CHAPTER 7

SIGNAL TRANSDUCTION REACTION MONITORING DECIPHERS SITE-SPECIFIC PI3K-mTOR/MAPK PATHWAY DYNAMICS

Manuscript submitted
Chapter 7

SIGNAL TRANSDUCTION REACTION MONITORING DECIPHERS SITE-SPECIFIC PI3K-mTOR/MAPK PATHWAY DYNAMICS

Erik L. de Graaf1,2,*, Joanna Kaplon3,*, Shabaz Mohammed1,2,4, Lisette A.M. Vereijken1,2, Daniel P. Duarte3, Laura Redondo Gallego1,2, Albert J.R. Heck1,2, Daniel S. Peeper3, A.F. Maarten Altelaar1,2

1Biomolecular Mass Spectrometry and Proteomics, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands. 2Netherlands Proteomics Centre, Padualaan 8, 3584 CH Utrecht, The Netherlands. 3Division of Molecular Oncology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands. 4Present address: Chemistry Research Laboratory, Department of Chemistry and Department of Biochemistry, University of Oxford, South Parks Road, OX1 3QU, Oxford, United Kingdom. * These authors contributed equally to this work.

SUMMARY
Combining targeted quantitative mass spectrometry (MS) with highly selective phosphopeptide enrichment, we have monitored phosphorylation dynamics in the PI3K-mTOR and MAPK signaling networks, in the context of oncogene-induced senescence (OIS). Our analysis reveals site-specific phosphorylation of ERK 1/2, p70S6K and RPS6, which were previously undetectable by phospho-antibodies. This study illustrates the applicability of our MS-based approach for high-resolution phosphodynamics screening and provides novel insights in signaling network perturbations associated with OIS.

Alterations in cellular signaling networks are the cause of many diseases and determine highly variable and microenvironment-dependent drug target potency1. Therefore, understanding perturbations in cellular signaling networks is of great interest from a clinical point of view. Signal transduction is still mostly studied by immunoblot analysis using phosphosite-directed antibodies. However, the limited availability of these reagents and the low throughput of immunoblot analysis severely hinder the analysis of complete signaling pathways. Other limiting factors of antibody-based protein phosphorylation assays are the semi-quantitative and often ambiguous nature of the results obtained. Phosphosites resident in highly conserved sequences are frequently indistinguishable between closely related protein isoforms, with ERK1 Y204 and ERK2 Y187 serving as prime examples. Moreover, close-proximity sites such as T202/Y204 on ERK1, T185/Y187 on ERK2 or S235/S236 and S240/S244 on RPS6, are indistinguishable by antibody binding. In an attempt to address these problems, dual site-specific antibodies have been developed that recognize multiple isoforms and multiply phosphorylated domains. However, this approach does not allow for monitoring of possible mutually exclusive phosphorylation patterns and other
cross-talk that may occur\(^2\). The current lack of antibodies that are specific for close-proximity or protein isoform phosphosites, has left the dynamics of these potentially important phosphosite differences largely unexplored, demanding the development of methods with increased resolving power.

Global shotgun mass spectrometry (MS)-based phosphoproteomics can address many of these issues, allowing for the analysis of thousands of phosphorylation events with high specificity for protein isoform and phosphosite localization\(^3\). Currently, the main challenge of low phosphoprotein stoichiometry can be partially solved by enriching the phosphorylated peptides from the more abundant unphosphorylated peptides, using affinity-based techniques such as TiO\(_2\) or Fe\(^{3+}\)/Ti\(^{4+}\)-IMAC\(^5,6\). Nonetheless, the large dynamic range in the remaining phosphopeptide population greatly hampers global shotgun proteomics approaches.

Detection reproducibility and sensitivity can be optimized using targeted MS, referred to as selected or multiple reaction monitoring (SRM/MRM). Moreover, when combined with stable isotope standard (SIS) phosphopeptides, technical variation can be reduced, resulting in increased accuracy in quantification\(^7\). Although SRM has been established for monitoring protein abundance\(^8,9\), its applicability to monitor selected protein phosphorylation events is still in its infancy. Up till now, only low-throughput targeted phosphosite experiments have been described using SRM, analyzing a small number of phosphosites from high abundant proteins\(^7,10\) or protein complexes\(^11\). However, no monitoring strategy has been reported using SRM phosphorylation dynamics of all components, at differing expression levels and in entire signal transduction pathways.

