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Method Development and Application for Profiling Dynamic Tyrosine Phosphorylation-mediated Signaling Complexes

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ABSTRACT

Profiling the spatiotemporal dynamic behaviors of tyrosine phosphorylation (pTyr) sites and pTyr-mediated complexes in a high-throughput manner provides insights into intracellular signaling and facilitates drug screening. In this study, we established high-sensitive and highly integrated approaches for high-throughput pTyr-mediated interactome and tyrosine phosphoproteome analysis. Importantly, these approaches were successfully employed for elucidating tyrosine kinase inhibitor (TKI)-mediated signaling and profiling the dynamic responses of TKI treatment with different dosages.

Firstly, we immobilized the Src SH2 superbinder-engineered trifunctional probe to a streptavidin-coated 96-well microplate to steric hindrance-free photoaffinity capture of pTyr-mediated protein complexes, selectively enrich the biotinylated proteins at denaturing conditions, and efficiently in-well digest them in a fully integrated manner. The immobilized microplates can be stored at 4 °C for up to 1 month. Furthermore, a high-flow nano-LC separation and variable windows-based data independent acquisition (DIA) MS analysis was developed to increase the analysis throughput.

Finally, the dynamic pTyr protein complexes with EGF stimulation at five-time points were systematically profiled with high reproducibility, sensitivity, and throughput.

We further developed a 96-well microplate-based approach for high-throughput pTyr peptide enrichment. In this approach, we enriched 21% more pTyr sites than the traditional approach from 96-well microplate one well cultured cell. Benefiting from the tight conjugation between the biotinylated Src SH2 superbinder and streptavidin-immobilized microplate, this new approach could well avoid non-specific adsorption and significantly increase pTyr peptide enrichment efficiency and specificity. This approach, therefore, accomplishes high-sensitive and high-reproducible pTyr enrichment in a high-throughput manner.

Then, we established a two-dimensional proximity proteomic strategy to achieve time-resolved profiling of signaling complexes and pTyr sites in living cells. Taking advantage of its fast-labeling speed and short labeling radius, APEX2- and BP5-based proximity labeling was adopted for time-resolved profiling of dynamic and transient protein complexes in living cells. Part of the peptides from proximity proteomic analysis was employed for further pTyr peptide enrichment and the temporal pTyr changes of protein complexes after EGF stimulation was characterized. By correlating the time-course curves of interacting proteins and pTyr sites, we found that the interacting proteins and pTyr sites classified into the same cluster have a close functional relationship. More importantly, low abundant pTyr peptides could be well quantified in PRM-MS analysis due to the higher sensitivity. Thereby, this strategy

offers a choice for pTyr-mediated signaling pathway elucidation with high reproducibility and sensitivity.

Finally, we applied these approaches for profiling drug targets and downstream signaling pathways upon defined TKI treatment. For large-scale drug screening, the targeted proteins of corresponding TKIs can be selectively profiled by the high-throughput photo-pTyr-scaffold approach with both MS and chemiluminescence analysis. The effect of different EGFR inhibitors on distinct cell lines can be well differentiated. More importantly, the distinct EGFR signaling response to different EGFR TKI treatments on the pTyr level could be well differentiated.

Collectively, we established new proteomic approaches for high-throughput, high-sensitive, and high-reproducible profiling of pTyr-mediated protein complexes and associated pTyr sites. This thesis work is therefore valuable for pTyr signaling pathway elucidation and related drug screening.