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Using Peptide Arrays to Profile Phosphatase Activity in Cell Lysates

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Abstract: Phosphorylation is an important post-translational modification on proteins involved in many cellular processes; however, understanding of the regulation and mechanisms of global phosphorylation remains limited. Herein, we utilize self-assembled monolayers on gold for matrix-assisted laser desorption/ionization mass spectrometry (SAMDI-MS) with three phosphorylated peptide arrays to profile global phosphatase activity in cell lysates derived from five mammalian cell lines. Our results reveal significant differences in the activities of protein phosphatases on phospho- serine, threonine, and tyrosine substrates and suggest that phosphatases play a much larger role in the regulation of global phosphorylation on proteins than previously understood.

Protein phosphorylation is the most prominent reversible post-translational modification and is involved in the regulation of almost all cellular processes including signaling, migration, proliferation, apoptosis, differentiation, and metabolism.[1] The addition and removal of phosphorylation sites on proteins are regulated by kinases and phosphatases, respectively, and dysregulation of phosphorylation has been found to contribute to several diseases such as cancer, diabetes, and neurodegenerative and inflammatory disorders.[2]

Proteomic studies have shown that phosphorylation occurs on over 30% of cellular proteins, where it is most commonly found on serine (Ser, 86.4%), followed by threonine (Thr, 11.8%) and tyrosine (Tyr, 1.8%) residues.[3] The reasons and functional implications for this distribution of phosphosites remain poorly understood. Tyrosine phosphorylation is known to be an important regulator of dynamic signaling events, and the lower levels of phosphotyrosine (pTyr) are consistent with the comparable number of kinases (90) and phosphatases (107).[4] The functional roles of Ser/Thr phosphorylation are less understood, and the reasons for the abundance of phosphoserine (pSer) compared to phosphothreonine (pThr) are unclear. The number of serine/threonine kinases (428) is far greater than the number of phosphatases (only 30),[4] leading many researchers to postulate that Ser/Thr phosphatases play a "housekeeping" role. However, in recent years, it has been recognized that Ser/Thr phosphatases are important for regulating phosphorylation, and they act as holoenzymes that form various complexes with large numbers of regulatory subunits to gain specificity.[5] The interactions between phosphatases and their regulatory subunits are yet to be fully elucidated; however, several reviews comprehensively describe findings from past research and recent discoveries, notably the review by Cesareni and colleagues[6] and the special issue titled "Protein Phosphatases as Critical Regulators for Cellular Homeostasis" in *BBA—Molecular Cell Research*.[7]

The inherent challenges of studying the activities of Ser/Thr phosphatases that are regulated by interactions with many different regulatory subunits and the lack of assays that can quantitate both phosphatase activity and substrate specificity in complex samples, such as cell lysates, help to explain why the majority of work is directed towards the roles of kinases.[8] In fact, most explanations for the regulation and distribution of phosphorylation sites have often emphasized or solely addressed changes in kinase activity with little consideration for the roles that phosphatases play. However, as the extent of phosphorylation depends on the balance of kinase and phosphatase activities, increases in kinase activity or corresponding decreases in phosphatase activity can both lead to greater phosphorylation.

In this work, we used phosphopeptide arrays to profile total phosphatase activity from the three main protein phosphatase families (serine/threonine phosphatases, tyrosine phosphatases, and dual-specific phosphatases that dephosphorylate all three phosphosites) in cell lysates with the aim of identifying trends in the relative activities on pSer, pThr, and pTyr substrates. Biochemical arrays are valuable platforms for high-throughput experiments,[9] and peptide arrays have been important for the study of substrate specificity of enzymes, epitope mapping of antibodies, and protein binding interactions.[10] We prepared peptide arrays on surfaces presenting self-assembled monolayers (SAMs) that are well-suited for assays of cell lysates and compatible with matrix-assisted laser desorption/ionization mass spectrometry (SAMDI-MS), which provides a label-free assay of phosphatase activity on peptide substrates.[11] SAMDI-MS is particularly significant for analyzing phosphatase reactions because it can observe the reaction product directly, without the need for labels, and is applicable

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to all phosphopeptide substrates. The most striking insight from this work is that substrates containing pThr are globally more active for the Ser/Thr phosphatases than are substrates containing pSer. This work profiles protein phosphatase activity in cell lysates using hundreds of distinct peptide substrates and has revealed activity trends that will be important for understanding the roles these phosphatases play in cellular processes.