Here, we have substantially improved phospho-SRM methods to accurately, reproducibly and comprehensively quantify phosphorylation events in the MAPK and PI3K-mTOR pathways. The success of this approach is determined by the combination of the sensitive and quantitatively reproducible Ti\(^{4+}\)-IMAC enrichment strategy\(^6,12\) with SRM, using low-cost crude synthetic SIS phosphopeptides at two different concentrations (Figure 1). To measure the essential phosphosites selected, robust interference-free SRM assays were constructed using reproducible retention times, co-eluting SIS reference peptides for relative fragment ion intensity comparison together with SRM-triggered MS/MS for identification and phosphosite localization (Suppl. Figure 1-4. and Suppl. Data).

Our method, termed signal transduction reaction monitoring (STREM), was applied to characterize the dynamics of the MAPK and PI3K-mTOR signal transduction pathways in oncogene-induced senescence (OIS). OIS is a largely irreversible state of cell cycle arrest, which can be triggered by the unscheduled activation of oncogenes\(^13\). It has been shown to act alongside death programs to suppress tumorigenesis\(^14,15\). For this purpose, “control” cycling cells (Cycl), OIS cells (upon introduction of BRAF\(^V600E\), a common oncogene and strong inducer of OIS) and OIS-bypassing cells (OISb)\(^16,17\), were analyzed in biological triplicates.
Additionally, cycling, OIS and OISb cells treated with BEZ235 (a dual PI3K/mTOR inhibitor), were included to screen for mTOR-sensitive perturbations. In total, 51 phospho-patterns from 27 different signaling proteins could be reproducibly quantified in 18 samples.

Figure 1. Experimental workflow scheme of signal transduction reaction monitoring (STREM)
Biological triplicates of cycling (Cycl), undergoing oncogene-induced senescence (OIS) and OIS bypassing (OISb) cells were collected for mass-spectrometry (MS) analysis. After lysis and digestion, two concentrations (high and low) of stable isotope standard (SIS) phosphopeptides per each phosphosite to be analyzed were added, to achieve similar levels of endogenous and SIS peptides. Subsequently phosphopeptides were enriched using single stage Ti⁴⁺-IMAC, followed by a scheduled two hour LC-SRM run for each sample.

Several nodes in the MAPK pathway were expected to show changes in phosphorylation, as both OIS and OISb cells overexpress the constitutively active BRAF<sup>V600E</sup> mutant. Indeed, under each condition we observed a specific upregulation of the downstream ERK1/2 TEY dual phosphorylation motif, as seen in both immunoblot and STREM analyses. We also detected a strong BRAF<sup>S729</sup> phosphorylation in the STREM measurements (Figure 2a, b). Comparing the two ERK isoforms, similar trends in phosphorylation were observed in both immunoblot and STREM analyses, indicating a level of redundancy between ERK1/2. However, the STREM data showed a stronger induction of ERK1 dual phosphorylation. Moreover, STREM allowed for the analysis of the single tyrosine sites (Y204/Y187) of which phosphorylation is known to precede that of the threonine residues (T202/T185). Surprisingly, single tyrosine site phosphorylation on both Y204 and Y187 was downregulated in OIS cells compared to cycling cells for both ERK1 and 2, which contrasts with the increase observed for the dual phosphorylation in OIS (Suppl. Figure 3). These results highlight the superior resolving power of our method, revealing differences in TEY-motif phosphorylation on different ERK1/2 isoforms that would remain undetected with classical approaches.

