

# Unambiguous Phosphosite Localization using Electron-Transfer/Higher-Energy Collision Dissociation (ETHcD)

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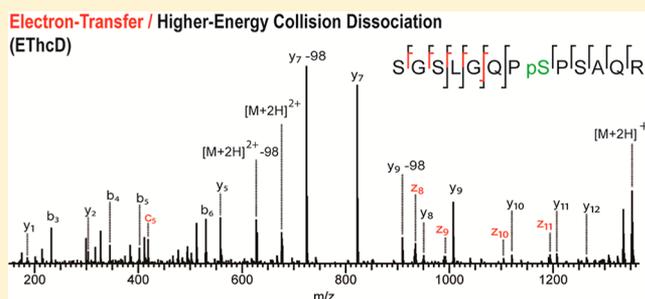
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## **S** Supporting Information

**ABSTRACT:** We recently introduced a novel scheme combining electron-transfer and higher-energy collision dissociation (termed ETHcD), for improved peptide ion fragmentation and identification. We reasoned that phosphosite localization, one of the major hurdles in high-throughput phosphoproteomics, could also highly benefit from the generation of such ETHcD spectra. Here, we systematically assessed the impact on phosphosite localization utilizing ETHcD in comparison to methods employing either ETD or HCD, respectively, using a defined synthetic phosphopeptide mixture and also using a larger data set of Ti<sup>4+</sup>-IMAC enriched phosphopeptides from a tryptic human cell line digest. In combination with a modified version of phosphoRS, we observed that in the majority of cases ETHcD generated richer and more confidently identified spectra, resulting in superior phosphosite localization scores. Our data demonstrates the distinctive potential of ETHcD for PTM localization, also beyond protein phosphorylation.

**KEYWORDS:** electron transfer dissociation, ETD, HCD, ETHcD, phosphorylation site localization, phosphoRS



## **I** INTRODUCTION

Reversible phosphorylation of proteins is a key regulatory mechanism in living cells.<sup>1</sup> Protein phosphorylation can modulate protein activity, turnover, subcellular localization, complex formation, folding and degradation. Dynamic phosphorylation plays a pivotal role in almost all biological processes including cell division, differentiation, polarization and apoptosis.<sup>2</sup> Moreover, it is an important switch in cellular signal transduction.<sup>3</sup> The importance of this post-translational modification (PTM) for cell biology has driven the development of novel mass spectrometric tools for sensitive and global detection of phosphorylation.<sup>4,5</sup> However, the analysis of phosphorylated peptides by mass spectrometry is still not as straightforward as for “regular”, unmodified peptides. One of the major challenges in phosphoproteomics is to improve MS level representation since phosphopeptides are usually present at substoichiometric levels. Hence, an enrichment step is necessary to enable deeper penetration of the phosphoproteome. Enrichment is typically performed by chromatography,<sup>6</sup> antibodies<sup>7</sup> or metal-ion/metal oxide affinity-based<sup>8,9</sup> techniques. Two other main challenges are the identification of phosphopeptides and confident localization of the corresponding phosphosite.<sup>10</sup> The challenge is caused by the higher lability

of the phosphate group when compared to the amide bond. A number of strategies have been proposed to circumvent poor fragmentation and improve sequence and site diagnostic fragmentation, including the use of neutral loss-triggered MS/MS<sup>11</sup> and multistage activation (MSA)<sup>12</sup> in ion traps, the use of beam type CID fragmentation,<sup>13</sup> and electron capture/transfer dissociation<sup>14</sup> or a combination of some of these approaches.<sup>9,15</sup>

Once phosphopeptide identification is feasible through sufficient peptide backbone fragments, it can still be challenging to pinpoint the true phosphosite. This becomes more difficult as the number of potential phosphorylation sites within the peptide sequence increases. In principle, unambiguous phosphosite localization requires site-determining fragment ions.<sup>16</sup> Direct validation is feasible through detection of a fragment ion that carries the phosphate group. Neutral loss fragment ions can be used as well; however, since they exhibit the same mass as a water loss from an unmodified residue they do not directly confirm the correct site.<sup>17</sup> Diagnostic phosphosite-specific fragments facilitate pinpointing the correct

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phosphosite.<sup>18–20</sup> Several algorithms and programs have been developed to enable automatic phosphosite localization.<sup>3,16,21–26</sup> These software tools are based on distinct but similar approaches and they all aim to provide a metric that allows for assessment of the confidence in phosphosite localization. Recently, Taus et al. have reported on a new algorithm, coined phosphoRS,<sup>27</sup> which presently is uniquely compatible with CID, HCD and ETD fragmentation and was optimized for both low- and high-resolution MS/MS spectra. phosphoRS provides individual localization probabilities for all potential phosphosites in a given peptide.

