Supporting Information

Xue et al. 10.1073/pnas.1119418109

SI Materials and Methods

Cell Culture and Sample Preparation. DG-75 cells (ATCC) were grown to 50% confluency in RPMI medium 1640 supplemented with 10% heat inactivated FBS, 1 mM sodium pyruvate, 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.05 mM 2-mercaptoethanol. The cells were washed with PBS, collected, and frozen at −80 °C for further use. To specifically inhibit Syk activity, cells were incubated with 100 μg/mL piceatannol in 37 °C for 30 min. Cells were lysed in lysis buffer containing 50 mM Tris•HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 1 mM sodium orthovanadate, 1× phosphatase inhibitor cocktail (Sigma), and 10 mM sodium fluoride for 20 min on ice. The cell debris was cleared by centrifugation at 16,000 × *g* for 10 min. The supernatant containing soluble proteins was collected. MDA-MB-231 (Syk-negative) and tetracycline-inducible MDA-MB-231 cells were generated and cultured as described (1). Syk-inducible cells were incubated with 1 μg/mL of tetracycline for 20 h at 37 °C to induce the expression of Syk-EGFP. Both sets of cells were then lightly stimulated with 20 μM sodium pervanadate for 15 min at 4 °C. Cells were collected, washed, and lysed as described above.

Peptide Preparation. Proteins in cell lysates were denatured in 0.1% RapiGest (Waters) and reduced with 5 mM dithiothreitol for 30 min at 50 °C. Proteins were alkylated in 15 mM iodoacetamide for 1 h in the dark at room temperature and then digested with proteomics grade trypsin at a 1 : 100 ratio overnight at 37 °C. The pH was adjusted below 3 and the sample was incubated for 45 min at 37 °C. The sample was centrifuged at 16,000 × *g* to remove RapiGest. The supernatant was collected.

Enrichment of Phosphotyrosine-Containing Peptides. The sample was adjusted to pH 7.5 with 100 mM Tris•HCl buffer (pH 8.0). Anti-phosphotyrosine antibodies (4G10, PT66, and PY20) conjugated to agarose beads were added and incubated overnight at 4 °C with agitation. The beads were washed twice with 500 μL of the lysis buffer and twice with water. Phosphopeptides were eluted with 100 μLof 0.1% TFA with 10 min of vigorous agitation and twice more with 100 μL of 0.1% TFA in 50% acetonitrile (10 min each). All eluents were combined and dried under vacuum using a Speedvac.

Kinase Reactions. Samples of phosphopeptides were resuspended in 200 μL of phosphatase buffer (Roche). Two units of phosphatase were added and incubated at 37 °C for 1 h. The phosphatase was deactivated by heating at 75 °C for 5 min. Samples were incubated in buffer containing 300 ng Syk (Sigma), 5 mM $MgCl_2$, and 1 mM ATP at 30 °C for 30 min. Reactions were quenched by the addition of 1% TFA to a pH below 3.

Phosphopeptide enrichment Using Polymer-Based Metal Ion Affinity Capture (PolyMAC) Reagent. The rephosphorylated samples were desalted using a C18 Nu-tip (Glygen) and dried. The peptide mixture was resuspended in 100 μL of loading buffer (100 mM glycolic acid, 1% trifluroacetic acid, 50% acetonitrile) to which 5 nmol of the PolyMAC-Ti reagent was added (2). The mixture was incubated for 5 min; 200 μL of 300 mM Hepes, pH 7.7, was added to the mixture to achieve a final pH of 6.3. The solution was transferred to a spin column (Boca Scientific) containing Affi-Gel Hydrazide beads (BioRad) to recover the polyMAC-Ti dendrimers. The column was gently agitated for 10 min and then centrifuged at 2,300 × *g* for 30 s to collect the unbound flow-through. The gel was washed once with 200 μL loading buffer, twice with 100 mM acetic acid, 1% trifluoroacetic acid, 80% acetonitrile, and once with water. The phosphopeptides were eluted from the dendrimers by incubating the gel twice with 100 μL of 400 mM ammonium hydroxide for 5 min. The eluates were collected and dried under vacuum.