We prepared phosphopeptide arrays on steel plates with 384 gold islands arranged in the standard microwell plate geometry, each modified with a SAM. The SAMs present a maleimide group against a background of protein-resistant tri(ethylene glycol) (EG3) groups, as described previously.[12] We used standard Fmoc-based solid-phase peptide synthesis to prepare a pSer peptide library based on the following sequence: Ac-GXS^{p}ZGRC (in which X and Z are variable positions and represent all natural amino acids except for cysteine). In this way, the peptides are designed not to identify specific substrates of individual phosphatases, but instead to represent a broad distribution of sequences that can resolve the general specificities of phosphatases in their many complexed forms. The cysteine-terminated peptides (suspended in 50 mM Tris buffer, pH 7.5) were robotically spotted onto the monolayer array, where peptides underwent immobilization to each spot through reaction of the cysteine thiol with the maleimide group. We evaluated the quality of each peptide using MALDI-MS, and the signal-to-noise ratios of each peptide are listed in Tables S1–S3, Supporting Information.

We next used the pSer peptide array to profile phosphatases that were present in lysates derived from cultured NIH/3T3 cells. We applied the lysate to each spot on the array (1.5 μL at 0.5 mg mL^{-1} total protein) and incubated for 15 minutes at 37 °C before the peptide array was rinsed and dried. During the incubation, endogenous phosphatase enzymes could dephosphorylate their corresponding active peptide substrates. We then applied MALDI matrix to the array plate and measured the extent of dephosphorylation of each peptide using SAMDI mass spectrometry (Figure 1).

The extent of dephosphorylation of each peptide was determined by dividing the area under the curve (AUC) of the dephosphorylated product peak by the sum of the AUC of the dephosphorylated product and phosphorylated substrate peaks. The extents of dephosphorylation of each peptide in the array are displayed in heatmaps, where each square represents an individual peptide with X and Z residues on the horizontal and vertical axes, respectively. The heatmaps report the percent of dephosphorylation on a color scale, where dark purple represents 100% dephosphorylation, and the standard deviation from three replicates for each peptide is displayed by circle size within each peptide square—where a smaller circle corresponds to a larger standard deviation.

To assess phosphatase activity on other residues, we similarly prepared pThr and pTyr arrays based on the sequences: Ac-GXT^{p}ZGRC, and Ac-GXY^{p}ZGRC (in which X and Z represent all natural amino acids except for cysteine). We treated the pThr and pTyr peptide arrays with the same NIH/3T3 lysate, and analyzed the extent of dephosphorylation of each peptide using MALDI-MS. The use of three phosphorylated peptide arrays containing the same sequence (apart from the central phosphorylated residue) allows for a direct assessment and comparison of broad phosphatase activities on pSer, pThr, and pTyr substrates. The phosphatase activity profiles on all three arrays from the NIH/3T3 cell lysate are shown in Figure 2.

Inspection of the heatmaps reveals striking differences in activity and specificity between the pSer, pThr, and pTyr arrays. While Ser/Thr phosphatases (and dual-specific phosphatases) act on both pSer and pThr substrates, the levels of activity observed on the arrays are notably different. The substrate specificities observed on these two arrays are quite similar, but the activity on the pThr array is clearly higher than on the pSer array. The pSer and pThr arrays show preferential Ser/Thr phosphatase activity on substrates containing hydrophobic and aromatic amino acids in both the X and Z positions (V, L, I, M, F, Y, and W). Additionally, we find disfavored Ser/Thr phosphatase activity on peptides with combinations of G, A, S, T, D, E, N, and Q in both X and Z positions. The pTyr array revealed low Tyr phosphatase specificity where most peptides were dephosphorylated between 40–60%; however, we note slightly lower Tyr phosphatase activity on substrates with lysine or arginine in either variable position, which is consistent with the findings of Pei and colleagues.[13] Additionally, on all three arrays, we observe very little activity on peptides containing proline in the Z position. While this observation may be related to phosphatase substrate specificity, we recognize that proline in the

