Supporting Information

An eco-friendly, low-cost, and automated strategy for phosphoproteome profiling

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Additional Experimental Section

Characterization

Scanning electron microscopy (SEM) and Energy Dispersive X-ray Spectrometry (EDX) images were obtained using an S-3400N-II scanning electron microscope (Hitachi, Japan). Fourier-transform infrared spectroscopy (FTIR) was performed on a Nicolet iS5 (Thermo, USA) using KBr pellets. Zeta potential measurements were performed on a Nano-ZS90 instrument (Malvern, UK) under acid condition (pH = 3.0) at 25 °C. For FTIR and zeta potential measurements, the monolith was ground into powder for preparation.

Enrichment ability test of the phos-trap column

The Enrichment ability of phos-trap column was tested by a simple manual connection of the syringe pump, sample loop, phos-trap column and 6-port valve. The syringe pump provided a constant boost. The 6-port valve and sample loop were used for manual injections. The end of phos-trap column was connected with an emitter inlet of the nano ESI for direct acquisition by nanoESI-MS. At a constant flow rate of 500 nL/min, the 20 mM $ZrCl_4$, sample buffer (protein digest dissolved in 80% ACN and 3% TFA), washing buffer (80% ACN and 3% TFA), and elution buffer (5% NH_4OH) were pushed through the phos-trap column in succession. The real-time mass spectrometry can show the effluent at different stages of peptide loading, washing, and elution.

Optimization and evaluation of the automated online platform

Standard protein digests were used for optimization and evaluation after the platform was built as described in the text. Due to the low complexity, the C18 analytical column, LC gradient, and MS2 were not used here. The source voltage was 1.8 kV. The temperature of the ion transfer tube was 320°C. The MS1 scans were acquired by the Orbitrap detector (m/z 600−2000, resolution 30K, RF lens 60%, AGC target 2.0E5, maximum ion injection time 100 ms). The background ion $(Si(CH_3)_2O)_6H^+$ of m/z = 445.1200 was used for internal calibration (lock mass).

1 M $NH_4H_2PO_4$, 50 mM NH_4HCO_3, and 100 mM $ATPNa_2$ were tested to elute the enriched phosphopeptides from the phos-trap column to a subsequent C18 pre-column for further RPLC-MS/MS analysis. In detail, the 20 mM $ZrCl_4$ solution, sample buffer (protein digest dissolved in 80% ACN and 3% TFA), washing buffer (80% ACN and 1% TFA) were injected and pushed through the phos-trap column to waste successively. Whereafter, the eluent was injected and pushed through the phos-trap column and C18 pre-column, with an extended 10 min of flush to remove salts. Finally, the adsorbates on the C18 pre-column were eluted by 50% ACN, 0.1% FA, and directly detected by the MS.

For the optimization of loading and washing conditions, a mixture of 100 ng α-casein digest and 10 μg BSA digest was used as the analyte for each run. Different loading and washing buffers, 50% ACN 1% TFA, 50% ACN 3% TFA, 80% ACN 1%

TFA, 80% ACN 3% TFA, 80% ACN 5% TFA, 80% ACN 3% TFA 200mg/mL DHB, and 80% ACN 3% TFA 1M LA were tested.

For the sensitivity and linear range assessment, different amounts of α-casein digest (0.2 ng, 2 ng, 20 ng, 50 ng, 100 ng, 150 ng, 200 ng) were analyzed in triplicate according to the order after a blank test. For the selectivity testing, 100 μg of α-casein digest mixed with different folds (1000, 500, 200) of BSA digest were analyzed in turn after a blank test.

Proteomic sample preparation

Tryptic digests of standard phosphoprotein (α-casein), standard non-phosphoprotein (BSA), HeLa cells, and maize seedlings were prepared by the following methods.

1 mg of α-casein was dissolved in 1 mL of 50 mM NH_4HCO_3, it was then incubated with trypsin (1/50, w/w) at 37 ℃ for 16 h. The digest was desalted by a desalting spin column. Samples were stored at −20 ℃ until use.

1 mg of bovine serum albumin (BSA) was dissolved in 0.12 mL of 50 mM NH_4HCO_3, 8 M urea and incubated with 10mM DTT for 30 min at 37 ℃, this was followed by alkylation with 40 mM iodoacetamide for 30 min in the dark. The solution was diluted to a volume of 1.0 mL with 50 mM NH_4HCO_3 and incubated with trypsin (1: 50, w/w) at 37 ℃ for 16 hours. The digest was desalted by a desalting spin column and stored at −20 ℃ until use.

HeLa cells were cultured to 90% confluence in 15 cm diameter dishes. After two

washes with ice-cold PBS buffer (pH 7.4), about 2×10^7 cells were suspended in 5 mL GdmCl lysis buffer (6M Guanidine hydrochloride, 100 mM Tris pH=8.5, 10 mM TCEP, 40 mM 2-Chloroacetamide) for 5 min at 95℃. [1] The obtained lysates were cooled on ice, sonicated, and precipitated overnight by incubating with four volumes of cold acetone at -20℃. Precipitated protein was collected by centrifugation (8000g, 4℃, 10min), washed with cold acetone, resuspended in 5mL of 50 mM NH_4HCO_3, and incubated with trypsin (1/100, w/w) at 37 ℃ for 16 h. The digest was acidified to pH ≤ 3 with TFA, desalted with peptide desalting spin columns, freeze-dried into powder and stored at −20 ℃ for further use.

The maize seedlings were ground into powders in liquid nitrogen by a mixer mill. 2 g of the powder was suspended in 10 mL of pre-cooled extraction buffer containing 0.1 M Tris-Cl (pH=8.0), 10 mM EDTA, 5 mM DTT, 0.9 M sucrose, protease and phosphatase inhibitors, followed by mixing with 10 mL of Tris-buffered phenol (pH=8.0). Proteins were extracted by sonication in an ice bath (10s on/10s off, 10 cycles). The phenol phase was isolated by centrifugation (8000g, 4℃, 20min), mixed with five volumes of pre-cooled methanol containing 0.1M ammonium acetate, and incubated at −20℃ for 3 hours. The precipitated protein was collected by centrifugation (8000g, 4℃, 20min), washed with cold methanol and acetone, followed by air drying for 10 min. The pellets were then resuspended in 50 mM NH_4HCO_3 and 8 M urea, incubated with 10mM DTT for 30 min at 37 ℃, followed by alkylation with 40 mM iodoacetamide for 30 min at room temperature in the dark. Before digestion, the

solution was diluted by 50 mM NH_4HCO_3 to reduce the urea concentration to 1 M. The protein concentration was measured using the BCA Protein Assay. The proteins were incubated with trypsin (1/100, w/w) at 37 ℃ for 16 h. The digest was acidified to pH ≤ 3 with TFA, desalted with peptide desalting spin columns, freeze-dried into powder and stored at −20 ℃ for further use.