Generally, all scoring tools depend on the quality of the MS/MS spectra. The more site-determining ions are detected, the higher the confidence in phosphosite localization. We have recently introduced a novel fragmentation scheme combining electron-transfer and higher-energy collision dissociation, termed EThcD.<sup>28</sup> This method employs dual fragmentation to generate both b/y and c/z ions which leads to very fragment ion- and thus data-rich MS/MS spectra. Compared to HCD and ETD, we found a substantial increase in peptide backbone fragmentation, which translated into a remarkable average peptide sequence coverage of ~94% for tryptic peptides. We reasoned that localization of post-translational modifications could also highly benefit from EThcD spectra. Here, we systematically assessed the impact on phosphosite localization using EThcD. In this work we evaluate the performance of EThcD in comparison to ETD and HCD using a defined synthetic phosphopeptide mixture and also on a larger data set of Ti<sup>4+</sup>-IMAC enriched phosphopeptides, all in combination with a modified version of phosphoRS.

## ■ EXPERIMENTAL SECTION

### Materials

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. Formic acid and ammonia were obtained from Merck (Darmstadt, Germany). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands).

### Sample Preparation

Protein from HeLa cells was harvested and digested with trypsin, as previously described.<sup>29</sup> Ti<sup>4+</sup>-IMAC beads were prepared as reported elsewhere.<sup>30,31</sup> Phosphopeptides were enriched as previously described.<sup>32</sup> Briefly, Gel-loader tips that were plugged with C8 material (3M, Zoeterwoude, The Netherlands) were filled up to 1 cm with Ti<sup>4+</sup>-IMAC beads. Columns were equilibrated with loading buffer (80% ACN, 6% TFA). Peptides were reconstituted in loading buffer, loaded onto the columns and washed with washing buffer 1 (50% ACN, 0.5% TFA, 200 mM NaCl) and subsequently washing buffer 2 (50% ACN, 0.1% TFA). Phosphopeptides were eluted with elution buffer 1 (10% NH<sub>3</sub> in H<sub>2</sub>O) followed by elution buffer 2 (80% ACN, 2% FA). Eluate was acidified and diluted with formic acid to a final acetonitrile concentration of <5%, split into three equal amounts and directly analyzed by single run LC-MS/MS utilizing ETD, HCD and EThcD, respectively.

### Mass Spectrometry

All data was acquired on an ETD enabled Thermo Scientific LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Thermo Scientific EASY-nLC 1000 (Thermo Fisher Scientific, Odense, Denmark) was

connected to the LTQ Orbitrap Velos mass spectrometer. ETD, HCD and EThcD methods were set up as previously described.<sup>28</sup> Briefly, all spectra were acquired in the Orbitrap at a resolution of 7500. For HCD the normalized collision energy was set to 40%. The ETD reaction time was set to 50 ms for ETD and EThcD. Supplemental activation was enabled for ETD. HCD normalized collision energy was set to 30% for EThcD (calculation based on precursor *m/z* and charge state). The anion AGC target was set to 4e5 for both ETD and EThcD.

### Data Analysis

Peak lists were generated using Thermo Scientific Proteome Discoverer 1.3 software (Thermo Fisher Scientific, Bremen, Germany). The nonfragment filter was used to simplify ETD spectra with the following settings: the precursor peak was removed within a 4 Da window, charged reduced precursors were removed within a 2 Da window, and neutral losses from charge reduced precursors were removed within a 2 Da window (the maximum neutral loss mass was set to 120 Da). MS/MS spectra were searched against a database containing the synthetic phosphopeptide sequences and the human Uniprot database (version v2010–12), respectively, including a list of common contaminants using SEQUEST or Mascot (Matrix Science, UK). The precursor mass tolerance was set to 10 ppm, the fragment ion mass tolerance was set to 0.02 Da. Enzyme specificity was set to Trypsin with 2 missed cleavages allowed. Data from the synthetic phosphopeptide mixture was searched with no enzyme specificity. Oxidation of methionine and phosphorylation (S,T,Y) were used as variable modification and carbamidomethylation of cysteines was set as fixed modification. Percolator<sup>33</sup> was used to filter the PSMs for <1% false-discovery-rate. Phosphorylation sites were localized by applying a custom version of phosphoRS<sup>27</sup> (v3.0 – EThcD enabled) that has been expanded to allow analysis of EThcD data.<sup>28</sup> Briefly, the algorithm considers both HCD- and ETD-type fragment ions at the same time. While singly and doubly charged b- and y-type fragment ions including neutral loss of phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) are considered for site localization, only singly charged c-, z-radical and z-prime ions are scored.