Mass Spectrometric Data Acquisition and Data Analysis. Peptide samples were dissolved in 8 μL of 0.1% formic acid and injected into an Eksigent NanoLC Ultra 2D high-pressure liquid chromatography (HPLC) system. The reverse phase C18 was performed using an inhouse C18 capillary column packed with 5 μm C18 Magic beads resin (Michrom; 75 μm i.d. and 12 cm of bed length). The mobile phase buffer consisted of 0.1% formic acid in ultrapure water with an eluting buffer of 0.1% formic acid (buffer A) in 100% CH_3CN (buffer B) run over a shallow linear gradient over 90 min with a flow rate of 300 nL/ min. The electrospray ionization emitter tip was generated on the prepacked column with a laser puller (Model P-2000; Sutter Instrument Co.). The Eksigent Ultra2D HPLC system was coupled online with a high-resolution hybrid duel-cell linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap Velos; Thermo Fisher). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS (from m/z 300–1,700 with the resolution of 60,000 at m/z 400) was followed by 20 MS/MS scans of the most abundant ions. Ions with charge state of +1 were excluded. The mass exclusion time was 90 s. All mass spec analyses were performed with at least two biological replicates and data were combined.

The LTQ-Orbitrap raw files were searched directly against *Homo sapiens* database with no redundant entries (67,250 entries; human International Protein Index v.3.64) using SEQUEST algorithm on Proteome Discoverer (Version 1.2; Thermo Fisher). Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included a static modification of cysteine residues of +57.0214 Da and a variable modifications of +15.9949 Da to include potential oxidation of methionines and a modification of +79.996 Da on serine, threonine, or tyrosine for the identification of sites of phosphorylation. Searches were performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. False discovery rates (FDR) were set for 1% for each analysis. Proteome Discoverer generates a reverse "decoy" database from the same protein database, and any peptide passing the initial filtering parameters that were derived from this decoy database is defined as a false-positive. The minimum cross-correlation factor (Xcorr) filter was readjusted for each individual charge state separately in order to optimally meet the predetermined target FDR of 1% based on the number of random false-positive matches from the reversed decoy database. Thus, each dataset had its own passing parameters. The number of unique phosphopeptides and nonphosphopeptides identified were then manually counted and compared. Phosphorylation site localization from collision-induced dissociation mass spectra was determined by Sequest Xcorr scores and only one phosphorylation site was counted using the top-scored phosphopeptide for any phosphopeptide with potentially ambiguous phosphorylation sites.

Immunoprecipitation and Western Blotting Experiments. Cells were collected and lysed in lysis buffer containing protease and phosphatase inhibitors [50 mM Tris•HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1× phos-

phatase inhibitor cocktail (Sigma), 10 mM sodium fluoride, 1× Mini Complete protease inhibitor cocktail (Roche)] for 20 min on ice. Samples were cleared of debris and normalized based on protein concentration. One milligram of total lysate was used for immunoprecipitation experiments. One milligram of lysate was preincubated with 20 μL protein A/G agarose beads (Thermo Fisher Scientific) for 20 min at 4 °C to remove nonspecific binding. Then the lysates were incubated with 2 μg of the selected antibody [anti-microtubule-associated protein RP/EB family member 1 (MAPRE1), anti-nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS), anti-POU domain class 2-associating factor 1 (BOB1), anti-hepatocellular carcinoma-associated antigen 59 (HCA59), or anti-methylosomesubunit pICln (CLNS1A)] for 30 min at 4 °C. Finally, the samples were incubated with 20 μL of protein A/G agarose beads for 45 min at 4 °C. The beads were washed and the bound proteins were eluted by boiling the beads in SDS loading buffer with 50 mM DTT for 5 min. The eluents were separated on a 12% SDS-polyacrlyamide gel and transferred onto a PVDF membrane. The membranes were probed using antibodies against the proteins of interest. Then the membranes were stripped and reprobed using the 4G10 antiphosphotyrosine antibody.

Cloning, Purification, and in Vitro Kinase Assay of Nek9 Full Length, Truncated, and Mutant. To construct vectors for Nek9 protein fragments for overexpression, Nek9 DNA truncated (Nek9-t) and full length (Nek9-fl) were amplified by PCR from Nek9 cDNA (Thermofisher). The amplified DNA fragments, after digestion with SalI and HindIII, were then cloned into the same restriction sites on pGEM-KG vector for the target vectors. cDNA for the expression of site-directed mutants were generated using the transformer mutagenesis kit (Clontech). Primers used in PCR are as followed:

Nek9-t fwd: ATGCGTCGACAACTTCTAGATCGCCCTC TTCTCAGG.

Nek9-t rev: ATGCAAGCTTCTACCGAGGCCATGAGGTA CAGATATC.