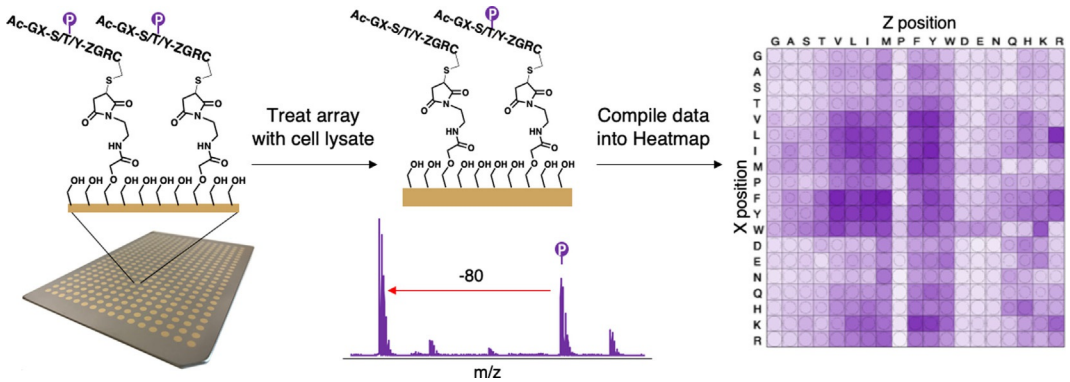

Figure 1. Profiling phosphatase activity from cell lysates on peptide arrays. Phosphopeptide libraries were immobilized onto SAMDI arrays. The arrays were treated with cell lysates and the extent of dephosphorylation of each peptide was measured using SAMDI-MS.

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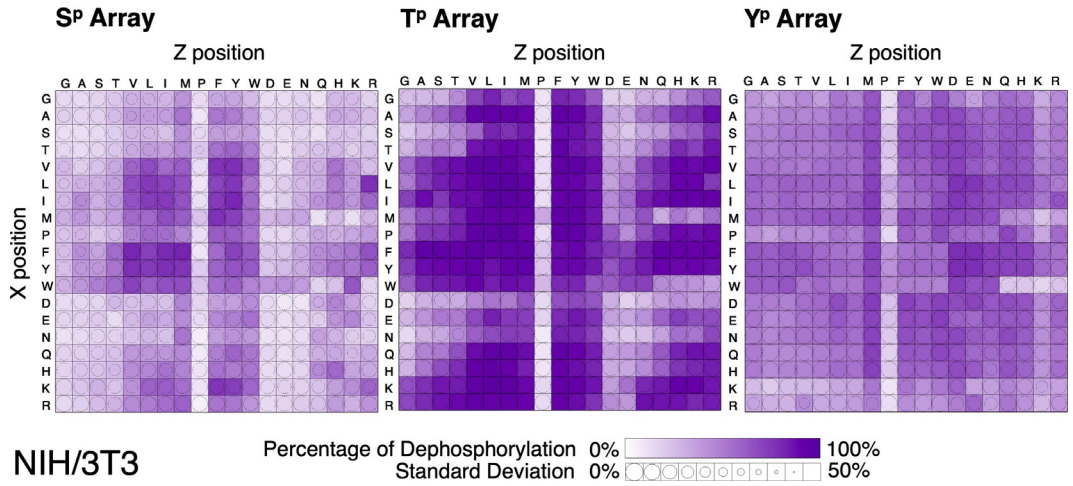

Figure 2. Heatmaps showing global phosphatase activity in cell lysate from NIH/3T3 cells. The average extent of dephosphorylation of each peptide is plotted in the heatmap, in which complete dephosphorylation is denoted by dark purple, and each square represents a peptide of sequence Ac-GX-S^P/T^P/Y^P-ZGRC, in which the X and Z residues are denoted on the vertical and horizontal axes, respectively. The standard deviation of the dephosphorylation of each peptide is displayed by circle size in each peptide square, with larger standard deviations resulting in smaller circles.

Z position may introduce steric interactions of the phosphorylated residue with the surface and result in our observed low phosphatase activity.

We note that our measurements are not calibrated, and there are differences in ionization efficiency between unphosphorylated and phosphorylated peptides; however, these differences are consistent between peptides of various sequences and, therefore, have a minimal perturbation on the trends in activity and sequence specificity that we observe (Figure S1, Supporting Information). Additionally, we observe no kinase activity in our cell lysates without the addition of ATP, a required co-factor for kinase activity (Figure S2, Supporting Information).