## ■ RESULTS AND DISCUSSION

Increasing the confidence in phosphosite localization is a key challenge in phosphoproteomics. Site-determining fragment ions are required to unambiguously pinpoint the correct phosphosite. Observing all possible peptide backbone cleavages in a single MS/MS spectrum substantially simplifies phosphosite localization. Recently, we showed that EThcD enables complete peptide sequencing through dual fragmentation.<sup>28</sup> In EThcD, the peptide precursor is initially subjected to an ion/ion reaction with fluoranthene anions in a linear ion trap, which generates c- and z-ions. However, the unreacted precursor and the charge-reduced precursor remain highly abundant after ETD. In the second step HCD all-ion fragmentation is applied to all ETD derived ions. This generates b- and y-ions from the unreacted precursor and simultaneously increases the yield of c- and z-ions by fragmentation of the charge reduced precursor. Since the remaining unreacted precursor population is higher charged than the ETD-derived fragment ions one can apply a level of energy that fragments the precursor but does not induce secondary fragmentation of c- and z-ions. Here, we continue to explore the benefits of this novel fragmentation mode for the analysis of phosphopeptides.

### Evaluation of Phosphosite Localization by EThcD using a Defined Phosphopeptide Mixture

To evaluate the potential added value of phosphopeptide analysis by EThcD we initially used a defined mixture of well-characterized synthetic phosphopeptides. This mixture consists of 30 phosphopeptides of varying length with up to four phosphorylated residues (see Supplementary Table 1 for a complete list, Supporting Information). We analyzed this mixture by LC–MS/MS employing ETD, HCD and EThcD fragmentation, respectively. We used identical instrument settings with the only exception being the parameters for peptide dissociation, which were set to the for each method optimized values. The data was searched with SEQUEST and the PSMs were manually validated and filtered (7 ppm peptide mass tolerance, search engine rank 1, absolute Xcorr threshold 0.4). Additionally, we considered only PSMs for which the injection time did not max out (<500 ms), that is, the target number of ions was reached. Note that this precaution was taken to exclude the number of ions as a variable that might impair the quality of fragmentation. We calculated the average precursor ion purity (PIP)<sup>34</sup> for each data set and found similar values, which were approximately 95% for all three techniques. Together, these stringent criteria ensure that the activation technique is the only variable that controls the fragmentation behavior. A summary of the data from this direct comparison is given in Table 1. Similar numbers of PSMs were identified for

**Table 1. Analysis of 30 Synthetic Phosphopeptides**

	ETD	HCD	EThcD
#PSM	216	237	248
# unique peptides	21/30	22/30	24/30
average Xcorr	1.5	1.9	2.5
% PSM with correctly localized phosphosite (SEQUEST)	79%	78%	95%
# phosphosites with phosphoRS site probability >99%	478	410	423
% phosphosites with phosphoRS site probability >99%	96%	95%	97%

all three fragmentation techniques. We found that EThcD provided 248 PSMs while these numbers were 237 and 216 for HCD and ETD, respectively. Out of the 30 unique synthetic phosphopeptides injected ETD, HCD and EThcD identified 21, 22 and 24, respectively. We found the average SEQUEST Xcorr being highest for EThcD (2.5) followed by HCD (1.9) and ETD (1.5), which is in line with our previous results for nonmodified peptides.<sup>28</sup> The SEQUEST algorithm correctly annotated the known phosphosites in 79% of ETD and 78% of HCD data. Significantly, for EThcD this was over 95% (of all PSMs), which directly reflects the higher spectral quality, due to the generation of both b/y and c/z ions. This initial data suggests that EThcD provides even more extensive backbone fragmentation of phosphorylated peptides than ETD or HCD alone, facilitating sensitive phosphosite localization with very high confidence. It should be noted that the application of a site localization algorithm would be prudent for real-life samples since the true phosphorylation sites are unknown.

