PNAS
www.pnas.org

Supplementary Information for

Phosphorylation-mediated activation of mouse Xkr8 scramblase for phosphatidylserine exposure

Takaharu Sakuragi, Hidetaka Kosako, and Shigekazu Nagata

Shigekazu Nagata
Email: snagata@ifrec.osaka-u.ac.jp

This PDF file includes:

Supplementary text
Figs. S1 to S3
References for SI reference citations

www.pnas.org/cgi/doi/10.1073/pnas.1820499116

Extended Materials and Methods

Cell lines, recombinant proteins, and materials. The mouse pro-B cell line, Ba/F3 (1), was grown in RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco) and 45 units/ml mouse IL-3. Mouse WR19L cells expressing Fas (W3) (2) and human PLB985 cells (PLB) were cultured in RPMI containing 10% FCS and 50 μM β-mercaptoethanol. Human HEK293T cells were cultured in DMEM containing 10% FCS. The *TMEM16F*$^{-/-}$Ba/F3 and *ATP11A*$^{-/-}$*ATP11C*$^{-/-}$*TMEM16F*$^{-/-}$W3 (*TKO*-W3) cell lines were described previously (3, 4). Recombinant IL-3 was prepared as described previously (5). Staurosporine (STS) was a gift from Dr. Satoshi Omura (Kitasato Institute for Life Science, Kitasato University). Cy5-labeled Annexin V was from BioVision. The 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-PC) and 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl}-*sn*-glycero-3-phosphoserine (NBD-PS) were from Avanti Polar Lipids. The *O*,*O'*-Bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N'*,*N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) was from Dojindo.

Expression plasmids, and real time reverse-transcription-PCR. pPEF and pNEF were derivatives of pEF-BOS-EX (6), and carried the puromycin or neomycin-resistance gene, respectively. pPEF-mEGFP-FLAG was a derivative of the pPEF vector designed to express proteins tagged with Flag and monomeric EGFP (mEGFP) at the C-terminus.

Expression plasmids for mXkr8, mXkr8-2DA (caspase-resistant mXkr8), and mouse Basigin were described previously (7, 8). The cDNAs coding for the mXkr8 mutants T356A, S361A, T375A, T356A/S361A/T375A (S/T-3A), T356D, S361D, T375D, and T356D/S361D/T375D (S/T-3D) were generated by PCR using the mXkr8 cDNA template and primers carrying the mutated nucleotides. The resultant cDNAs were introduced into pPEF-mEGFP-FLAG using In Fusion HD Cloning Kits (Takara Clontech). The primers used to generate mutants were: T356A, 5'-GGATGGGGCCCTAGGACTCCTTTCTCCCCATC-3' and 5'-CCTAGGGCCCCATCCACGAGGTCAGGGTC-3'; S361A, 5'-CTCCTTGCTCCCCATCGTCCTCCTAAGCTG-3' and 5'-ATGGGGAGCAAGGAGTCCTAGGGTCCCATCC-3'; T375A, 5'-CGTGCCGCCCTGTTAGCAGAGAACTTCTTCGCC-3'and 5' TAACAGGGCGGCACGCCTGTTATAAATCAGC-3'; T356D, 5'-GATGGGGACCTAGGACTCCTTTCTCCCCATCG-3' and 5'-TCCTAGGTCCCCATCCACGAGGTCAGGGTC-3'; S361D, 5-'CTCCTTGATCCCCATCGTCCTCCTAAGCTG-3' and 5' ATGGGGATCAAGGAGTCCTAGGGTCCCATCC 3'; T375D, 5'-CGTGCCGACCTGTTAGCAGAGAACTTCTTCGCC-3' and 5'-TAACAGGTCGGCACGCCTGTTATAAATCAGC-3'.

For real-time RT-PCR, total RNA was prepared from Ba/F3 cells with Isogen (Nippon Gene) followed by the RNeasy Mini Kit (Qiagen), and was reverse-transcribed with a High Capacity RNA-to-cDNA^{TM} Kit (Life Technologies, Applied Biosystems). Specific cDNAs were amplified using LightCycler 480 SYBR Green I Master (Roche Diagnostics). Primers for mouse ATP8A2, ATP11A, and ATP11C were described previously (9).