Table S1. Performance comparison of the platform and recent offline methods.

Operation Mode	Materials	Detection Limit	Sample Type	Amount of Digest	Phospho-peptides	Phospho-sites	Multi-phosphopeptides	Specificity	Instrument	Source
Online	Zr-IMAC	10 fmol	HeLa cells	100 μg	5228	6575	64%	82%	Orbitrap Fusion	This work
Offline (centrifuge)	DMSNs@PDA-Ti^{4+}	40 fmol	HeLa cells	200 μg	2422	6689	41%	96%	Q Exactive	Ref[2]
	$B_{0.15}F_{0.15}TiO_2$	~200 fmol	liver cancer tissue	NA	116	223	69%	75%	NA	Ref[3]
	DZMOF–FDP	40 fmol	HeLa cells	200 μg	2669	~5800	70%	NA	Orbitrap Elite	Ref[4]
Offline (magnet)	Fe_3O_4@H-TiO_2@f-NiO	40 fmol	HeLa cells	NA	972	1477	45%	92%	Q Exactive	Ref[5]
	TiO_2NTs@Fe_3O_4NPs	NA	Jurkat T cells	500 μg	3541	4361	8%	80%	Q Exactive	Ref[6]
	mP5SOF-Arg	10 fmol	A594 cells	NA	450	~750	38%	83%	Orbitrap Fusion	Ref[7]
	Zr-IMAC	NA	HepG2/C3A cells	200 μg	5173	~6500	23%	97%	Q Exactive HF	Ref[8]
	Fe_3O_4@ZrO_2/TiO_2	200 fmol	HeLa cells	100 μg	1177	1260	12%	NA	Orbitrap Fusion	Ref[9]

Please note: the data in this table is for reference only. In actual experiments, the performance is affected by many other factors such as sample types, pretreatment methods, analytical column, analytical gradient, instrument, acquisition parameters, database, search software, parameters, etc.

Table S2. Summary information of the identification results by different methods

Method	Sample	Database source	Phosphoproteins/ Proteins (Master)	Phosphopeptides/ Peptides	Phosphorylated sites
Online Platform	HeLa Cell	Swiss-prot	1695/2020	5229/6469	6575
	Maize	EPSD	1423/2099	2926/6124	3530
	Maize	Uniprot	1383/2661	2678/7311	3272
Offline TiO_2	HeLa Cell	Swiss-prot	465/719	898/1148	1238

The data in the table corresponds to Figure 4

Supplemental Figure S1

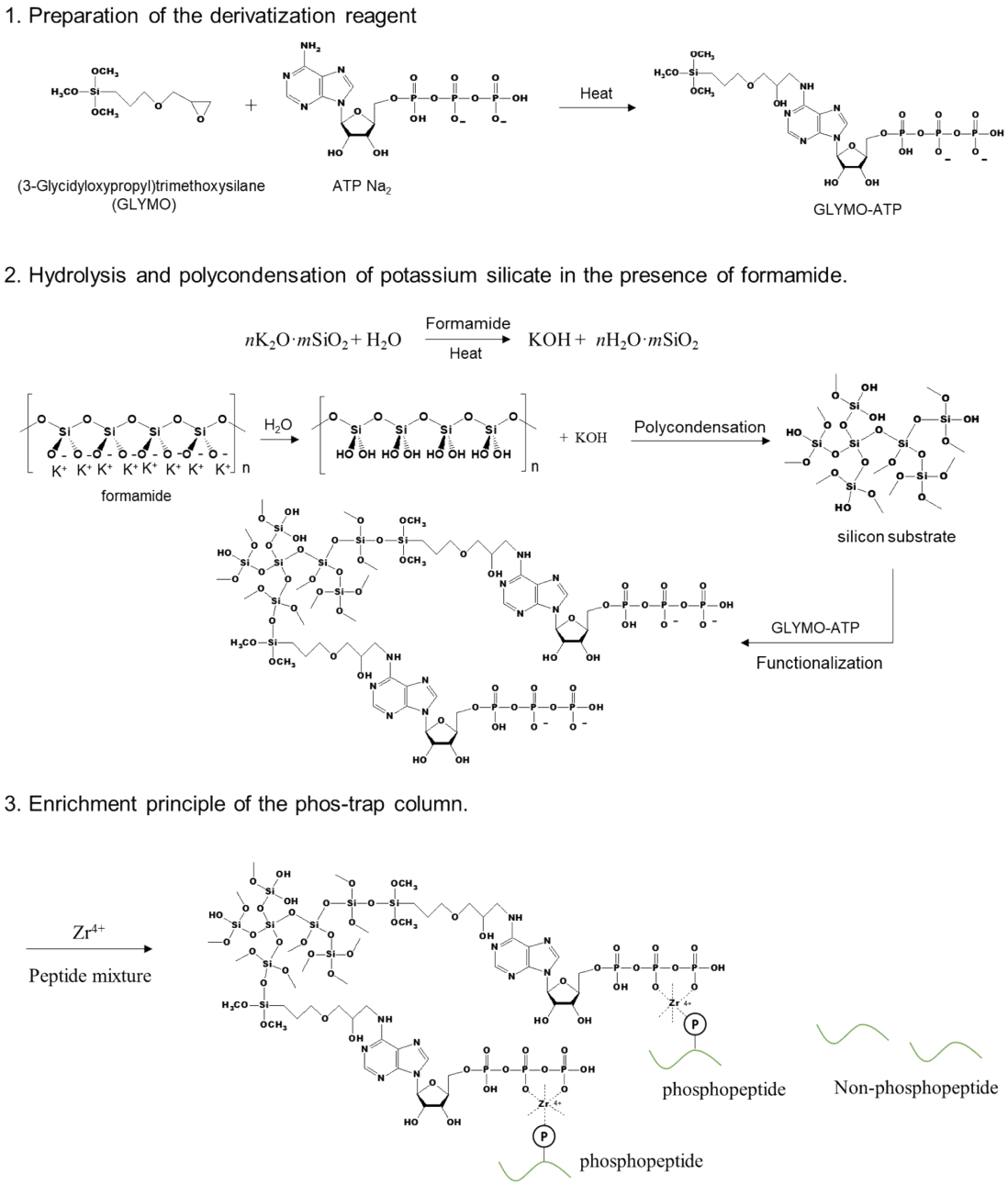


Figure S1. Fabrication process and enrichment mechanism of the phos-trap column.