Recently, Taus et al. described phosphoRS, a novel tool to improve confident localization of phosphosites.<sup>27</sup> The software is based on validated peptide identifications provided by database search engines and calculates site probabilities for each potential phosphosite in the peptide sequence. For this study we used a modified version of phosphoRS that also enables

assessment of individual phosphosite probabilities for EThcD fragmentation. We analyzed each data set using phosphoRS and found that it performs equally well for all three fragmentation techniques. Of all true phosphosites, 96% (ETD), 95% (HCD) and 97% (EThcD) were assigned a site probability >99%, which corresponds to a very high confidence in site localization (Table 1). Together, these findings suggest that EThcD generates MS/MS spectra that contain sufficient fragment ions for the unambiguous and sensitive phosphorylation site localization.

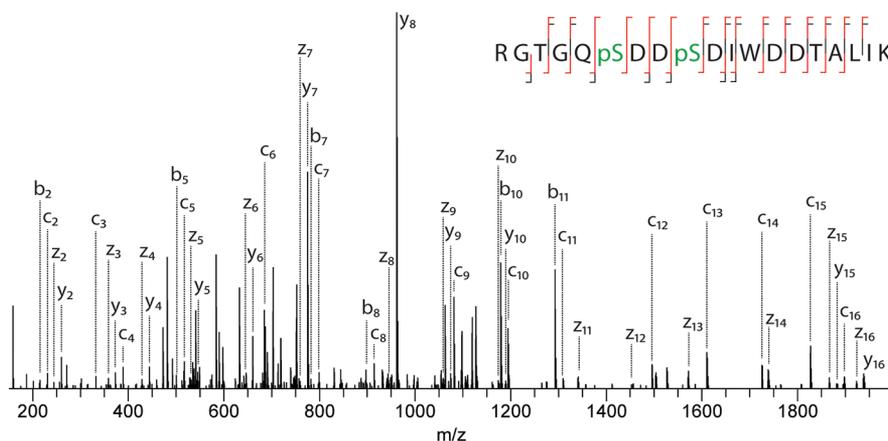
### Phosphosite Localization of Ti<sup>4+</sup>-IMAC Enriched Phosphopeptides by EThcD

Next, we assessed the performance of EThcD for phosphosite localization on a larger data set. We used Ti<sup>4+</sup>-IMAC material for the enrichment of phosphopeptides from a tryptic digest of HeLa cells and analyzed equal amounts (corresponding to enriched phosphopeptides from 100 μg of protein) by LC–MS/MS with ETD, HCD and EThcD, respectively (Supplementary Figure 1A, Supporting Information). All three methods generated a similar number of MS/MS spectra. All spectra were searched with SEQUEST. The ETD data was also searched with Mascot because we found SEQUEST to perform poorly for doubly charged phosphopeptides. Note that other search engines such as OMSSA or SpectrumMill might provide larger number of identifications for ETD data.<sup>35</sup> However, these algorithms are currently not compatible with EThcD data and phosphoRS analysis within the Proteome Discoverer software environment. All identified PSMs were then filtered for <1% FDR using percolator to ensure consistency. In total we identified 2217 (ETD), 4179 (HCD) and 3594 (EThcD) phospho-PSMs (Table 2). Our initial analysis of a defined

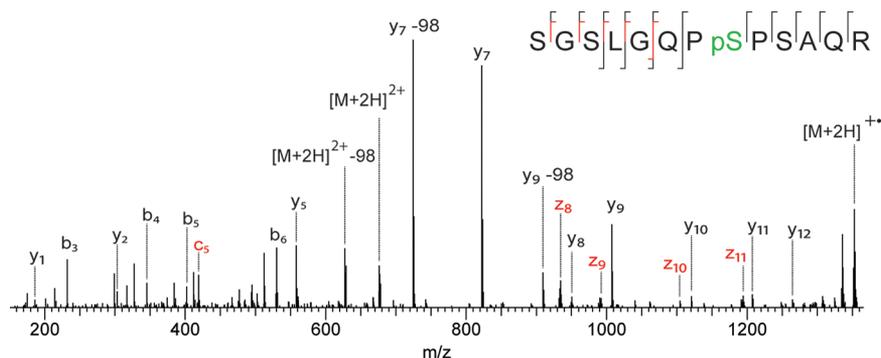
**Table 2. LC–MS/MS Analysis of Ti<sup>4+</sup>-IMAC Enriched Tryptic Phosphopeptides Originating from a Cellular Lysate using ETD, HCD and EThcD**