Another major proliferation-controlling pathway is the one in which PI3K and mTOR are the main actors. Activation of PI3K-mTOR triggers protein synthesis via the phosphorylation of 4E-binding protein 1 (4EBP1) and the ribosomal protein S6 kinase (p70S6K). Both immunoblot and STREM assays confirmed a strong mTOR-dependent regulation of 4EBP1 S65, as evidenced by the total loss of S65 phosphorylation upon treatment with the dual PI3K/mTOR inhibitor BEZ235 (Figure 2a,b). Interestingly, a strong reduction of
growth-stimulating 4EBP1 phosphorylation was observed in OIS and OISb, albeit less pronounced in the latter. A similar trend in signaling perturbation was observed for p70S6K phosphorylation on T412 (often referred to as T389) and S452, however not for S441 and S447 (Figure 2, 3a). Phosphorylation of p70S6K T412 is often used as a read-out for mTOR activity in immunoassays. For p70S6K S452, no site-specific antibody is available and no responsible kinase or biological role has been reported. By STREM analysis, we show an identical regulation of S452 and T412, including a total loss of phosphorylation upon BEZ235 treatment, indicating S452 as a novel putative mTOR phosphorylation site.

Figure 2. Phosphosite dynamics of several nodes in the MAPK and PI3K-mTOR signaling
Using STREM (a) protein isoform phosphorylation domains as well as close-proximity phosphosites could be dissected, untraceable by immunoblot analysis (b).

Another well-studied downstream target of both the PI3K-mTOR and MAPK pathways is the ribosomal protein S6 (RPS6). RPS6 functions to integrate proliferation promoting signals from both pathways; its phosphorylation is increased in many types of cancer and is proposed to be a marker for drug resistance. RPS6 is primarily known as a downstream mTOR target and is phosphorylated on S235, S236, S240 and S244 by p70S6K. However, S235 and S236 can be phosphorylated also by RSKs (RSK1/2) from the MAPK pathway. In all studies on RPS6 signaling antibodies were used detecting only dual phosphorylation on S235/S236 or S240/S244. As this highly conserved and confined domain is functionally important and phosphorylated by two different kinases from two pathways, phosphorylation patterns are most likely more complex than merely two separate dual phosphorylations. The immunoblot data on RPS6 S235/S236 and S240/S244 phosphorylation showed no substantial differences in the level of phosphorylation between cycling, OIS and OISb cells.
Treatment with BEZ235 revealed a strong dependence on mTOR activity in cycling cells (Figure 2b). Interestingly, the immunoblot data already reveal an altered response of these sites to BEZ235 treatment, showing only partial loss of phosphorylation in OIS and to a lesser extent in OISb. Exploiting the specificity of the STREM approach, we were able to dissect different patterns for S235, S236 and S240 phosphorylation, associated with different biological conditions (Figure 2a). For example, a strong mTOR-independent induction of S236 phosphorylation was observed in OIS. When interpreting the results obtained by the S235/S236 antibody one might falsely conclude that the phosphorylation of both sites is mainly dependent on mTOR. However, STREM revealed that S236 is strictly phosphorylated by RSK1/2, specifically in OIS. STREM analysis on single S240 phosphorylation showed comparable results to dual S240/S244 phosphoantibody analysis. Similar to immunoblot analysis, STREM showed no difference in the level of S240 phosphorylation between control, OIS and OISb cells. In addition, both analyses revealed only partial loss of phosphorylation in BEZ235-treated OIS cells when compared to BEZ235-treated control or OISb cells. Interestingly, the phosphorylation of the dual (S236/S240) and the triply phosphorylated domain (S235/S236/S240) were strongly (albeit not exclusively) dependent on mTOR. In samples without BEZ235 treatment, both dual and triple phosphorylation showed a significant downregulation in OIS compared to cycling and OISb, a pattern that was reversed when mTOR was inhibited (Suppl. Figure 3). The complex differential phosphorylation patterns on RPS6, which was found by STREM analysis only, indicate an important role of multiple signaling pathways in regulation of a confined domain on a single protein. Taken together, STREM analysis revealed a strong and specific regulation of different mTOR substrates in cells that undergo OIS, which is associated with, not reported before, remarkably high phosphosite pattern differentiation.