Supplemental Figure S2

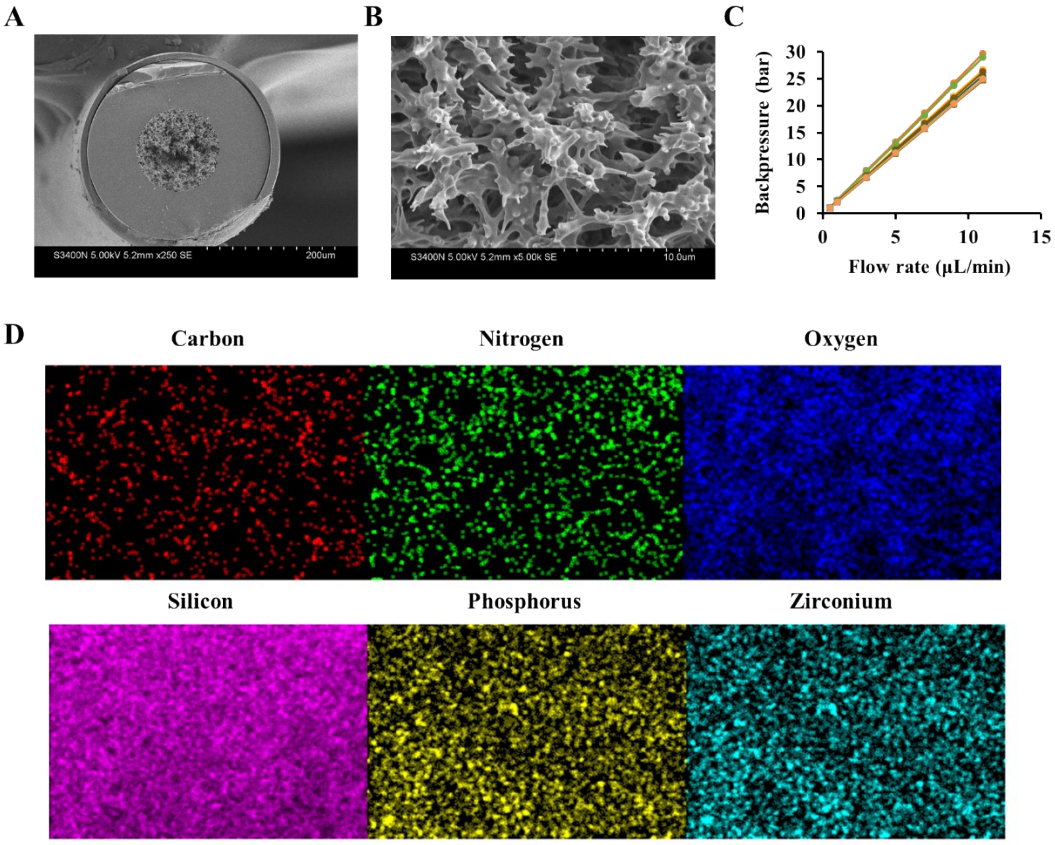


Figure S2. SEM and EDX characterization of a prepared phos-trap column. (A) SEM image of the column in the scale of 200 µm. (B) SEM image of the monolith in the scale of 10 µm. (C) Backpressure of phos-trap columns synthesized in the same batch at different flow rates. (D) EDX elemental mapping images of the monolith.