To induce and overexpress Nek9 protein fragments, *Escherichia coli* BL21 (DE3) cells harboring a plasmid containing pGST-Nek9 were grown at 37 °C in 2YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter) and induced with 0.4 mM IPTG when the culture had reached an optical density at 550 nm of 0.6–0.8; the cells were harvested 4 h after IPTG induction by centrifugation at 4,000 × *g* for 15 min and then homogenized by sonication followed by centrifugation at 12,000 × *g* for 10 min.

GST-Nek9 and GST-Nek9 mutants (truncation and Y520Y521 mutants) were isolated from lysates by adsorption to glutathione-Sepharose. GST was purified from bacteria transformed with the GST-expressing vector pGEX-4T2. B cells were lysed in 50 mM Tris•HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM NaVO3, and 1× protease inhibitor cocktail (Roche). The lysates were centrifuged at 14,000 × *g* for 1 min.

For kinase reaction in vitro, GST-Nek9 or mutants were incubated with or without purified GST-Syk (Sigma) in 25 mM Hepes (pH 7.5), 2 mM MnCl2, 1 mM Na3VO4, 1× protease inhibitor cocktail, and 100 μM ATP, at 30 °C for 30 min. All kinase assay reactions were stopped with 2× SDS loading buffer and boiled at 99 °C for 5 min. Proteins were separated by 12% or 4–12% gradient SDS-PAGE and transferred onto polyvinylidene difluoride membranes, blocked with 1% BSA, and incubated with antipY (4G10, Millipore; all other were from Santa Cruz Biotechnology) primary antibodies and detected using ECL detection reagents (GE Healthscience).

1. Zhang X, Shrikhande U, Alicie BM, Zhou Q, Geahlen RL (2009) Role of the protein tyrosine kinase Syk in regulating cell-cell adhesion and motility in breast cancer cells. *Mol Cancer Res* 7:634–644

2. Iliuk AB, Martin VA, Alicie BM, Geahlen RL, Tao WA (2010) In-depth analyses of kinasedependent tyrosine phosphoproteomes based on metal ion functionalized soluble nanopolymers. *Mol Cell Proteomics* 9:2162–2172.

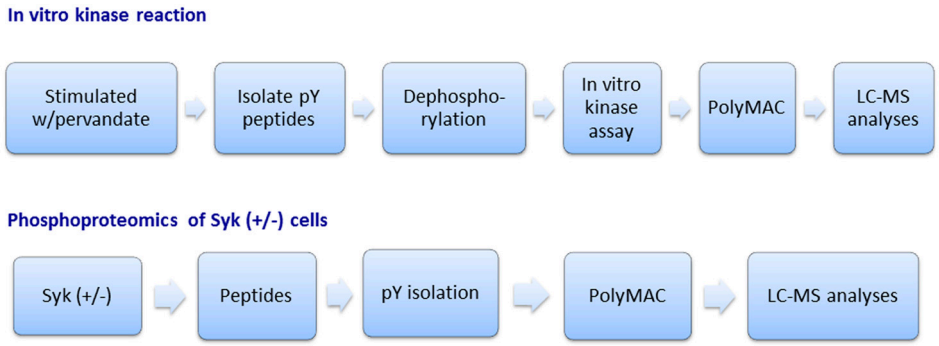
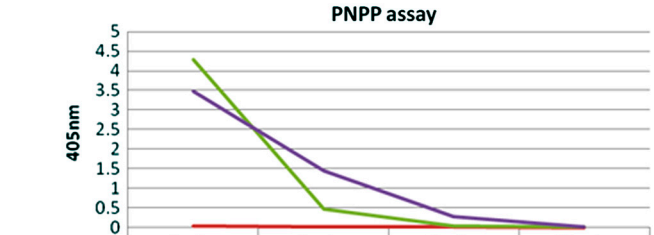


Fig. S1. Flowchart for the identification of phosphopeptides after in vitro kinase assay and endogenous phosphoproteomics. LC, liquid chromatography.