Besides obtaining specific phosphosite pattern quantifications, STREM also enables comprehensive monitoring of pathway dynamics in a single run per sample, illustrated here for the full PI3K-mTOR and MAPK pathways (Figure 3a). It has been reported previously that phosphorylation of PRAS40 at S183 and T246 by mTOR and AKT, respectively, represses its inhibitory function on mTORC1 signaling. Interestingly, our pathway analysis revealed that both S183 and T246 phosphorylation sites of PRAS40 are reduced in OIS, hinting at an important role of PRAS40 in the reduced mTORC1 activity associated with OIS. In line with this, PDK1, an upstream AKT-PRAS40 kinase, showed lower S241 phosphorylation in OIS that has been previously associated with decreased PDK1 activity. Next, we systematically searched for pathway components specifically regulated by the PI3K-mTOR pathway (Figure 3b). Analysis of BEZ235-treated cells confirmed that phosphorylation of specific sites on p70S6K and 4EBP1 is mTOR-dependent whereas all measured sites on RSK1/2 are not. Other potential direct or indirect mTOR substrates showing regulation upon BEZ235 treatment include TSC2 S1388 and S1411, MEK2 T394 and eIF4B S445.
In conclusion, we report here a new strategy to comprehensively monitor pathway signal transduction dynamics in a site-specific manner. Precise and phosphosite-specific quantification with increased throughput is demonstrated, allowing the characterization of complex protein phosphorylation patterns associated with a particular biological phenotype. The need for such a comprehensive full pathway analysis method is illustrated by highlighting previously unknown and undetectable changes in protein phosphorylation in OIS before and after pharmacological inhibition of mTOR.

Figure 3. Regulations in the PI3K-mTOR and MAPK pathway measured by STREM
a. Phosphosites specifically regulated in OIS. b. Phosphosites sensitive to PI3K-mTOR inhibition by BEZ235 treatment. Significantly up- or downregulated phosphosites are color-coded in green and red circles, respectively. Protein interaction and kinase substrate relationships were extracted from the UniprotKB and phosphositeplus databases and manually curated using literature.

ACKNOWLEDGEMENTS
This work was supported by the Netherlands Proteomics Center, and the PRIME-XS project (grant agreement number 262067) funded by the European Union 7th Framework Programme. A.F.M.A. was supported by the Netherlands Organization for Scientific Research.
(NWO) with a VIDI grant (723.012.102) and D.S.P. with a NWO VICI grant and a Queen Wilhelmina Award grant from the Dutch Cancer Society (KWF Kankerbestrijding). This work is part of the project Proteins At Work, financed by the Netherlands Organization for Scientific Research (NWO) as part of the National Roadmap Large-scale Research Facilities of the Netherlands (project number 184.032.201).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

ONLINE METHODS

Cell culture

The human diploid fibroblast (HDF) cell line Tig3 expressing the ecotropic receptor, hTERT and sh-p16INK4A (Tig3(et)-16i) was maintained in DMEM with 4.5 mg/ml glucose and 0.11 mg/ml sodium pyruvate, supplemented with 9% fetal bovine serum (PAA), 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (GIBCO). The Phoenix packaging cell line was used for the generation of ecotropic retroviruses. For infections, filtered (pore size 0.45 mm) viral supernatant, supplemented with 4–8 μg/ml polybrene was used. In general, a single infection round of 6 h was sufficient to infect at least 90% of the population. For senescence experiments, cells were infected with shCEBP/β-encoding or control retrovirus, selected pharmacologically (puromycin) and subsequently infected with BRAFV600E-encoding or control virus. After 9 days of selection (blastomycin), cells were collected for mass-spectrometry (MS) and immunoblot assays. For screening for mTOR-sensitive perturbations, cells were pre-treated with 500 nM mTOR inhibitor BEZ235 for 24 h. Results represent data from three independent experiments.