Establishment of the *TMEM16F*$^{-/-}$*Xkr8*$^{-/-}$Ba/F3 cell line. The mXkr8 gene in Ba/F3 cells was edited with the CRISPR (clustered regularly interspersed short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system (10) as described previously (9). Briefly, the target sequences were selected using the CRISPR Design Tool at the Zhang laboratory (http://tools.genome-engineering.org). The following oligonucleotides containing the target sequences were annealed and inserted into the px459 V2.0 vector (Addgene) (11): 5'-CACCGGATGCCATCCGAGTGTGACC-3' and 5'-AAACGGTCACACTCGGATGGCATCC-

3'. The plasmid DNA was electroporated twice into the *16F*$^{-/-}$Ba/F3 cells (3) using the NEPA21system (NEPAgene), and the cells were subjected to limiting dilution. Clones carrying the *Xkr8*-null mutation were identified by sequencing the genomic DNA.

Establishment of transformants expressing mutant mXkr8. *16F*$^{-/-}$Ba/F3 or *16F*$^{-/-}$*Xkr8*$^{-/-}$Ba/F3 cells were transfected with Ahd1-cleaved plasmid DNA by electroporation using NEPA21, and selected with 0.5-1 mg/ml G418 for Basigin or 1.0 μg/ml puromycin for mXkr8 expression. PLB and W3 cells were transformed by infection with a pantropic retrovirus as described (7). Briefly, HEK293T cells were transfected with a pMX-puro or pCX4-bsr vector carrying the respective cDNA, pGP for the gag-pol fusion protein (Takara Bio), and pCMV-VSV- G-RSV-Rev (provided by Dr. H. Miyoshi, RIKEN). The virus was concentrated by centrifugation at 6,000 x *g* for 16 h, and used to infect PLB and W3 cells. Transformants were selected with 1 μg/ml puromycin for mXkr8 and 10 μg/ml blasticidin for Basigin. To analyze the cellular localization, the stable transformants expressing mXkr8-GFP-Flag were observed by confocal microscopy (FV-1000D; Olympus).

Induction of apoptosis and treatment with inhibitors. Apoptosis was induced by incubating cells (1.0 x 10^6 cells/ml) with 10 μM STS for 2 h at 37°C. For BAPTA-AM treatment, cells (5.0 x 10^5 cells/ml) were incubated at 37℃ for 15 min with 25 μM BAPTA-AM. Pervanadate, a protein tyrosine-phosphatase inhibitor, was prepared as described (12). Briefly, 10 mM vanadate was incubated for 5 min at room temperature with 50 mM hydrogen peroxide in 10 mM Hepes-NaOH (pH 7.4), and the excess hydrogen peroxide was degraded by incubation at room temperature for a few minutes with 100 μg/ml catalase. To examine the effect of phosphatase inhibitors, cells (5.0 x 10^5 cells/ml) were incubated at 37℃ for 30 min with 100 μM pervanadate or 50 nM calyculin A. The effect of kinase inhibitor was examined by incubating the cells (5.0 x 10^5 cells/ml) at 37°C for 30 min with 10 μM STS.

Assays for scramblase and flippase activity. The phospholipid scrambling activity was measured by the PtdSer exposure or the internalization of NBD-PC as described previously (4). In brief, cells were washed with cold Annexin V buffer [10 mM Hepes-NaOH buffer (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$], incubated on ice for 15-30 min with 1,000-fold-diluted Cy5-Annexin V (BioVision) and 5 μg/ml propidium iodide (PI), and analyzed by flow cytometry with a FACSCanto Ⅱ (BD Biosciences). For the incorporation of NBD-PC, 1.0 x 10^6 cells were washed with Annexin V buffer, and incubated with 0.5 μM NBD-PC in 1 ml of Annexin V buffer. A 150-μl aliquot was mixed with an equal volume of Annexin V buffer containing 5 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich) and 5 nM SYTOX red (Thermo Fisher Scientific), and analyzed by FACSCanto II.

To assay the flippase activity, 1.0 x 10^6 cells were washed with HBS [10 mM Hepes-NaOH buffer (pH 7.4), 140 mM NaCl], and incubated at 20°C with 0.5 μM NBD-PS in 1 ml of HBS. After incubation, a 150-μl aliquot was mixed with an equal volume of HBS containing 5 mg/ml fatty acid-free BSA and 5 nM SYTOX Red, and analyzed by FACSCanto II.