	0 min	2 min	5 min	15 min
Promega	0.042	0.018	0.002	0
Roche	4.293	0.474	0.031	0.005
Sigma	3.476	1.446	0.276	0.015

Fig. S2. Comparisons of phosphatase activity using *p*-nitrophenyl phosphate (PNPP) assay. Rate of hydrolysis of substrate is plotted against increasing phosphatase incubation time and readings were taken on a microtiter plate spectrophotometer (405 nm). Thermosensitive Alkaline Phosphatase (TSAP) from Promega, 100 units (1 molecular biology unit/μL) M9910 (red); rAPid Alkaline Phosphatase from Roche, 1,000 units 1 unit/μL, 04898133001 (green). Calf intestinal alkaline phosphatase from Sigma Aldrich, 10 units/μL, P4978 (purple). The curve indicates phosphatases from Roche and Sigma have higher activity than Promega's. Phosphatases from Roche and Promega can be efficiently deactivated after 5 min heating, whereas the Sigma's needs longer heating time to be deactivated.

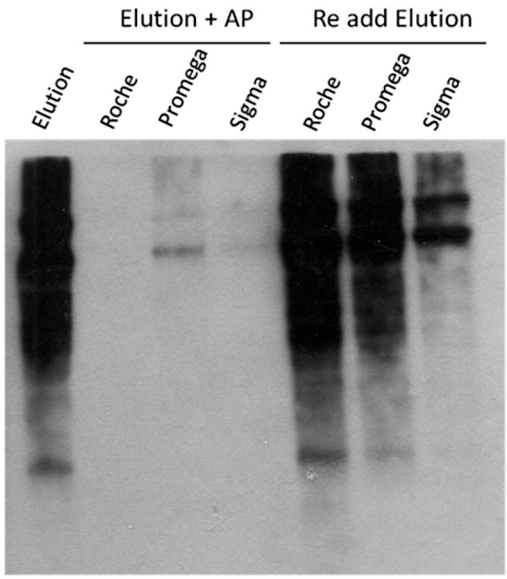


Fig. S3. Western blotting to monitor the phosphatase activity in complex biological sample. Three commercial phosphatases (as Fig. S2 mentioned) were added to PT66 enriched DG75 lysates and incubated at 37 °C for 2 h to see dephosphorylation activity (lanes: 1, pTyr containing proteins; 2–4, pTyr proteins incubated with phosphatases). All samples were heated for 5 min to deactivate phosphatase. The residual phosphatase activity was examined by readding PT66-enriched pY phosphoproteins. (Lanes: 5–7, pTyr proteins incubated with deactivated phosphatases.) The anti-pY Western blotting shows that phosphatases from Roche and Sigma have higher dephosphorylation efficiency than Promega's. On the other hand, only the phosphatases from Roche and Promega can be efficiently deactivated after heating.

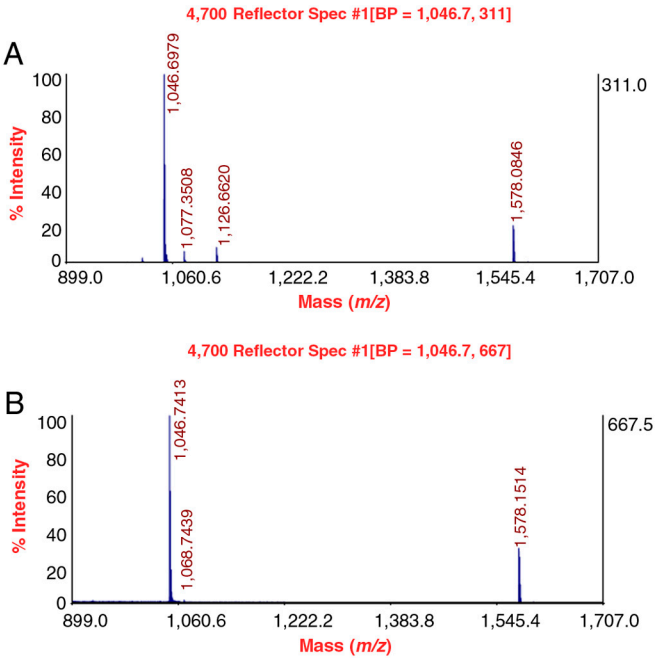


Fig. S4. Dephosphorylation of phosphopeptides by alkaline phosphatase. A peptide mixture consisting equal amount of angiotensin II (*m/z* 1,046) and phosphoangiotensin II (*m/z* 1,126) was analyzed by MALDI-TOF-MS at zero time (*A*) or after 30 min (*B*) incubation with rAPid Alkaline Phosphatase from Roche. Reactions containing peptide mixture in Roche phosphatase buffer were initiated by the addition of 1 unit of phosphatase. At the time indicated, aliquots were removed, diluted 10-fold with 0.1% TFA, prior to mixing 1:1 with DHB (2,5-dihydroxybenzoic acid) matrix in 50% acetonitrile containing 2% phosphoric acid. Aliquots (0.3 μL) were spotted onto the MALDI plate and spectra were acquired. Note the decrease in mass of 80 units corresponding to single site dephosphorylation. The higher ratio of angiotensin II (*m/z* 1,046) versus phosphoangiotensin II (*m/z* 1,126) after incubation showed virtually complete dephosphorylation. BP, base peak.