	ETD	HCD	EThcD
#PSM	2266	4282	3679
ID success rate	25%	51%	44%
average Xcorr	1.9	2.5	3.2
% average peptide sequence coverage	83%	81%	92%
# phospho-PSM	2217	4179	3594
# phospho-sites >99% pRS probability	2002	4291	3942
% phospho-sites >99% pRS probability	81%	89%	95%

synthetic phosphopeptide mixture demonstrated that EThcD performs at least on the same level as HCD in terms of peptide identification. However, the overall identification success rate in the Ti<sup>4+</sup>-IMAC data set was slightly lower for EThcD compared to HCD. This can be attributed to the rigid automatic FDR filtering. The MS/MS spectra from the synthetic phosphopeptide mixture were manually validated whereas the Ti<sup>4+</sup>-IMAC data set was computationally filtered to <1% FDR. The application of EThcD, in comparison to ETD or HCD alone, significantly increases the number of fragment ions observed in the MS/MS scans. On the one hand EThcD spectra contain more sequence information, which is beneficial for inferring the peptide sequence and PTM localization. On the other hand, these additional fragment ions may also match to random peptide sequences, increasing their score and hampering the differentiation between correct and incorrect matches. Consequently, the chance for a high scoring random match will be elevated. Similar to the increased average score of decoy hits



**Figure 1.** EThcD MS/MS spectrum of a doubly phosphorylated peptide. RGTGQSDSDIWDDETALIK is doubly phosphorylated and contains in total four potential phosphorylation sites. EThcD generates dual ion series that enable phosphorylation site localization with very high confidence (phosphoRS site probabilities: T(3), 0.0%; S(6), 100.0%; S(9), 100.0%; T(15), 0.0%). SEQUEST Xcorr 7.79.



**Figure 2.** EThcD spectrum of a proline-containing phosphopeptide. This EThcD spectrum of a doubly charged peptide that contains four serine residues, one of which is phosphorylated. ETD does not cleave the N-C $\alpha$  bond N-terminal to proline and the phosphorylation site probability is only 50% based on c- and z-ions alone. Dual fragmentation by EThcD generates complementary sequence information from c/z- and b/y-ions (SEQUEST Xcorr 4.10). Here, the exact phosphosite is revealed by y-ions that cover the corresponding phosphosite (phosphoRS site probabilities: S(1): 0.0; S(3): 0.0; S(8): 99.5; S(10): 0.5). SEQUEST Xcorr 4.10.

also the true hits are likely to provide on average higher scores. Depending on whether the distance between the two score distributions decreases or increases, the identification success rate will be higher or lower. Since the ID success rate is slightly lower for EThcD compared to HCD alone, the negative effect of higher-scoring random matches might be more pronounced. Thus, higher score cut-offs need to be applied in order to reach the desired FDR. A standard target-decoy approach<sup>36</sup> against a reversed concatenated database revealed the FDR for EThcD (2.6%) being almost twice as high compared to HCD (1.4%), which provides further evidence for this hypothesis.

Next, we calculated the average peptide sequence coverage for all PSM. As expected, EThcD provided a substantial increase in sequence coverage (92%) compared to HCD (81%) and ETD (83%). Obtaining near-complete peptide sequence coverage tremendously simplifies phosphosite localization. We used the extended phosphoRS algorithm to validate our assumption. Remarkably, EThcD provided for 95% of all phosphosites a confident site localization probability of >99%. In the HCD data set we found that 89% of all phosphosites were assigned with a confident site localization probability >99%, while this was only 81% for ETD data set. We recalculated these number for all peptides that contain >2 residues that can be phosphorylated because singly phosphorylated peptides with only one potential phosphorylation site

could bias the results toward HCD. Of all phosphosites from this subset of peptides 97% (ETCaD), 93% (EThcD) and 87% (HCD), respectively, were assigned a localization probability >99%.