Plasmids

The plasmids pMSCV-blast and pMSCV-blast-BRAFV600E as well as pRS-puro and pRS-puro-C/EBPβ#1 were previously described14,16

Antibodies

Antibodies used for immunoblotting were: phospho-p44/42 MAPK (ERK1/2) (T202,Y204/T185,Y187; E10, #9106; Cell Signaling); total p44/42 MAPK (ERK1/2) (#9102; Cell Signaling); phospho-p70S6 (T389/T412; #9208; Cell Signaling); total p70S6 (#9202; Cell Signaling);
phospho-4EBP1 (S65; #9456; Cell Signaling); total 4EBP1 (#9453; Cell Signaling); phospho-
RPS6 (S235/S236; #2211; Cell Signaling); phospho-RPS6 (S240/S244; #2215; Cell Signaling);
total RPS6 (#2217; Cell Signaling).

Sample preparation
Frozen HDF cell pellets were lysed by sonication in lysis buffer (8M Urea in 50 mM ammonium
bicarbonate, supplemented with 1 tablet Complete mini EDTA-free Cocktail (Roche) and 1
tablet PhosSTOP phosphatase inhibitor Cocktail (Roche) per 50 mL of lysis buffer). After
centrifugation (20,000x g 30min at 4°C), the supernatant was collected and assayed for protein
content using the bicinchoninic acid assay (BCA) kit following manufacturer instructions
(Pierce, IL USA). Protein reduction and alkylation were performed using final concentrations
of 5 mM dithiothreitol and 10 mM iodoacetamide, respectively. The first enzymatic digestion
step was performed with Lys-C at 37 °C for 4 h in lysis buffer (enzyme:substrate 1:75). For the
second digestion, samples were diluted to 2 M Urea using 50 mM ammonium bicarbonate
and incubated overnight with trypsin (Promega, USA) at 37 °C (enzyme:substrate 1:100). The
resulting endogenous peptides were acidified with 10% formic acid (FA) and split into two
aliquots of 500 µg peptides for each sample. Next, aliquots were spiked with either 10 pmol
or 0.1 pmol of heavy stable isotope standard (SIS) peptides. Crude synthetic SIS peptides
containing one C-terminal heavy Lysine (6C13,2N15: +8Da) or heavy Arginine (6C13, 4N15:
+10Da) amino acid, were purchased from JPT technologies (Berlin, Germany). The SIS stock
was created by mixing equimolar quantities of all peptides in 50% acetonitrile (ACN), 1% FA
and divided into 100 pmol aliquots stored at -20°C. Prior to phosphopeptide enrichment,
peptide mixtures were desalted on Sep-Pak C18 columns (Waters, USA, Massachusetts),
dried to completion in vacuum and stored at -80°C. Conventionally, highly purified synthetic
peptides are used for high precision absolute quantification. In our novel approach we used
less pure crude synthetic phosphopeptides for each site and in two different concentrations
per sample, to allow for high-throughput, sensitive and reproducible relative quantification.

Phosphopeptide enrichment
Two Ti4+-IMAC columns for each sample were prepared and processed in parallel using a
micro centrifuge as described previously6. Briefly, microcolumns were created by loading
500 µg Ti4+-IMAC beads onto GELoader tips (Eppendorf) with a C8 plug. Ti4+-IMAC columns
were pre-equilibrated two times with 30 µL of loading buffer (80% ACN, 6% trifluoroacetic
acid (TFA)). Next, all samples were reconstituted in 250 µL loading buffer and 100 µL was
loaded onto two equilibrated Ti4+-IMAC columns (corresponding to 200µg natural peptides
(NAT) and 4 or 0.04 pmol SIS per column).
After loading of samples, Ti4+-IMAC columns were washed with 60 µL washing buffer A
(50% ACN, 0.5% TFA, 200 mM NaCl) and subsequently with 40 µL washing buffer B (50%
ACN, 0.1% TFA). Bound peptides were eluted by 30 µL of 10% ammonia into 30 µL of 10%
FA. Finally, the remainder of the peptides was eluted with 5 µL of (80% ACN, 2% FA). The
collected eluate was further acidified by adding 20 μL of 10% TFA and subsequently desalted using homemade reversed phase tips loaded with 10 μL of Aqua C18 5μm beads. Tips were washed twice with 30 μL 0.1% TFA, followed by elution of peptides with 20 μL 80% ACN, 1%FA. Prior to liquid chromatography (LC)-MS analysis, peptide mixtures were dried to completion in vacuum and stored at -80°C. For each sample only one Ti⁴⁺-IMAC column was analysed. The second loaded column served as a backup and was only used when primary column failed.