Supplemental Figure S3

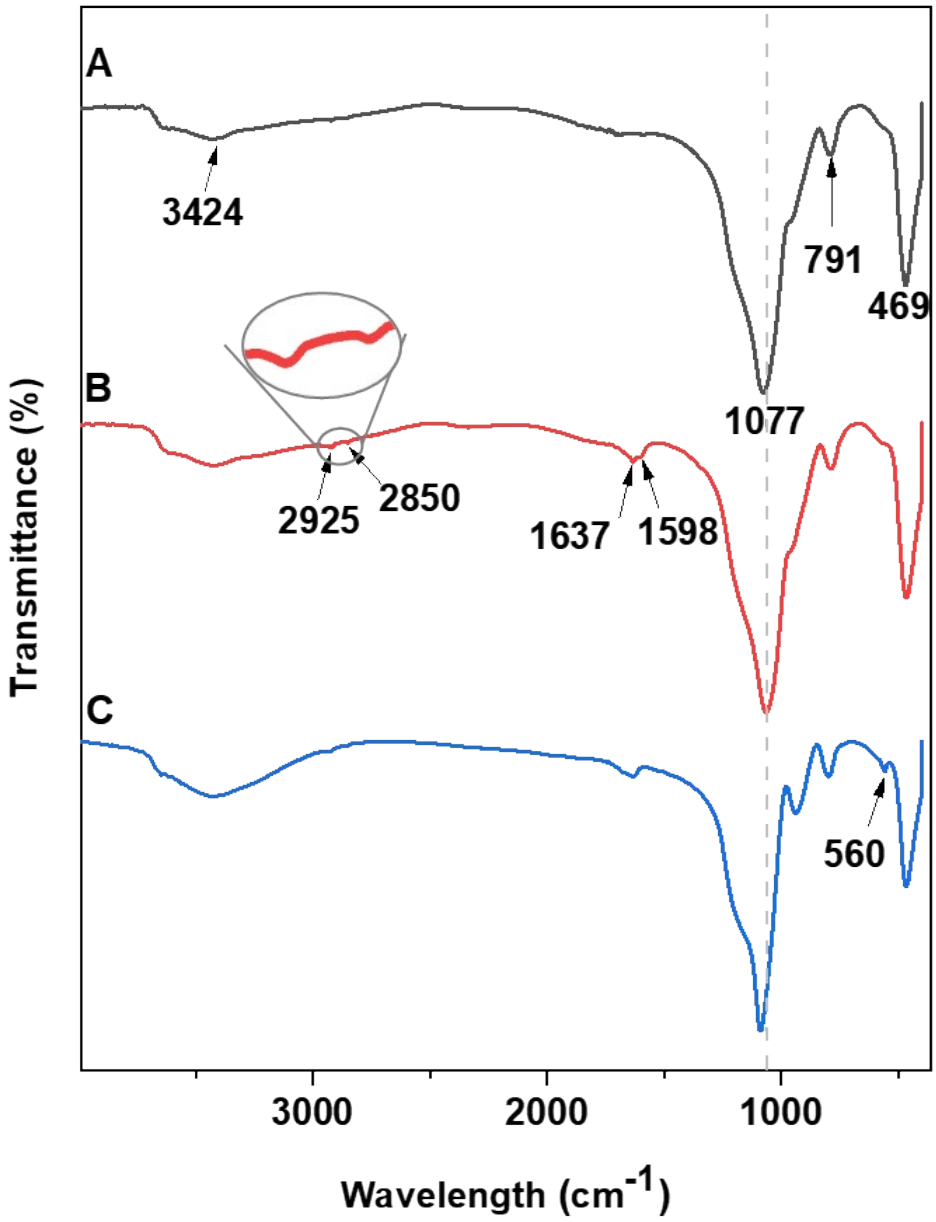


Figure S3. FTIR spectra of (A) Silica, (B) Silica−GLYMO−ATP, and (C) Silica−GLYMO−ATP−Zr^{4+}.

Supplemental Figure S4

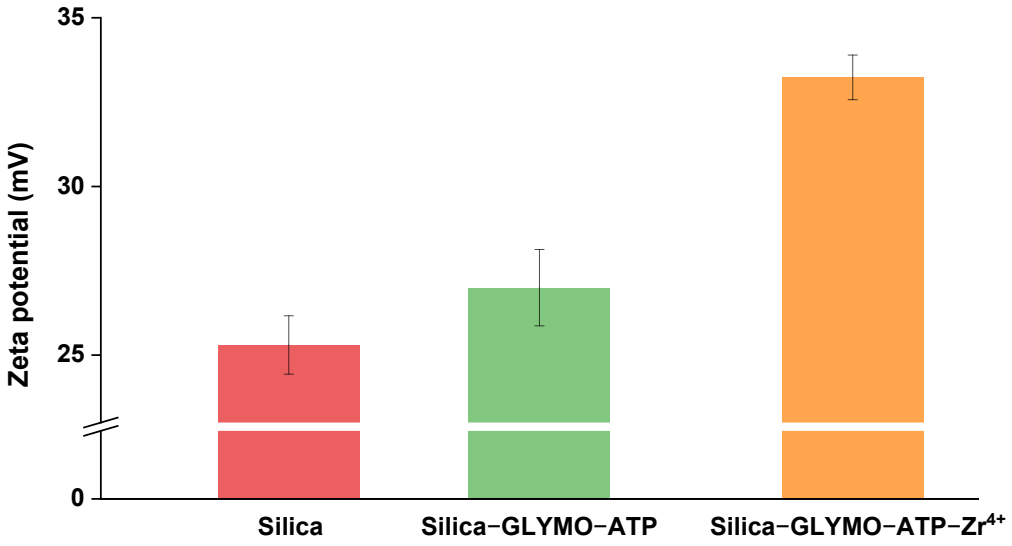


Figure S4. Zeta potential of Silica (25.3 mV), Silica−GLYMO−ATP (27.0 mV), and Silica−GLYMO−ATP−Zr^{4+} (33.2 mV).

Supplemental Figure S5

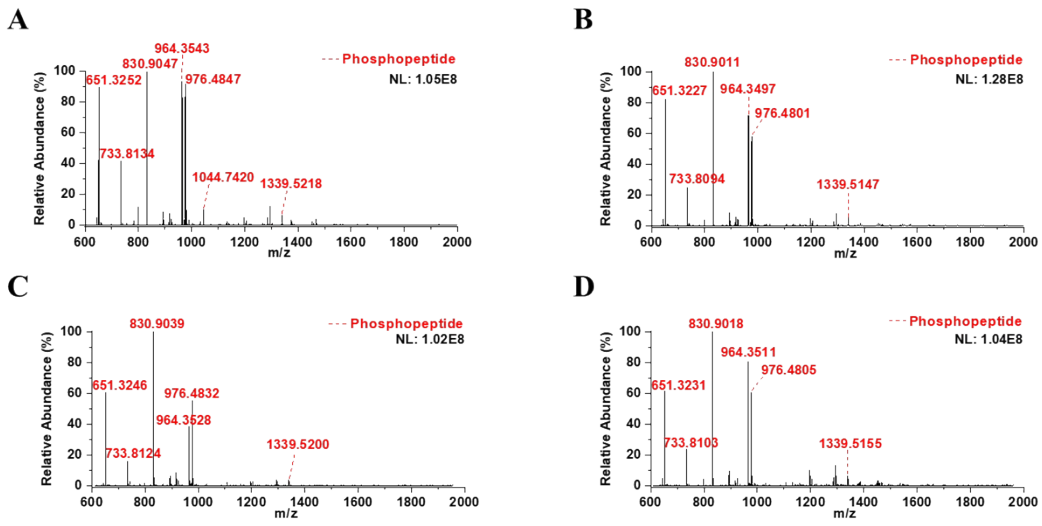


Figure S5. Recyclability test of a phos-trap column forenriching phosphopeptides from an α-casein digest. A phos-trap column was used for 50 consecutive cycles of phosphopeptide enrichment from an α-casein digest, followed by a two-month application using different complex biological samples. Mass spectra show the results at different time points: (A) the 1st, (B) the 50th cycle, (C) one month, and (D) two months after being applied to biological samples.