For multiply phosphorylated peptides site localization becomes more challenging. Figure 1 shows an MS/MS spectrum of a doubly phosphorylated peptide upon EThcD fragmentation. The overall sequence coverage is 89% taking b/y- and c/z-ions into account. Six out of 18 amino acid bond cleavages are represented by c- and b-ions (referred to as "golden pairs"<sup>37</sup>). Additionally, we observed 11 z/y-ion pairs, which strengthens the argument that EThcD provides extensive sequence information that facilitates pinpointing the correct phosphorylation site. More than 95% of the phosphosites from all doubly phosphorylated peptides were assigned with a site localization probability >99%, highlighting that EThcD performs equally well with singly and doubly phosphorylated peptides. A known limitation of ETD is its inability to cleave the N-C $\alpha$  bond N-terminal to proline.<sup>38,39</sup> This can hamper phosphosite localization for proline-rich peptides. Generation of dual ion series in EThcD can overcome this issue. Figure 2 shows the EThcD spectrum of a singly phosphorylated peptide that contains four serine residues. The c- and z-ions derived from the ETD step cover only the N-terminal part of the peptide and the site probability is only 50%. The additional y-

ions derived from the subsequent HCD activation provide supporting sequence information and cover also the two serine residues next to the prolines which enables unambiguous phosphosite localization.

## CONCLUSIONS

Here we have evaluated the potential of EThcD in improving the analysis of phosphopeptides. Our data highlights the benefit of dual ion series as generated by EThcD fragmentation. We observed for a defined phosphopeptide mixture average higher SEQUEST Xcorr values, higher peptide sequence coverage and more confident phosphosite localization in EThcD compared to ETD and HCD. This finding was confirmed when we analyzed a complex phosphopeptide sample resulting from a  $Ti^{4+}$ -IMAC enrichment of peptides from a cellular lysate. This is in line with recent reports that showed that confidence in phosphorylation site localization increases when multiple separately acquired MS/MS spectra (e.g., ETD/CID or MSA/ETD) are combined for scoring.<sup>25,26</sup> For this larger data set, we observed that the identification success rate was slightly lower for EThcD compared to HCD. This can be attributed to the use of conventional database search engines that are not optimized for spectra that contain dual ion series.<sup>40</sup> However, the fact that both peptide sequence coverage and the percentage of localized phosphosites are higher for EThcD than for HCD suggests that once a peptide was identified, further analyses such as site localization benefit from the more data-rich EThcD spectra. In EThcD often c/b- and z/y-ion pairs are observed that increase the confidence in a particular peptide backbone cleavage.<sup>41</sup> We speculate that the identification success rate of EThcD for phosphopeptides can be improved by novel or optimized data analysis tools. Finally, we reason that EThcD can also be beneficial and used to improve the localization of other post-translational modifications such as ubiquitination, glycosylation or acetylation.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