SDS-PAGE, Zn^{2+} Phos-tag SDS-PAGE, and western blotting. Cell lysates were prepared by incubating the cells on ice for 10 min in radioimmunoprecipitation assay (RIPA) buffer [50 mM Hepes-NaOH buffer (pH 8.0), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and 150 mM NaCl] containing a mixture of protease inhibitors (cOmplete EDTA-free, Roche Diagnostics) and 10 mM NaF. After removing insoluble materials by spinning at 4°C at 20,000 x *g* for 10 min, the lysates were mixed with a 0.25 volume of 5 x SDS sample buffer [200 mM Tris-HCl buffer (pH 6.8), 10% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.05% bromophenol blue]. For SDS-PAGE, proteins were separated by electrophoresis on a 10-20% polyacrylamide gel (Nacalai

Tesque), and transferred to a PVDF membrane (Millipore). The membranes were probed with HRP-labeled rabbit anti-GFP Ab (anti-GFP pAb-HRP-DirecT, MBL) using the "Can Get Signal" system (Toyobo Life Science). The bound HRP was detected with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore)

For Zn^{2+} Phos-tag SDS-PAGE (13), proteins were separated by electrophoresis on an 8% polyacrylamide gel containing 30 μM Phos-tag acrylamide (Wako) that had been prepared according to the manufacturer's instructions. After electrophoresis, the gel was incubated twice for 10 min each at room temperature with 5 mM EDTA in 25 mM Tris buffer (pH 9.5) containing 40 mM 6-amino-caproic acid and 20% methanol. Proteins were transferred to a PVDF membrane, and membranes were probed with the anti-GFP Ab as described above.

Identification of phosphorylation sites in mXkr8. To determine the phosphorylation sites in mXkr8, GFP-Flag-tagged mXkr8 was immunoprecipitated from the membrane fractions of Ba/F3 transformants expressing mXkr8-GFP-Flag, with anti-GFP nanobody-coupled magnetic agarose beads (GFP-Trap_MA, ChromoTek). In brief, 1 x 10^9 cells in 800 ml of culture medium were treated with 100 μM pervanadate for 1 h at 37°C and resuspended in 16 ml of cold TBS [20 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl] containing cOmplete EDTA-free, phosSTOP, 10 mM NaF, and 5 mM Na_3VO_4. The cells were homogenized using a Dounce homogenizer with a tightly fitting pestle. After centrifugation at 6,000 x *g* for 25 min to remove cell debris and large organelles, the membrane fractions were collected by centrifugation at 100,000 x *g* for 1 h. The membranes were suspended in 4 ml of TBS containing 10% (v/v) glycerol, cOmplete EDTA-free, phosSTOP, 10 mM NaF, and 5 mM Na_3VO_4, and homogenized using a Dounce homogenizer. To the homogenates, lauryl maltose neopentyl glycol (LMNG, Anatrace), and cholesteryl hemisuccinate (CHS, Sigma-Aldrich) were added to final concentrations of 1% and 0.1%, respectively, and the mixture was incubated at 4°C for 3 h. After removing insoluble materials by centrifugation at 20,000 x *g* for 10 min, the lysates were incubated with GFP-Trap_MA for 1.5 h at 4°C. The beads were washed four times with RIPA buffer and twice with 50 mM ammonium bicarbonate. Proteins on the beads were digested with 200 ng of Asp-N or 200 ng of chymotrypsin (Promega) for 16 h at 37°C or 25°C, respectively. The digests were reduced, alkylated, acidified, and desalted using GL-Tip SDB (GL Sciences). The eluates were evaporated in a SpeedVac concentrator and dissolved in 0.1% trifluoroacetic acid.

LC-MS/MS analysis of the resultant peptides was performed on an EASY-nLC 1200 UHPLC connected to a Q Exactive Plus mass spectrometer through a nanoelectrospray ion source (Thermo Fisher Scientific). The peptides were separated on a 75 μm inner diameter × 150 mm C18 reversed-phase column (Nikkyo Technos) with a linear gradient from 4–28% acetonitrile (ACN) for min 0–100 followed by an increase to 80% ACN during min 100–110. The mass spectrometer was operated in a data-dependent acquisition mode with a top 10 MS/MS method. MS1 spectra were measured with a resolution of 70,000, an automatic gain control (AGC) target of 1×10^6 and a mass range from 350 to 1,500 *m/z*. HCD MS/MS spectra were acquired at a resolution of 17,500, an AGC target of 5×10^4, an isolation window of 2.0 *m/z*, a maximum injection time of 60 msec and a normalized collision energy of 27. Dynamic exclusion was set to 10 sec. Raw data were directly analyzed against the SwissProt database restricted to *M. musculus* using Proteome Discoverer version 2.2 (Thermo Fisher Scientific) with Mascot search engine version 2.5 (Matrix Science). The search parameters were as follows: (a) Asp-N_ambic or chymotrypsin as an enzyme with up to one or three missed cleavages; (b) precursor mass tolerance of 10 ppm; (c) fragment mass tolerance of 0.02 Da; (d) carbamidomethylation of cysteine as a fixed modification; and (e) acetylation of protein N-terminus, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine as variable modifications. Peptides were filtered at a false-discovery rate of 1% using the percolator node.