Supplemental Figure S6

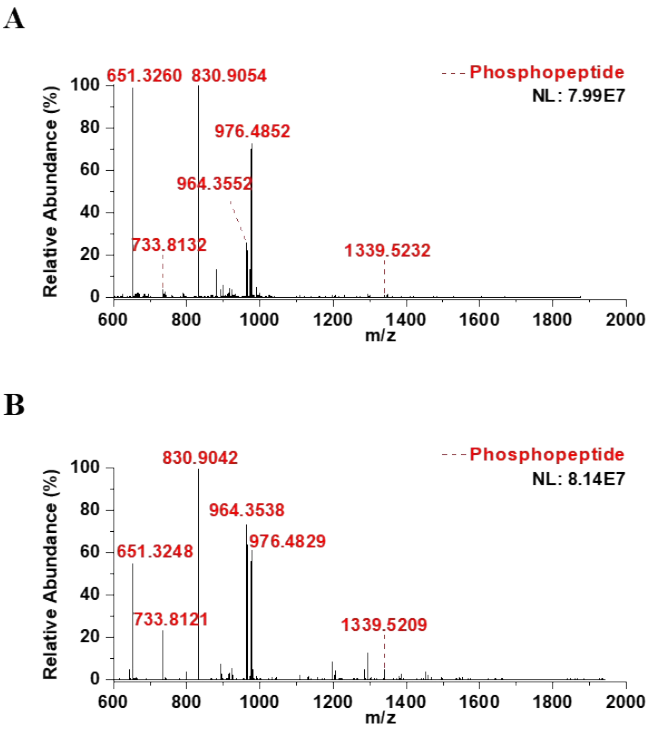


Figure S6. Shelf life test of the phos-trap columns. Mass spectra in (A) and (B) show the phosphopeptides enriched from an α-casein digest by two phos-trap columns of the same batch over a nine-month interval.

Supplemental Figure S7

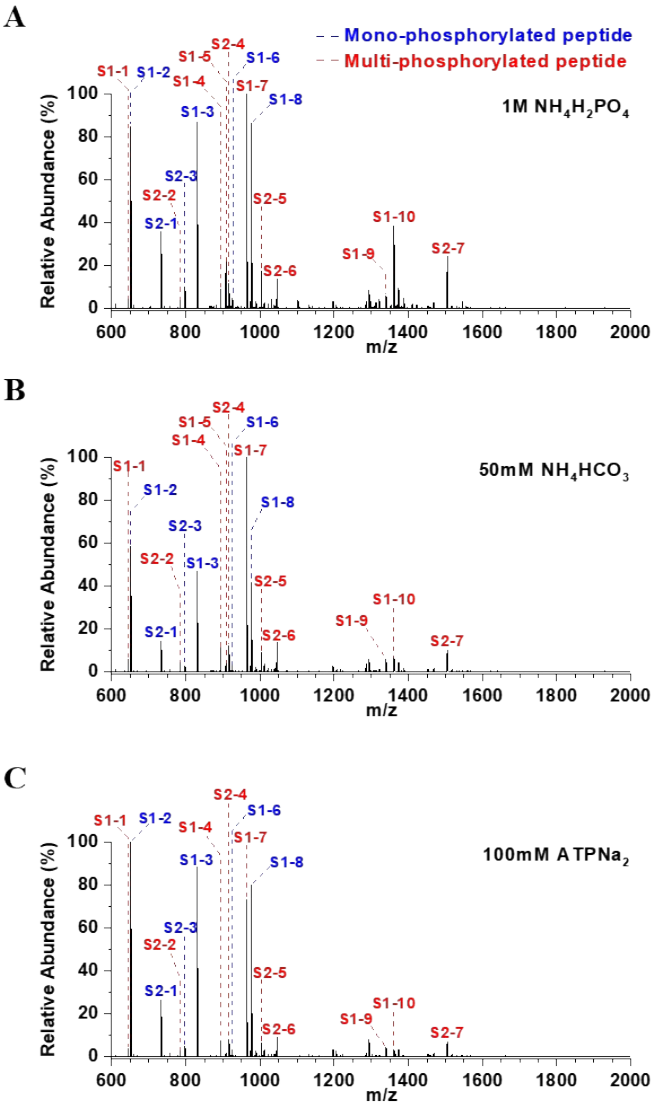


Figure S7. The screening of a feasible eluent for online phosphoproteomics. 1 M $NH_4H_2PO_4$, 50 mM NH_4HCO_3 and 100mM $ATPNa_2$ are candidates. # The mono- and multi-phosphorylated peptides are shown in blue and red respectively, with detailed qualitative information listed in Table 1.

Supplemental Figure S8

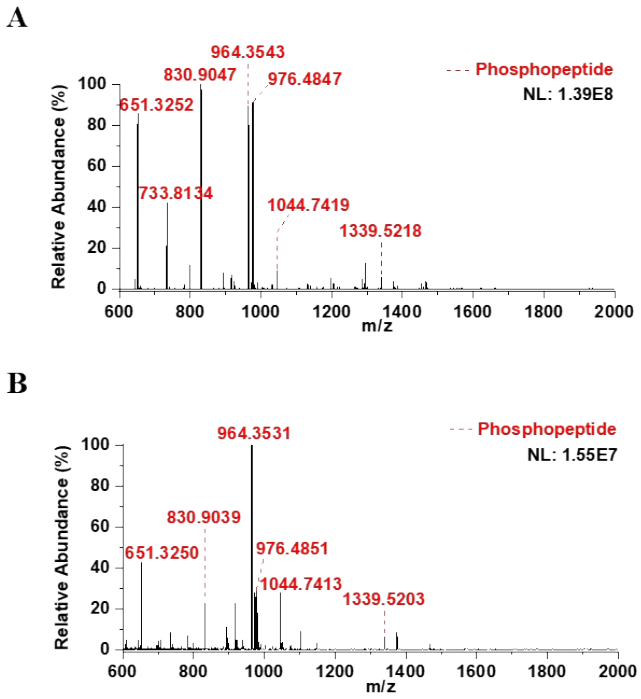


Figure S8. The efficiency test of 1 M $NH_4H_2PO_4$ as the eluent. (A) Mass spectrum of the eluate by the first elution, showing the intensity of 1.39E8. (B) Mass spectrum of the eluate by the second elution, showing the intensity of 1.55E7. The efficiency of 1 M $NH_4H_2PO_4$ can be calculated as 90%.