Following our analysis of global phosphatase activity in NIH/3T3 cell lysates, we extended our study to measure phosphatase activity in cell lysates from four additional mammalian cell lines: HT-1080, MCF-7, BT474, and MDA-MB-231. We chose these five cell lines because we were interested in comparing the phosphatase profiles between 1) different species (i.e. NIH3T3 from mouse and the four others from human), 2) different cancer cell types (i.e. breast cancer and fibrosarcoma), and 3) similar cancer cell types with altering metastatic levels (three breast cancer cell lines with different receptor profiles). The chosen cell lines met these three comparison criteria and are relatively simple to culture. Each cell lysate was applied to the pSer, pThr, and pTyr peptide arrays, and the arrays were analyzed by MALDI-MS and reported in heatmaps (Figure 3). The data values for each peptide for all five cell lysates are listed in Tables S1–S3, Supporting Information.

The global phosphatase profiles for each lysate again show preferential activity on pThr compared to pSer substrates. As mentioned earlier, several proteomic studies have found that in various cellular conditions, phosphorylation occurs with ≈86% on Ser, ≈12% on Thr, and ≈2% on Tyr residues.[3] Proteomic studies for the detection of phosphorylated sites offer an unbiased view of the in vivo proteome; however, they do not provide mechanistic information such as the enzymes responsible for the addition and removal of the phosphorylation modification or the stability and regulatory roles of each phosphorylation site. Previous reports suggest the phosphoproteome distribution across Ser, Thr, and Tyr residues likely results from the higher number of Ser/Thr kinases and their preferences for Ser and Thr as phosphoacceptor residues.[14] While this may be true, our results suggest that phosphatases, and more specifically the Ser/Thr phosphatases (and the dual-specific phosphatases), may play a more significant role in determining the phosphoproteome distribution than previously believed and can help explain the high levels of pSer.

The high specificity and lower reactivity of phosphatases on the pSer array may indicate that phosphorylation on Ser is more regulated than on Thr and/or that Ser/Thr phosphatases generally have a stronger preference for pThr over pSer substrates. Either of these conclusions could explain why proteomic studies report the highest level of phosphorylation on Ser. In fact, Merlevede and co-workers found that members of the PP2A family of Ser/Thr phosphatases preferred pThr over pSer substrates and that phosphatase activity on pSer could be increased by changing the proximal amino acid sequences surrounding the pSer, while activity on pThr was less affected by changes in proximal amino acid sequences.[15] Additionally, Burgess and co-workers observed in a proteomic study that pThr sites with proline in the +1 position were dephosphorylated at a higher rate than pSer-proline motifs.[16] These results are consistent with our findings that phosphatase activity is lower on pSer than on pThr substrates.

Our observation that Ser/Thr phosphatases are more active on pThr than pSer substrates is significant because it establishes that the prevalence of pSer in the proteome could reflect generally greater phosphatase activities on pThr. This example is a significant reminder that kinases and phosphatases both play important roles in determining global phosphorylation.

