prepared as previously described.

Development of SRM assays. For each peptide one precursor-to-fragment ion transition was calculated for each of the two main charge states (doubly and triply charged), corresponding to the first fragment ion of the y-ion series with m/z greater than m/z precursor + 20 thomson (Th). Microsoft Access and Excel macros and in-house–written Perl scripts were used to automate the process. The precursor-to-fragment ion transitions were used to detect, by SRM, the peptides of interest in the peptide mixtures and to trigger acquisition of the corresponding full fragment ion spectra. In detail, peptide samples were analyzed on a hybrid triple quadrupole–ion trap mass spectrometer (4000QTrap; ABI/MDS-Sciei) equipped with a nanoelectrospray ion source. Chromatographic separations of peptides were performed on a Tempo nano LC system (Applied Biosystems) coupled to a 15 cm fused silica emitter, 75 μm diameter, packed with a Magic C18 AQ 5 μm resin (Michrom BioResources). Peptides were loaded on the column from a cooled (4 °C) Tempo autosampler and separated with a linear gradient of acetonitrile and water, containing 0.1% formic acid, at a flow rate of 300 nl min\(^{-1}\). A gradient from 5 to 30% acetonitrile in 30 or 45 min was used. The mass spectrometer was operated in multiple reaction monitoring mode, triggering acquisition of a full MS/MS spectrum upon detection of an SRM trace (threshold 300 ion counts). SRM acquisition was performed with Q1 and Q3 operated at unit resolution (0.7 m/z half maximum peak width) with 200 or 300 transitions (dwell time 10 or 7 ms per transition, respectively) per run. MS/MS spectra were acquired in enhanced product ion (EPI) mode for the highest SRM transitions, using dynamic fill time, Q1 resolution low, scan speed 4,000 Da s\(^{-1}\), m/z range 300–1,400, 2 scans summed. Collision energies (CEs) used for both SRM and MS/MS analyses were calculated according to the formulas: CE = 0.044 × m/z + 5.5 and CE = 0.051 × m/z + 0.55 (m/z, mass-to-charge ratio of the precursor ion) for doubly and triply charged precursor ions, respectively. The declustering potential (DP) was calculated according to the formula DP = 0.129 × m/z + 1.

Fragment ion spectra collected in the QQQ MS were used to validate peptide identities and to extract optimal fragment ions for SRM analysis. MS/MS data were searched with Mascot (MatrixScience) against a subset of the yeast SGD database (version dated 01/26/2007). The database consisted of all proteins that could give rise to any of the synthetic peptides in the test set. A decoy database was generated from this subset by reverting amino acid sequences between tryptic cleavage sites and appended to the target database. Precursor mass tolerance was set to 2.0 Da and fragment mass tolerance to 0.8 Da. Data were searched allowing only fully tryptic termini and no missed cleavages. The search results were validated and assigned probabilities using a cutoff for the Mascot ion score for which the cut-off was defined by the proportion of assignments to decoy peptides according to ref. 16.

Spectral library creation. A spectral library was created from the spectrum–peptide matches. The spectrum with the highest ion score was selected in cases in which several spectra matched to a peptide. If more than one charge state was detected the highest scoring spectrum for both charge states were selected. These fragment ion spectra were used as a reference to derive the optimal coordinates of each SRM assay (for example, best responding fragments, fragment relative intensities and peptide elution time). For each spectrum, the most intense five peaks were selected as optimal SRM transitions. Fragments resulting from neutral loss from precursor were excluded. Fragments with m/z values close to the precursor ion m/z ([m/z\(Q1\) – m/z\(Q3\)] ≤ 5 Th) were discarded, as such transitions result in high noise levels. Collision energies associated to each transition were derived from the formulas given above. Additional features, such as fragment relative intensities and peptide elution times were extracted for each peptide from the corresponding MS/MS data.