Supplemental Figure S9

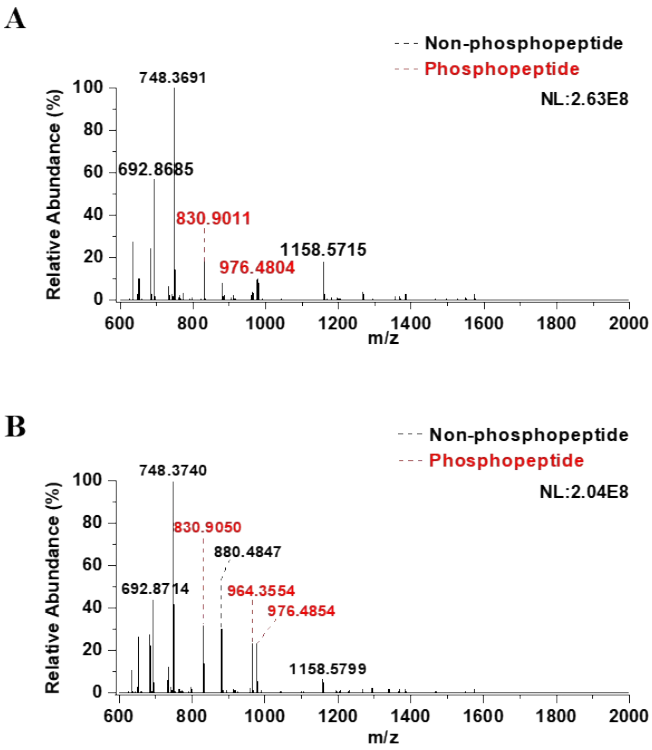


Figure S9. The interference test of 1 M $NH_4H_2PO_4$ for peptides loading on the C18 pre-column. The α-casein digest dissolved in different buffers (A) 0.1% FA and (B) 1 M $NH_4H_2PO_4$ was loaded on the C18 pre-column and detected by MS after desalting. There is no apparent difference in the intensity of phosphopeptides, especially, the phosphopeptides, indicating the 1 M $NH_4H_2PO_4$ has no interference of peptides loading on the C18 pre-column.

Supplemental Figure S10

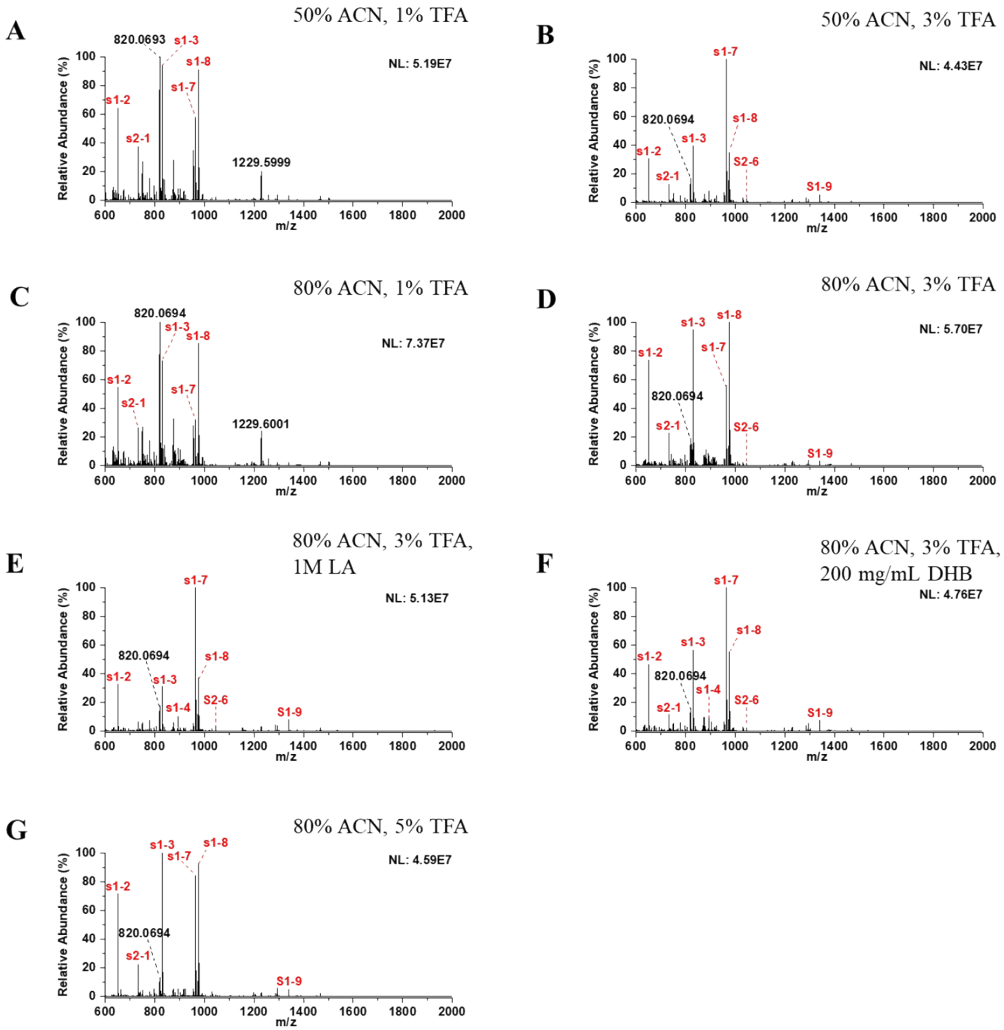


Figure S10. Optimization of the loading and washing conditions for the platform. The mass spectra are the platform analyzing a mixture of α-casein and BSA digest (1:100, w/w) under different loading and washing conditions. The phosphopeptides are shown in red, with detailed information listed in Table 1. A non-phosphopeptide (m/z 820.07, and 1229.60, corresponding to different charge states of the same peptide, DAIPENLPPLTADFAEDKDVCK) possessing strong nonspecific adsorption due to its six acidic residues can be used as a reference for selectivity assessment in the spectra.