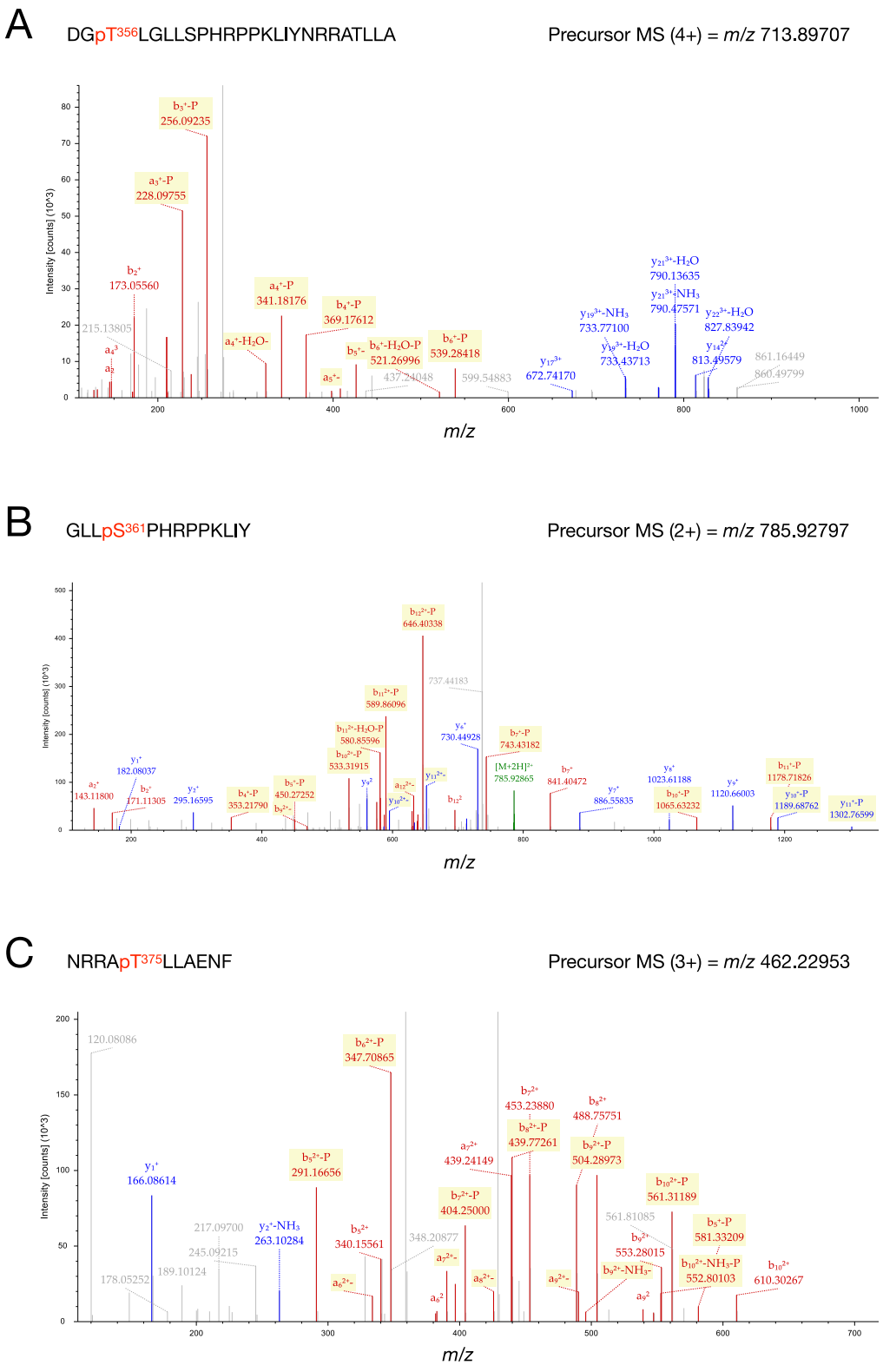

Fig. S1. Identification of mXkr8 phosphorylation sites by LC-MS/MS analysis. Phosphorylation of Thr-356 (A), Ser-361 (B), and Thr-375 (C) was demonstrated by the MS/MS spectra of *m/z* 713.89707, 785.92797, and 462.22953 ions in digested phosphopeptides, respectively.