- (1) Cohen, P. The regulation of protein function by multisite phosphorylation—a 25 year update. *Trends Biochem. Sci.* **2000**, *25* (12), 596–601.
- (2) Hunter, T. Signaling—2000 and beyond. *Cell* **2000**, *100* (1), 113–27.
- (3) Olsen, J. V.; Blagoev, B.; Gnäd, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **2006**, *127* (3), 635–48.
- (4) Eyrich, B.; Sickmann, A.; Zahedi, R. P. Catch me if you can: mass spectrometry-based phosphoproteomics and quantification strategies. *Proteomics* **2011**, *11* (4), 554–70.
- (5) Mann, M.; Jensen, O. N. Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* **2003**, *21* (3), 255–61.
- (6) Di Palma, S.; Hennrich, M. L.; Heck, A. J.; Mohammed, S. Recent advances in peptide separation by multidimensional liquid chromatography for proteome analysis. *J. Proteomics* **2012**, *75* (13), 3791–813.
- (7) Rush, J.; Moritz, A.; Lee, K. A.; Guo, A.; Goss, V. L.; Spek, E. J.; Zhang, H.; Zha, X. M.; Polakiewicz, R. D.; Comb, M. J. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **2005**, *23* (1), 94–101.
- (8) Ficarro, S. B.; McClelland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **2002**, *20* (3), 301–5.
- (9) Zhou, H.; Low, T. Y.; Hennrich, M. L.; van der Toorn, H.; Schwend, T.; Zou, H.; Mohammed, S.; Heck, A. J. Enhancing the identification of phosphopeptides from putative basophilic kinase substrates using  $Ti$  (IV) based IMAC enrichment. *Mol. Cell. Proteomics* **2011**, *10* (10), M110 006452.
- (10) Boersema, P. J.; Mohammed, S.; Heck, A. J. Phosphopeptide fragmentation and analysis by mass spectrometry. *J. Mass Spectrom.* **2009**, *44* (6), 861–78.
- (11) Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villen, J.; Li, J.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (33), 12130–5.
- (12) Schroeder, M. J.; Shabanowitz, J.; Schwartz, J. C.; Hunt, D. F.; Coon, J. J. A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry. *Anal. Chem.* **2004**, *76* (13), 3590–8.
- (13) Olsen, J. V.; Macek, B.; Lange, O.; Makarov, A.; Horning, S.; Mann, M. Higher-energy C-trap dissociation for peptide modification analysis. *Nat. Methods* **2007**, *4* (9), 709–12.
- (14) Chi, A.; Huttenhower, C.; Geer, L. Y.; Coon, J. J.; Syka, J. E.; Bai, D. L.; Shabanowitz, J.; Burke, D. J.; Troyanskaya, O. G.; Hunt, D. F. Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (7), 2193–8.
- (15) Molina, H.; Matthiesen, R.; Kandasamy, K.; Pandey, A. Comprehensive comparison of collision induced dissociation and electron transfer dissociation. *Anal. Chem.* **2008**, *80* (13), 4825–35.
- (16) Beausoleil, S. A.; Villen, J.; Gerber, S. A.; Rush, J.; Gygi, S. P. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* **2006**, *24* (10), 1285–92.
- (17) Stensballe, A.; Jensen, O. N.; Olsen, J. V.; Haselmann, K. F.; Zubarev, R. A. Electron capture dissociation of singly and multiply phosphorylated peptides. *Rapid Commun. Mass Spectrom.* **2000**, *14* (19), 1793–800.
- (18) Kelstrup, C. D.; Hekmat, O.; Francavilla, C.; Olsen, J. V. Pinpointing phosphorylation sites: Quantitative filtering and a novel site-specific x-ion fragment. *J. Proteome Res.* **2011**, *10* (7), 2937–48.
- (19) Shin, Y. S.; Moon, J. H.; Kim, M. S. Observation of phosphorylation site-specific dissociation of singly protonated phosphopeptides. *J. Am. Soc. Mass Spectrom.* **2010**, *21* (1), 53–9.
- (20) Gehrig, P. M.; Roschitzki, B.; Rutishauser, D.; Reiland, S.; Schlapbach, R. Phosphorylated serine and threonine residues promote

site-specific fragmentation of singly charged, arginine-containing peptide ions. *Rapid Commun. Mass Spectrom.* **2009**, *23* (10), 1435–45.

(21) Lu, B.; Ruse, C.; Xu, T.; Park, S. K.; Yates, J. R., 3rd Automatic validation of phosphopeptide identifications from tandem mass spectra. *Anal. Chem.* **2007**, *79* (4), 1301–10.

(22) Bailey, C. M.; Sweet, S. M.; Cunningham, D. L.; Zeller, M.; Heath, J. K.; Cooper, H. J. SLoMo: automated site localization of modifications from ETD/ECD mass spectra. *J. Proteome Res.* **2009**, *8* (4), 1965–71.

(23) Savitski, M. M.; Mathieson, T.; Becher, I.; Bantscheff, M. H-score, a mass accuracy driven rescoring approach for improved peptide identification in modification rich samples. *J. Proteome Res.* **2010**, *9* (11), 5511–6.

(24) Rutenber, B. E.; Pisitkun, T.; Knepper, M. A.; Hoffert, J. D. PhosphoScore: an open-source phosphorylation site assignment tool for MSn data. *Journal of Proteome Research* **2008**, *7* (7), 3054–9.

(25) Hansen, T. A.; Sylvester, M.; Jensen, O. N.; Kjeldsen, F. Automated and high confidence protein phosphorylation site localization using complementary collision-activated dissociation and electron transfer dissociation tandem mass spectrometry. *Anal. Chem.* **2012**, *84* (22), 9694–9.