Supplemental Figure S11

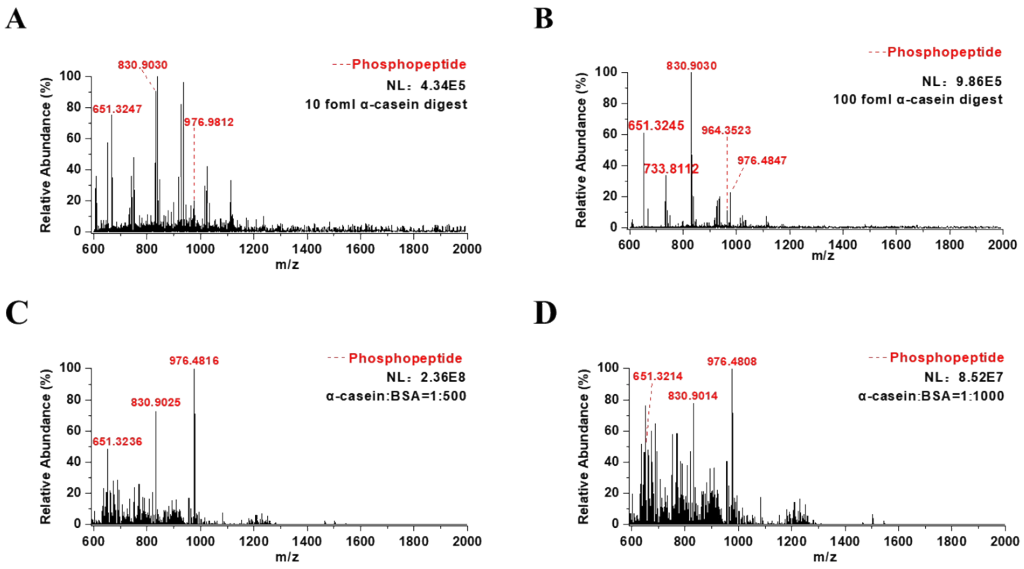


Figure S11. (A, B) Detect limit and (C, D) selectivity tests of the platform.

Supplemental Figure S12

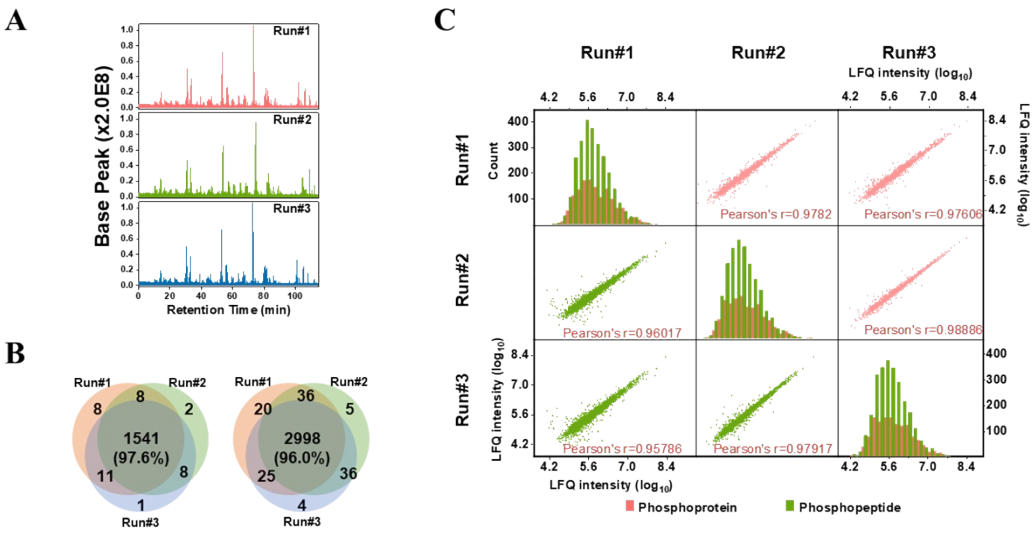
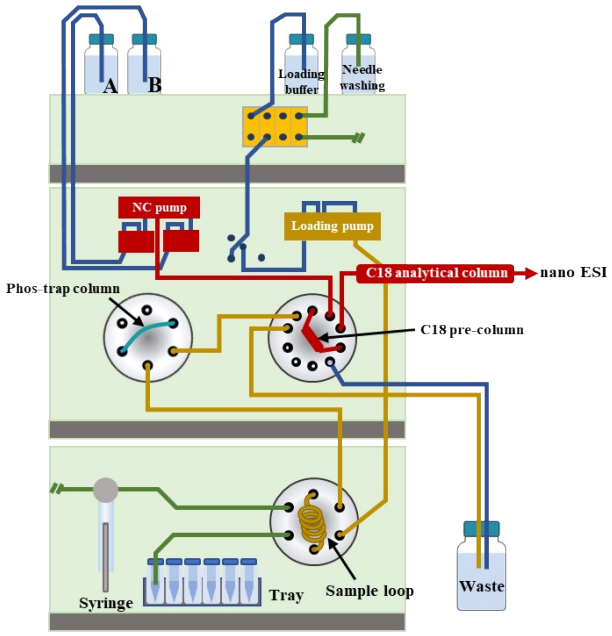


Figure S12. The reproducibility performance of the online platform for maize samples. (A) Base peak comparison of the three parallel runs. (B) Two Venn diagrams of identified phosphoproteins and phosphopeptides in maize by three parallel runs, respectively. (C) Scatter plots of abundances of the phosphoproteins (upper-right) and phosphopeptides (lower-left) in $\log_{10}$ scale between replicates, and histograms of the abundances in $\log_{10}$ scale of each replicate (middle line).