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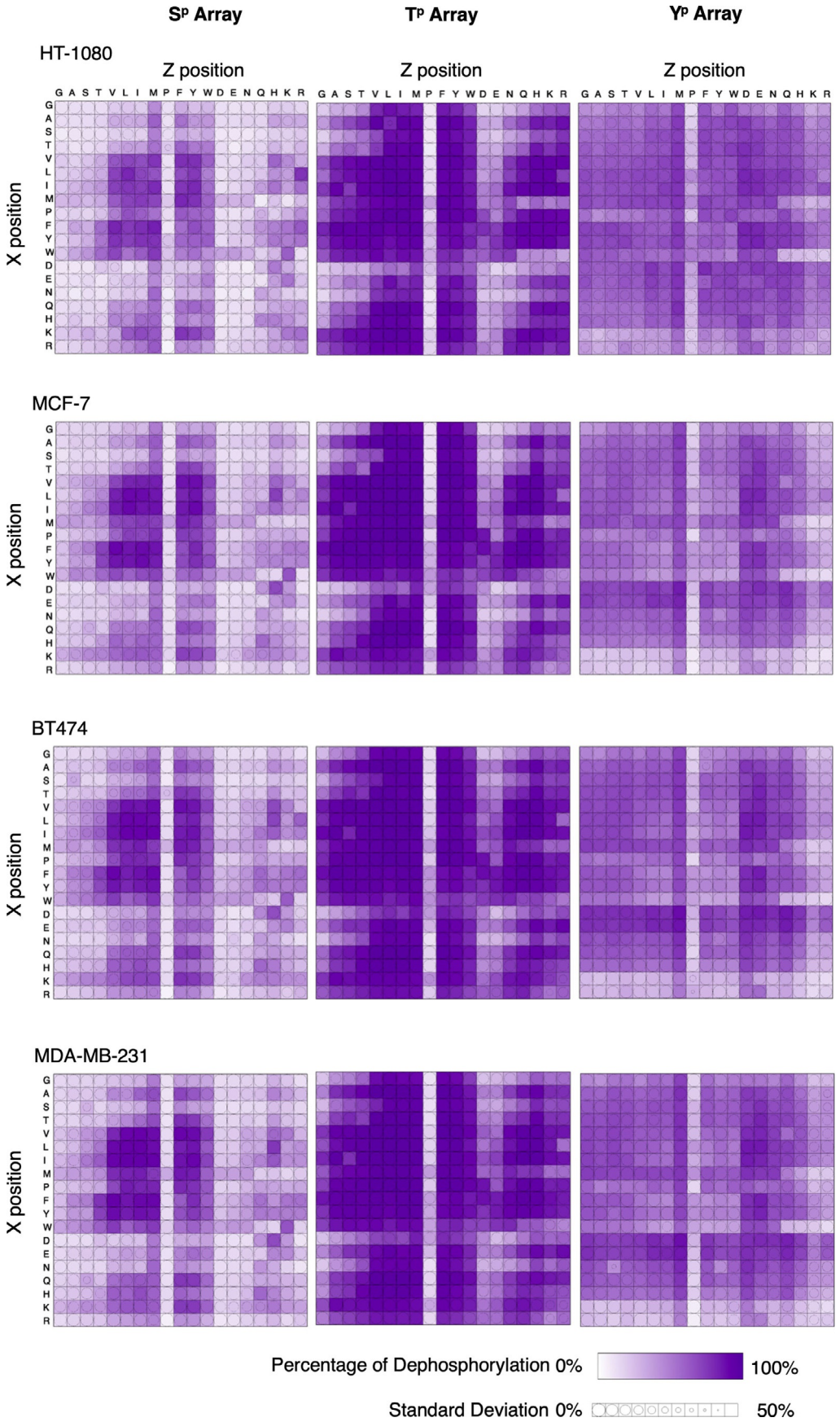

Figure 3. Heatmaps showing the global phosphatase activity in cell lysates from HT-1080, MCF-7, BT474, and MDA-MB-231 cell lines. The experiments and analysis were performed as described earlier and shown in Figure 2. Larger heatmaps for each of these cell lines are shown in Figures S3–S6, Supporting Information.

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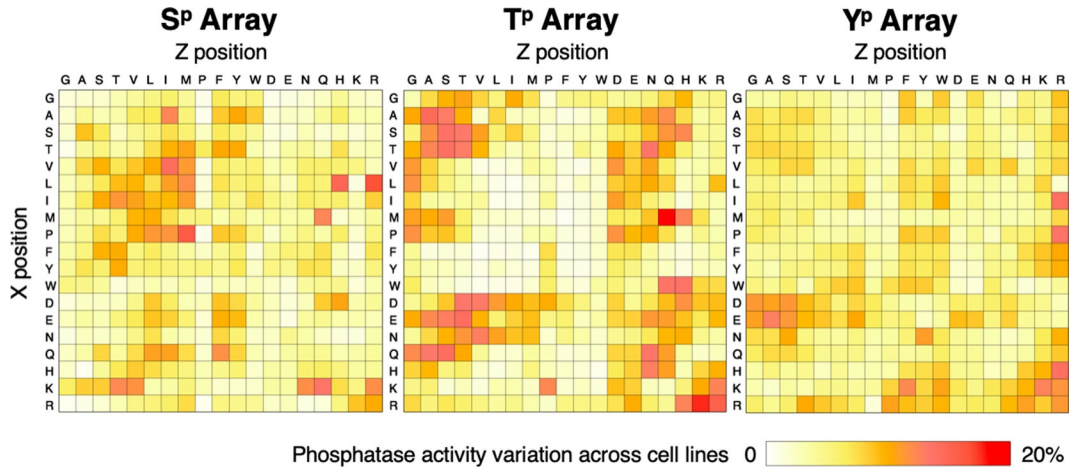

Figure 4. Heatmaps displaying the variation of phosphatase activity for experiments performed with lysates prepared from five different cell lines. The standard deviation of the average percentages of dephosphorylation from each cell lysate was calculated for each peptide and are shown on a color scale range of white–yellow–orange–red, in which white represents 0% and red represents 20% standard deviation.