(26) Vandenberg, M.; Hourdel, V.; Jardin-Mathe, O.; Bigeard, J.; Bonhomme, L.; Legros, V.; Hirt, H.; Schwikowski, B.; Pflieger, D. Automated phosphopeptide identification using multiple MS/MS fragmentation modes. *J. Proteome Res.* **2012**, *11* (12), 5695–703.

(27) Taus, T.; Kocher, T.; Pichler, P.; Paschke, C.; Schmidt, A.; Henrich, C.; Mechtler, K. Universal and confident phosphorylation site localization using phosphoRS. *J. Proteome Res.* **2011**, *10* (12), 5354–62.

(28) Frese, C. K.; Altelaar, M.; van den Toorn, H. W.; Nolting, D.; Griep-Raming, J.; Heck, A. J.; Mohammed, S. Toward full peptide sequence coverage by dual fragmentation combining electron-transfer and higher-energy collision dissociation tandem mass spectrometry. *Anal. Chem.* **2012**, *84* (22), 9668–73.

(29) Altelaar, A. F.; Frese, C. K.; Preisinger, C.; Hennrich, M. L.; Schram, A. W.; Timmers, H. T.; Heck, A. J.; Mohammed, S. Benchmarking stable isotope labeling based quantitative proteomics. *J. Proteomics* **2012**, DOI: 10.1016/j.jprot.2012.10.009.

(30) Zhou, H.; Ye, M.; Dong, J.; Han, G.; Jiang, X.; Wu, R.; Zou, H. Specific phosphopeptide enrichment with immobilized titanium ion affinity chromatography adsorbent for phosphoproteome analysis. *J. Proteome Res.* **2008**, *7* (9), 3957–67.

(31) Yu, Z.; Han, G.; Sun, S.; Jiang, X.; Chen, R.; Wang, F.; Wu, R.; Ye, M.; Zou, H. Preparation of monodisperse immobilized Ti(4+) affinity chromatography microspheres for specific enrichment of phosphopeptides. *Anal. Chim. Acta* **2009**, *636* (1), 34–41.

(32) Zhou, H.; Ye, M.; Dong, J.; Corradini, E.; Cristobal, A.; Heck, A. J. R.; Zou, H.; Mohammed, S. Robust phosphoproteome enrichment using monodisperse microspheres-based immobilized titanium (IV) ion affinity chromatography. *Nat. Protoc.* **2012**, accepted.

(33) Kall, L.; Canterbury, J. D.; Weston, J.; Noble, W. S.; MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* **2007**, *4* (11), 923–5.

(34) Mertins, P.; Udeshi, N. D.; Clauser, K. R.; Mani, D. R.; Patel, J.; Ong, S. E.; Jaffe, J. D.; Carr, S. A. iTRAQ labeling is superior to mTRAQ for quantitative global proteomics and phosphoproteomics. *Mol. Cell. Proteomics* **2012**, *11* (6), M111 014423.

(35) Kandasamy, K.; Pandey, A.; Molina, H. Evaluation of Several MS/MS Search Algorithms for Analysis of Spectra Derived from Electron Transfer Dissociation Experiments. *Anal. Chem.* **2009**, *81* (17), 7170–80.

(36) Elias, J. E.; Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **2007**, *4* (3), 207–14.

(37) Horn, D. M.; Zubarev, R. A.; McLafferty, F. W. Automated de novo sequencing of proteins by tandem high-resolution mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (19), 10313–7.

(38) Cooper, H. J.; Hudgins, R. R.; Hakansson, K.; Marshall, A. G. Secondary fragmentation of linear peptides in electron capture dissociation. *Int. J. Mass Spectrom.* **2003**, *228* (2–3), 723–8.

(39) Li, W.; Song, C.; Bailey, D. J.; Tseng, G. C.; Coon, J. J.; Wysocki, V. H. Statistical analysis of electron transfer dissociation pairwise fragmentation patterns. *Anal. Chem.* **2011**, *83* (24), 9540–5.

(40) Kim, M. S.; Zhong, J.; Kandasamy, K.; Delanghe, B.; Pandey, A. Systematic evaluation of alternating CID and ETD fragmentation for phosphorylated peptides. *Proteomics* **2011**, *11* (12), 2568–72.

(41) Nielsen, M. L.; Savitski, M. M.; Zubarev, R. A. Improving protein identification using complementary fragmentation techniques in fourier transform mass spectrometry. *Mol. Cell. Proteomics* **2005**, *4* (6), 835–45.