Supplemental Figure S13

A



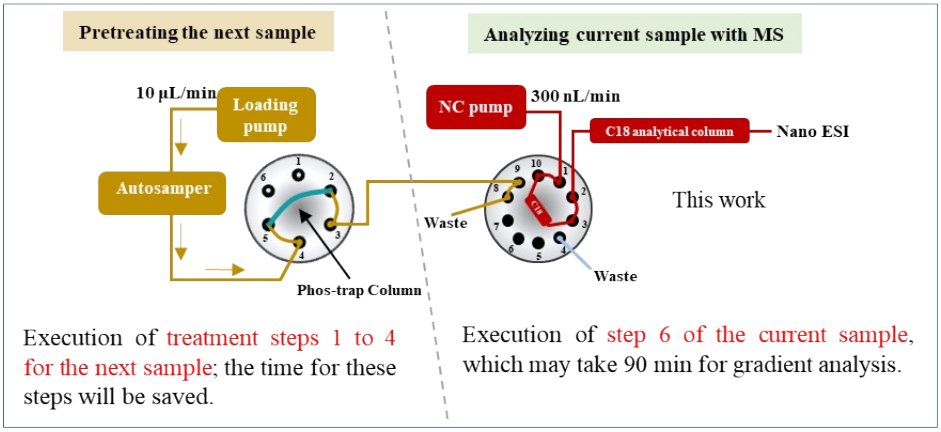
B

Steps involved in the automated analysis by this platform

Step	Task	Autosamper injection	Left Valve Position	Right Valve Position	Time-consuming
1	Regeneration	50μL of (40% ACN, 5% NH_4OH)	1-6	1-10	8 min
2	Loading Zr^{4+}	40 μL of 20 mM $ZrCl_4$	1-6	1-10	8 min
3	Loading peptides	50 μL of peptides	1-6	1-10	8 min
4	Washing 3 times	125 μL of (80% ACN, 3% TFA)	1-6	1-10	45min
5	Elution	50 μL of 1M $NH_4H_2PO_4$	1-6 (0-10 min) 1-2 (10-15 min)	1-2	15 min
6	Gradient analysis		1-6	1-10	90min

C

The following fluidic design allows the platform to enrich the next sample while analyzing the current one, therefore, steps 1-4 would not require additional time:

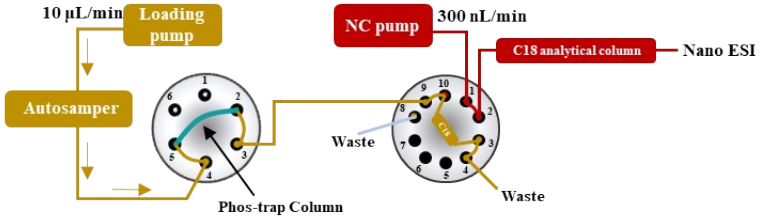


#The two parts can work independently without interference.

This design allowing online elution and fast desalting in step 5 (15 min):

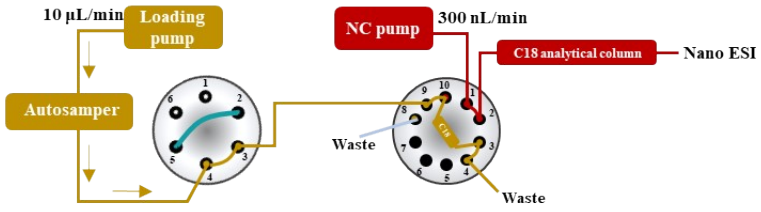
Steps 5

0-10 min:



The enriched phosphopeptides is eluted from the phos-trap column to a subsequent C18 pre-column.

10-15 min:



The remaining salt on C18 pre-column is quickly removed.

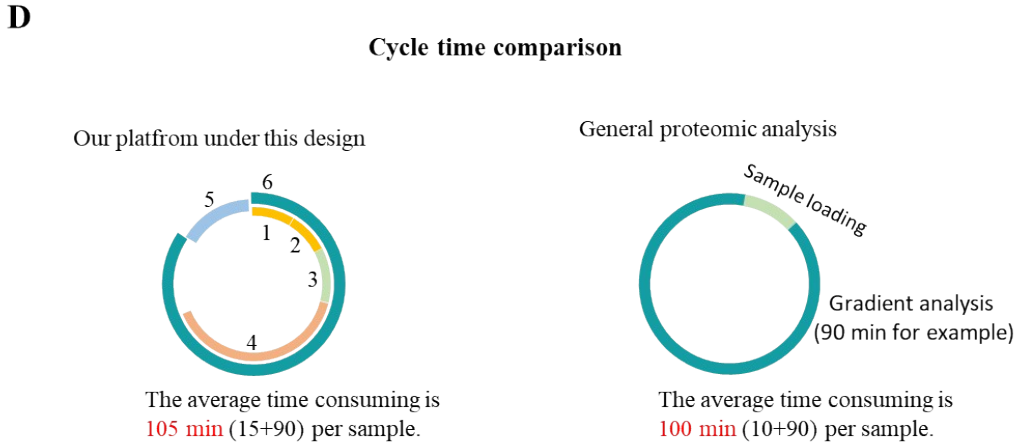


Figure S13. Next-generation design for simultaneous enrichment and gradient LC-MS analysis. (A) In the new fluid connection design, there is a separate 6-port valve controlling the connection of the phos-tarp, helping to reduce desalting time in step 5. (B) Steps involved in a complete analysis. (C) Details about achieving simultaneous enrichment and gradient LC-MS analysis. (D) Comparison of cycle times between this platform and conventional proteomic analysis.

Supplemental Figure S14

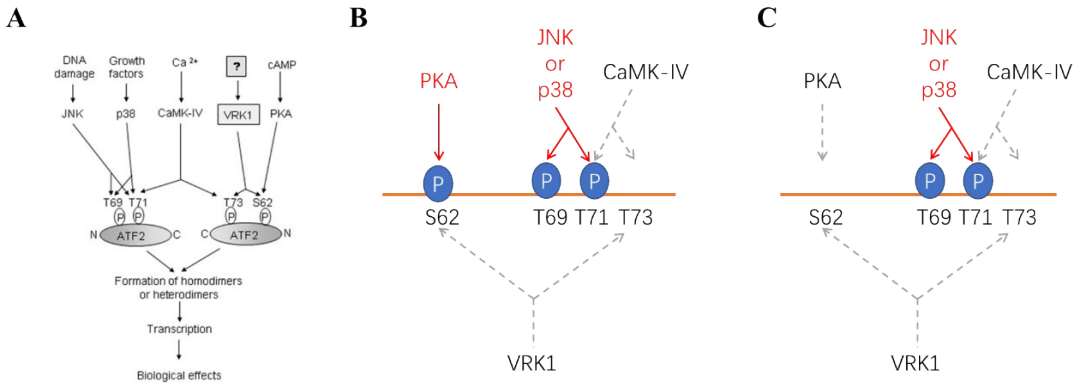


Figure S14. Prediction of the kinases involved in phosphorylation of ATF2 by peptides in different phosphorylation states. (A) Activation of ATF2 by different Ser-Thr kinases summarized in the literature.[10] (B) The peptides (position 60-74) phosphorylated on S62, T69, and T71 means PKA, JNK/p38 are involved in the phosphorylation of a part of ATF2. (C) The peptides (position 60-74) phosphorylated on T69, and T71 suggest only JNK/p38 is involved in the phosphorylation of some other ATF2.

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