**Signal transduction reaction monitoring (STREM) assay development**

To confirm SIS peptide sequence and phosphosite localization and consistent LC-MS observability, all ordered crude SIS peptides were run separately on different LC-MS set-ups. Crude peptides were characterized by data-dependent acquisition (DDA) LC-MS runs on an Orbitrap Q-Executive (Thermo Scientific) because we have demonstrated previously that this type of MS/MS spectra are more beneficial for selected reaction monitoring (SRM) assay development than conventional ion trap CID spectra. Peptides that failed to be identified or for which phosphosites were not localized by DDA LC-MS runs, were re-analyzed by targeted ETD fragmentation on an Orbitrap Elite (Thermo Scientific) for better phosphopeptide identification and localization, or by SRM triggered MS/MS on a TSQ Vantage (Thermo Scientific) for increased sensitivity. Subsequently, all beam-type MS/MS spectra (i.e. HCD and TSQ spectra) were used for SRM assay development. Assay development was performed using Skyline to pick the 8 most abundant fragment ions for SRM-mode validation and collision energy optimization. Collision energy optimization was achieved by ramping the collision energy (CE) from 7.5 eV lower to 7.5 eV higher than the calculated CE (standard skyline equation) in steps of 1.5 eV. For the final assay the 5 most abundant peptide fragment ions were manually picked that showed no (auto-)interference in standard (SIS) or endogenous (NAT) peptides measured in HeLa or HDF cell lysates enriched by Ti⁴⁺-IMAC. Peptide ID was confirmed by matching retention time (RT), relative fragment ion intensities and SRM triggered MS/MS runs that were scheduled in-between sample batches. Exemplary phosphopeptide RT reproducibility and XICs are shown in Supplementary Figure 1. Out of 49 phosphosites (on 51 phosphopeptides), 48 could be pinpointed with a localization certainty higher than 95% resulting in a total average localization probability of 98.6%. In some cases tryptic phosphopeptides inevitably contained miscleavages (MC) or methionine residues. MC in phosphopeptides are frequently observed when negatively charged phosphoieties are in close proximity to positively charged arginine or lysine residues thereby hampering cleavage by trypsin. Our data and other studies show that quantification standard deviations are similar between MC peptides and non MC peptides, indicating MC peptides are reproducibly formed under our sample preparation conditions and therefore can be used for reproducible phosphosite quantification. In the case of methionine containing peptides, SRM assays for both the normal and oxidized forms were generated and measured.
in the final sample. However, when one of the two oxidation states was not present in a natural or SIS peptide, the peptide isoform was not taken into account for further analysis. In only two cases both oxidation states were present on both the SIS and NAT peptide. SRM analysis of these peptides showed identical regulation of both isomers with small deviations between biological replicate samples (Suppl. Fig. 2), indicating oxidation was similar on both SIS and NAT peptides in all samples.

STREM LC-MS data acquisition
All samples were reconstituted in 10 μL 10% FA and 30% was analysed on a TSQ Vantage coupled to an Easy nLC-1000 LC system configured with a single easy spray analytical column (ES803; 50 cm x 75 μm ID, 2 μm particles, 100 Å pore size) (Thermo Scientific) using 2 hour runs. Briefly, samples were loaded with 6 μL buffer A (0.1% FA) at 800 bar limited to a maximum of 300 nl/min, column equilibration was performed with 3 μL buffer A at 800 bar, peptides were separated on a gradient of 0-65 min 3-25% buffer B (0.1% FA, 99,9% ACN) and 65-75 min 25-40% buffer B at 200 nl/min, followed by a column wash for 10 min at 200 nl/min 100% buffer B. The TSQ Vantage spray voltage was set to 2.3 kV and was further configured to select peptides in Q1 at 0.7 FWHM and fragment them at 1.5 mTorr Argon in the second quadrupole. In total 89 phosphopeptides were measured in light and heavy resulting in 884 transitions. All transitions were measured using polytyrosine tuned S-lens values, transition optimized CE values, scheduled peptide RT window of 1.75 - 3 minutes and a cycle time of 1.8 seconds, resulting in a maximum of ~60 concurrent transitions.