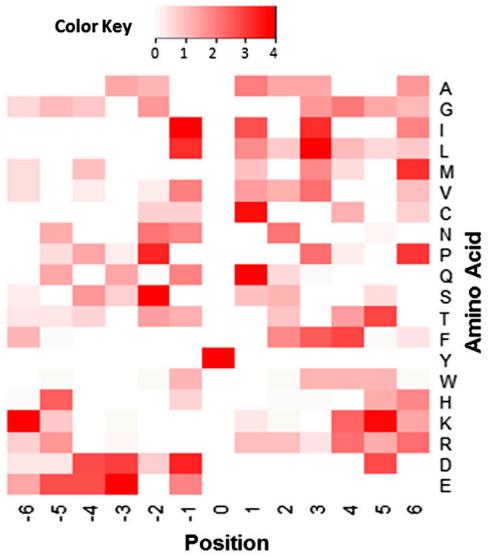


Fig. S5. Consensus motif analysis of cellular-Src kinase in vitro substrates. Phosphorylation sites were examined for the relative enrichment (red) or depletion (white) of amino acids flanking the phosphorylation site. The amino acid residue distribution (derived by application of Pearson's χ^2 test) is depicted by the heat map.

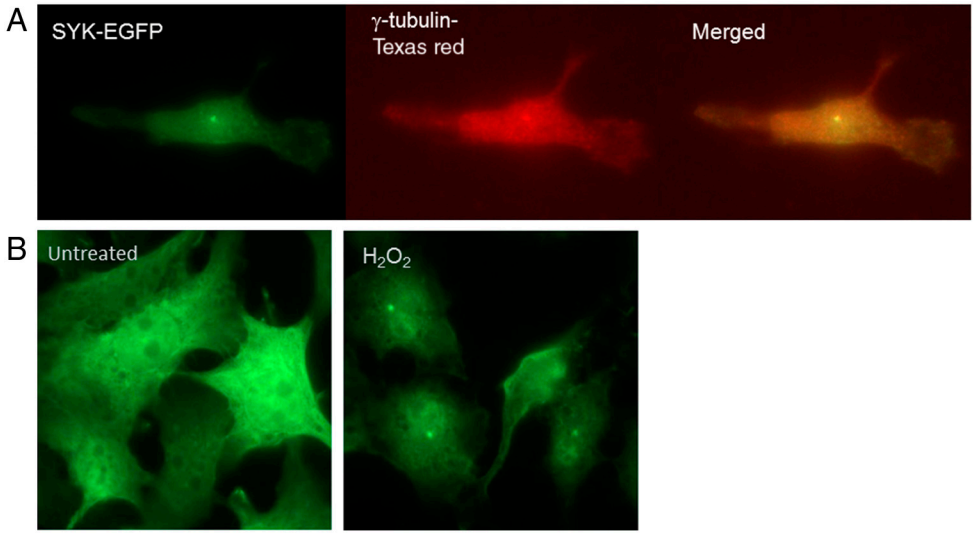


Fig. S6. (*A*) Centrosomal colocalization of GFP fused Syk with γ-tubulin. (*B*) The subcellular location of GFP-Syk fusion protein under oxidation stress.

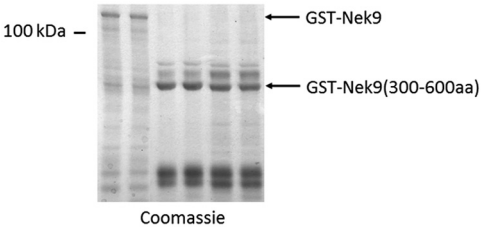


Fig. S7. Coomassie staining of in vitro kinase assays containing GST-Nek9, Nek9 truncations, or Nek9-Y520IY521D mutant, with or without GST-Syk.

Other Supporting Information Files.

Table S1 (DOCX)
Table S2 (DOCX)
Dataset S1 (XLSX)