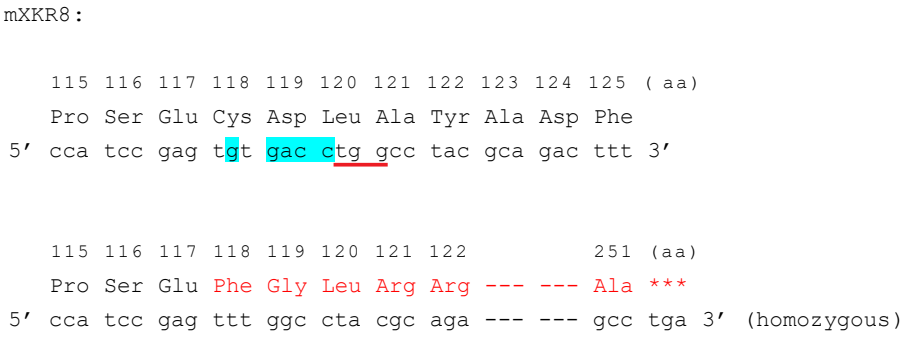

Fig. S2. Mutagenesis of the mouse *Xkr8* gene in *16F*$^{-/-}$Ba/F3 cells by the CRISPR-Cas9 system. The wild-type (upper) and mutated (lower) sequence of the mouse *Xkr8* gene are shown. The protospacer-adjacent motif (PAM) is underlined in red, and deleted residues are highlighted in light blue. The deletions caused homozygous truncations. Numbers at the top are amino acid positions. The red triple asterisks are stop codons.

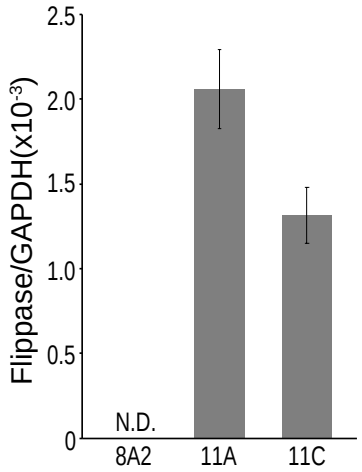

Fig. S3. Expression of *ATP8A2*, *ATP11A,* and *ATP11C* mRNAs in Ba/F3 cells. Using mRNA from Ba/F3 cells, the mRNA levels for ATP8A2, ATP11A, and ATP11C were quantified in triplicate by real-time RT-PCR. The mean values with S.E. (bar) relative to the *GAPDH* mRNA are shown. N.D., not detected.

References

1. Palacios R, and Steinmetz M (1985) Il-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. *Cell* 41:727-734.
2. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, and Nagata S (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314-317.
3. Gyobu S, Miyata H, Ikawa M, Yamazaki D, Takeshima H, Suzuki J, and Nagata S (2016) A role of TMEM16E carrying a scrambling domain in sperm motility. *Mol. Cell Biol.* 36:645-659.
4. Gyobu S, Ishihara K, Suzuki J, Segawa K, and Nagata S (2017) Characterization of the scrambling domain of the TMEM16 family. *Proc. Nat. Acad. Sci. USA* 114:6274-6279.
5. Fukunaga R, Ishizaka-Ikeda E, and Nagata S (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. *J. Biol. Chem.* 265:14008-14015.
6. Murai K, Murakami H, and Nagata S (1998) Myeloid-specific transcriptional activation by murine myeloid zinc finger protein-2. *Proc. Natl. Acad. Sci. USA* 95:3461-3466.
7. Suzuki J, Denning DP, Imanishi E, Horvitz HR, and Nagata S (2013) Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* 341:403-406.
8. Suzuki J, Imanishi E, and Nagata S (2016) Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. *Proc. Nat. Acad. Sci. USA* 113:9509-9514.
9. Segawa K, Kurata S, Yanagihashi Y, Brummelkamp T, Matsuda F, and Nagata S (2014) Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* 344:1164-1168.
10. Hsu PD, Lander ES, and Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157:1262-1278.
11. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, and Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8:2281-2308.
12. Huyer G, Liu S, Kelly J, Moffat J, Payette P, Kennedy B, Tsaprailis G, Gresser MJ, and Ramachandran C (1997) Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. *J. Biol. Chem.* 272:843-851.
13. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, and Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell Proteomics* 5:749-757.