Comparison of transition intensities with MS/MS spectra. Relative fragment ion intensities in QQQ MS/MS spectra were compared to the corresponding relative intensities of transitions measured in SRM mode. Transitions were selected based on the criteria described above. Intensities of transitions were extracted manually using the software Analyst, version 1.5 (ABI/MDS-Sciei). As a measure of similarity a Pearson product–moment correlation coefficient was calculated for all MS/MS spectra–SRM comparisons (Supplementary Fig. 7).
Detecting protein kinases and phosphatases. The final SRM assays for all yeast protein kinases and phosphatases were used to detect the corresponding proteins in total yeast cell lysates. Briefly, *S. cerevisiae* cells, strain S288C, BY4741 (ATCC strain 201388, MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) were grown in yeast extract peptone dextrose (YEPD) liquid medium to an OD$_{660}$ of ~1 at 30 °C. Pelleted cells were resuspended in an ice-cold lysis buffer including 50 mM Hepes (pH 7.5), 5% glycerol, 15 mM dithiothreitol, 100 mM KCl, 5 mM EDTA and a complete protease inhibitors cocktail (Roche), and disrupted by vortexing in the presence of acid-washed glass beads. Yeast lysates were centrifuged to remove cellular debris, the supernatants were transferred to a fresh tube and the protein concentration in the extracts was determined by the RC DC protein assay (Bio-Rad). Proteins were precipitated by adding six volumes of cold (−20 °C) acetone and resolubilized in a digestion buffer containing 8 M urea and 0.1 M NH$_4$HCO$_3$. Proteins were reduced with 12 mM dithiothreitol for 30 min at 35 °C and alkylated with 40 mM iodoacetamide for 45 min at 25 °C, in the dark. Samples were diluted with 0.1 M NH$_4$HCO$_3$ to a final concentration of 1.5 M urea and sequencing grade porcine trypsin (Promega) was added to a final enzyme:substrate ratio of 1:100. Peptide mixtures were cleaned by Sep-Pak TC18 cartridges (Waters) and eluted with 60% acetonitrile. The resulting peptide samples were evaporated on a vacuum centrifuge to dryness, resolubilized in 0.1% formic acid and immediately analyzed. Peptide mixtures (corresponding to 3.5 µg protein digest) were analyzed on a 4000QTrap mass spectrometer, under the same chromatographic conditions described above. Analysis was carried out in scheduled SRM mode (retention time window: 300 s; target scan time: 3.5 s). Blank runs (water, 0.1% formic acid, injected) were performed before SRM measurements of biological samples to avoid and assess sample carryover. In these control analyses, the same SRM method was used as in the subsequent (sample) run (for example, a method in which the same set of transitions was measured). Blank runs were performed until no signal was detected for all transition traces, before analyzing the biological sample. Detection of a target peptide in the yeast digest was based on the following criteria: (i) appearance of a set of five ‘co-eluting’ transition traces associated with the target peptide (or 10 when both charge states, doubly and triply charged, were used), within the 5-min elution time window, (ii) matching of the elution time of the endogenous peptide to that of the corresponding synthetic analog; (iii) at least three co-eluting transitions traces for the peptide exceeding a signal-to-noise ratio of 3; (iv) matching of the relative intensities of the highest four SRM transitions peaks detected in SRM measurements with the relative intensities of the corresponding fragments in the MS/MS spectrum of the synthetic analog (within the limits of an instrumental error). In four borderline cases (for example, transitions with signal-to-noise ratio around 2.9, but all transitions correctly ‘co-eluting’, or only two out of three SRM peaks above signal-to-noise ratio of 3), we confirmed identification by spiking the corresponding unpurified synthetic standards (peptides FVSPVDTNENLSPK, LyEVIVTESK, AGGVLHDDENLWSFKG, and GQNNIPLTPLATNTHQR) into a tryptic digest from $^{15}$N–fully labeled yeast cells, grown under the conditions previously described. Similarity of the fragmentation pattern and co-elution with the (light) standard allowed us to confirm identification for the five endogenous target peptides in the (heavy) yeast digest. The complete set of SRM assays and the associated dataset containing QQQ MS/MS spectra that allowed their validation were uploaded to the database MRMAtlas and made publicly available. The SRM assays developed in this study are listed in Supplementary Table 5. The proteotypic peptides with the best signal response for each of the detected kinases is shown in Supplementary Table 7.