DDA LC-MS and STREM Data analysis
Peptide MS/MS spectra were identified using Proteome Discoverer 1.4 (Thermo Scientific, Bremen) and Mascot 2.4 (Matrix Science, London) and phosphosite localization was performed using the phosphoRS PD node and SRM data were analyzed using Skyline. For the final SRM data set, transitions with remaining interference, signal intensities above 13E6 or low signal-to-noise ratios were discarded for further analysis. To find samples or peptides with interfering irreproducible transitions, a t-test was performed comparing the average relative fragment intensity ratio between light and heavy peptides from biological triplicate runs. All transitions with a p-value below 0.1 indicated inequality between NAT and SIS relative fragment intensities and were therefore discarded. Additionally, all samples were inspected manually for transition interference and consistency in transition peak shape, peak integration and RT, using Skyline. In the end, only peptides with a minimum of 3 transitions for each SIS and NAT were kept for further analysis resulting in an average of 4,2 transitions per peptide isotopologue.

To achieve relative quantification, crude synthetic peptides were used for determining relative ratios. To prevent quantification problems regarding upper and lower limits of detection and signal response linearity, preferably samples with equal intensity for SIS and NAT versions are used (SIS/NAT ratio close to 1). Therefore, as described above in sample preparation section,
each sample was split in two aliquots and each was spiked with a different SIS concentration allowing for each peptide analyte to select one of the two (or both) spike-in concentrations, ideally obtaining a SIS-NAT fold-difference less than 10. However, due to strong regulations in NAT levels between different phenotypes the ratio NAT/SIS could inherently not always be kept less than 10-fold. To obtain phosphosite abundances relative to control, each SIS-normalized phosphopeptide abundance was divided by the average intensity of the control biological triplicate. Finally, two sided student t-tests with equal variances were used to calculate significant (p<0.05) changes between biological triplicate samples.

**Supplementary Data.** All annotated spectra and STREM quantification results are available for download at the PASSEL data repository (acc. nr.: PASS00552).
Supplementary Figure 1. Typical RT reproducibility and extracted ion chromatograms of two representative phosphopeptides

a. RT reproducibility over all measured samples. LNQP[+80]TPR (MEK2 T394) (top) and IDS[+80]TEVIYQPR (LKB1 S31) (bottom) measured in high and low SIS spiked samples, respectively.

b. Phosphopeptide chromatograms of endogenous (Red) and SIS (Blue) LNQP[+80]TPR from all 18 samples.

c. Overlaid phosphopeptide chromatograms for all 18 samples of the SIS peptide IDS[+80]TEVIYQPR.
Supplementary Figure 2. Peptide methionine oxidation

Two peptides (a and b) observed to be oxidized show identical regulation for their unoxidized and oxidized state (top and bottom panel). No significant differences were observed between both states in any sample (P-value below 0.05), indicating similar oxidative conditions for both SIS and endogenous peptide versions in all samples.
Supplementary Figure 3. All STREM acquired phosphosite measurements in the PI3K-mTOR and MAPK pathway.
Supplementary Figure 4. Manually annotated spectra of the doubly and triply phosphorylated RPS6 peptides

Spectra of RLS+[80]SLRAS+[80]TSK⁺⁺⁺ (a), RLS+[80]S+[80]LRAS+[80]TSK⁺⁺⁺ (b) and RLS+[80]S+[80]LRAS+[80]TSK⁺⁺⁺ (c) were recorded in SRM triggered MS/MS runs and the most abundant fragment ions were annotated using the Protein Prospector software package (Baker, P.R. and Clauser, K.R. http://prospector.ucsf.edu). Fragment ions that were used in the final interference-free SRM assay are indicated in bold red.