We were surprised by the similar phosphatase profiles that we observed across five different cell lines. To visualize the variance in phosphatase activity across the five cell lysates, we calculated the standard deviation of the average percentages of dephosphorylation from all five cell lysates for each peptide. We found the variation in phosphatase activity on all peptides was less than 20%, with most peptides having a standard deviation between 1–10% (Figure 4).

The functionality of the vast majority of phosphorylation sites have yet to be studied; however, several reports have speculated that many phosphorylation sites are nonfunctional.[17] Many proteomic studies that have monitored global phosphorylation in various cellular processes found small variations across a myriad of conditions. For instance, proteomic studies that examined global phosphorylation changes in various mouse tissues[18] and during mitotic exit,[16] epidermal growth factor stimulation,[3a] and DNA damage response[19] all found that global phosphorylation was only altered between 10–15% which is consistent with our observation of little variation in phosphatase activity in the five different cell lysates. The similar, but specific phosphatase profiles that we observed likely indicate that phosphatase activity is similarly regulated in different cell types and could also suggest that many phosphorylation sites are nonfunctional. The mechanisms behind phosphatase specificity remain largely unknown; however, it is clear from our results, as well as from others, that phosphatases are highly regulated enzymes.[20]

Herein, we used peptide arrays and SAMDI-MS to observe and differentiate phosphatase activity on Ser, Thr, and Tyr residues on more than 1,000 peptide substrates for lysates derived from five cell lines. We found that phosphatase activity is lower on pSer in comparison to pThr substrates, which may suggest that the phosphorylation distribution across Ser, Thr, and Tyr residues is largely impacted by phosphatase activity, rather than differential activity of kinases alone. It is clear that phosphatases have significant regulatory roles in the cell; however, further studies on the substrate specificities and dynamics between kinases and phosphatases are necessary to fully decipher the mechanisms behind phosphorylation and the regulatory roles of each site.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cell lysates · high-throughput screening · phosphatases · phosphorylation · SAMDI-MS

[1] a) G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* **2002**, *298*, 1912; b) G. Manning, G. D. Plowman, T. Hunter, S. Sudarsanam, *Trends Biochem. Sci.* **2002**, *27*, 514–520; c) J. A. Ubersax, J. E. Ferrell Jr., *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 530.

[2] C. S. H. Tan, B. Bodenmiller, A. Pasculescu, M. Jovanovic, M. O. Hengartner, C. Jørgensen, G. D. Bader, R. Aebersold, T. Pawson, R. Linding, *Sci. Signaling* **2009**, *2*, ra39–ra39.

[3] a) J. V. Olsen, B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, M. Mann, *Cell* **2006**, *127*, 635–648; b) S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt, F. M. White, *Nat. Biotechnol.* **2002**, *20*, 301.

[4] Y. Shi, *Cell* **2009**, *139*, 468–484.

[5] D. M. Virshup, S. Shenolikar, *Molecular Cell* **2009**, *33*, 537–545.

[6] F. Sacco, L. Perfetto, L. Castagnoli, G. Cesareni, *FEBS Lett.* **2012**, *586*, 2732–2739.

[7] P. Ruvolo, V. Janssens, *Biochim. Biophys. Acta* **2019**, *1866*, 1–174.

[8] a) A. K. Bose, K. A. Janes, *Mol. Cell. Proteomics* **2013**, *12*, 797–806; b) S. D. Killilea, Q. Cheng, Z.-X. Wang, in *Protein Phosphatase Protocols, Vol. 93* (Ed.: J. Ludlow), Humana, **1998**, pp. 23–33; c) T. McAvoy, A. C.

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Nairn, *Curr. Protoc. Mol. Biol.* **2010**, *92*, 18.18.1 – 18.18.11; d) X. Espanel, M. Huguenin-Reggiani, R. H. van Huijsduijnen, *Protein Sci.* **2009**, *11*, 2326 – 2334; e) Z. A. Knight, K. M. Shokat, *Chem. Biol.* **2005**, *12*, 621 – 637; f) J. R. Beck, A. Lawrence, A. S. Tung, E. N. Harris, C. I. Stains, *ACS Chem. Biol.* **2016**, *11*, 284 – 290.

[9] M. D. Disney, P. H. Seeberger, *Chem. Biol.* **2004**, *11*, 1701 – 1707.

[10] a) V. Stadler, T. Felgenhauer, M. Beyer, S. Fernandez, K. Leibe, S. Güttler, M. Gröning, K. König, G. Torralba, M. Hausmann, V. Lindenstruth, A. Nesterov, I. Block, R. Pipkorn, A. Poustka, F. R. Bischoff, F. Breitling, *Angew. Chem. Int. Ed.* **2008**, *47*, 7132 – 7135; *Angew. Chem.* **2008**, *120*, 7241 – 7244; b) L. K. Weber, A. Isse, S. Rentschler, R. E. Kneusel, A. Palermo, J. Hubbuch, A. Nesterov-Mueller, F. Breitling, F. F. Loeffler, *Eng. Life Sci.* **2017**, *17*, 1078 – 1087; c) L. C. Szymczak, H.-Y. Kuo, M. Mrksich, *Anal. Chem.* **2018**, *90*, 266 – 282; d) X. Meng, J. Wei, Y. Wang, H. Zhang, Z. Wang, *Anal. Methods* **2018**, *10*, 4614 – 4624.

[11] a) Z. A. Gurard-Levin, M. Mrksich, *Annu. Rev. Anal. Chem.* **2008**, *1*, 767 – 800; b) M. Mrksich, *ACS Nano* **2008**, *2*, 7 – 18; c) E. J. Berns, M. D. Cabezas, M. Mrksich, *Small* **2016**, *12*, 3811 – 3818; d) H.-Y. Kuo, T. A. DeLuca, W. M. Miller, M. Mrksich, *Anal. Chem.* **2013**, *85*, 10635 – 10642.

[12] a) B. T. Houseman, E. S. Gawalt, M. Mrksich, *Langmuir* **2003**, *19*, 1522 – 1531; b) L. C. Szymczak, C.-F. Huang, E. J. Berns, M. Mrksich, in *Methods Enzymology, Vol. 607* (Ed.: K. N. Allen), Academic Press, **2018**, pp. 389 – 403.

[13] L. Ren, X. Chen, R. Luechapanichkul, N. G. Selner, T. M. Meyer, A.-S. Wavreille, R. Chan, C. Iorio, X. Zhou, B. G. Neel, D. Pei, *Biochemistry* **2011**, *50*, 2339 – 2356.

[14] a) C. Chen, B. H. Ha, A. F. Thévenin, H. J. Lou, R. Zhang, K. Y. Yip, J. R. Peterson, M. Gerstein, P. M. Kim, P. Filippakopoulos, S. Knapp, T. J. Boggon, B. E. Turk, *Molecular cell* **2014**, *53*, 140 – 147; b) T. Hunter, *Curr. Opin. Cell Biol.* **2009**, *21*, 140 – 146; c) K. Sharma, R. C. J. D'Souza, S. Tyanova, C. Schaab, J. R. Wiśniewski, J. Cox, M. Mann, *Cell Rep.* **2014**, *8*, 1583 – 1594.

[15] P. Agostinis, J. Goris, L. A. Pinna, F. Marchiori, J. W. Perich, H. E. Meyer, W. Merlevede, *Eur. J. Biochem.* **1990**, *189*, 235 – 241.

[16] R. A. McCloy, B. L. Parker, S. Rogers, R. Chaudhuri, V. Gayevskiy, N. J. Hoffman, N. Ali, D. N. Watkins, R. J. Daly, D. E. James, T. Lorca, A. Castro, A. Burgess, *Mol. Cell. Proteomics* **2015**, *14*, 2194 – 2212.

[17] G. E. Lienhard, *Trends Biochem. Sci.* **2008**, *33*, 351 – 352.

[18] E. L. Huttlin, M. P. Jedrychowski, J. E. Elias, T. Goswami, R. Rad, S. A. Beausoleil, J. Villén, W. Haas, M. E. Sowa, S. P. Gygi, *Cell* **2010**, *143*, 1174 – 1189.

[19] M. V. Bennetzen, D. H. Larsen, J. Bunkenborg, J. Bartek, J. Lukas, J. S. Andersen, *Mol. Cell. Proteomics* **2010**, *9*, 1314.

[20] X. Li, M. Wilmanns, J. Thornton, M. Köhn, *Sci. Signaling* **2013**, *6*, rs10.

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