SUPPLEMENTAL MATERIAL

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Phogrin(C934S). Mutation primers used to generate Phogrin(C934S) were designed using the design guidelines provided by Stratagene and ordered from Operon:
Phogrin(C934S) for 5'-TGTCCGATAATTGTCCATAGCAGTGACGGCGCGGGCA-3'
Phogrin(C934S) rev 5 '-TGCCCGCGCCGTCACTGCTATGGACAATTATCGGACA-3'

Preparation and purification of GST fusion proteins. BL21 CodonPlus cells (Stratagene, La Jolla, CA) carrying the various engineered plasmids were grown overnight in LB medium supplemented with 100 μg/ml ampicillin. Isopropyl-β-D-thiogalactoside (IPTG) was added to 0.2 mM and incubation was continued for three hours at 37°C. Cells were spun down and resuspended in TBS buffer containing 1 mg/ml lysozyme and a cocktail of protease inhibitors including 2 mM PMSF and 20 μg/ml of aprotinin and leupeptin. After addition of DTT to 5 mM final concentration, the cells were incubated on ice for 30 min followed by 30 seconds sonication at 4°C on a Microson ultrasonic cell disrupter (Branson Sonifier 250, City, State). The cell lysate was incubated for 30 min at 4° C in the presence of 1% Triton X-100 with gentle rotation and spun at 18,000RPM for 30 min. Supernatant was applied to a glutathione-sepharose column (Amersham-Pharmacia, Biotech, Inc. Piscataway, NJ) equilibrated with 50 mM Tris, pH 8.0. The column was washed three times with the same buffer, and GST-Phogrin fusion protein was eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris, pH 8.0. The eluant was supplemented with 10% glycerol and 5mM DTT and the fusion protein was stored at -80°C.

Phogrin siRNA constructs – The following siRNA constructs targeting phogrin were evaluated:

PhoSI-161Forward
GATCCCCCGATGGTGTGTTTGGAAGATTCAAGAGATCTTCCAAACACACCATCGTTTTTA
PhoSI-161Reverse
AGCTTAAAAACGATGGTGTGTTTGGAAGATCTCTTGAATCTTCCAAACACACCATCGGGG
PhoSI-563Forward
GATCCCCCATCCTGACCTATGTGGCCTTCAAGAGAGGCCACATAGGTCAGGATGTTTTTA
PhoSI-563Reverse
AGCTTAAAAACATCCTGACCTATGTGGCCTCTCTTGAAGGCCACATAGGTCAGGATGGGG
PhoSI-2754Forward
GATCCCCGTGAACAAATGCTACCGAGTTCAAGAGACTCGGTAGCATTTGTTCACTTTTTA
PhoSI-2754Reverse
AGCTTAAAAAGTGAACAAATGCTACCGAGTCTCTTGAACTCGGTAGCATTTGTTCACGGG
ControlSI Forward
GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTA
ControlSI Reverse
AGCTTAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGG

SUPPLEMENTAL FIGURE LEGENDS

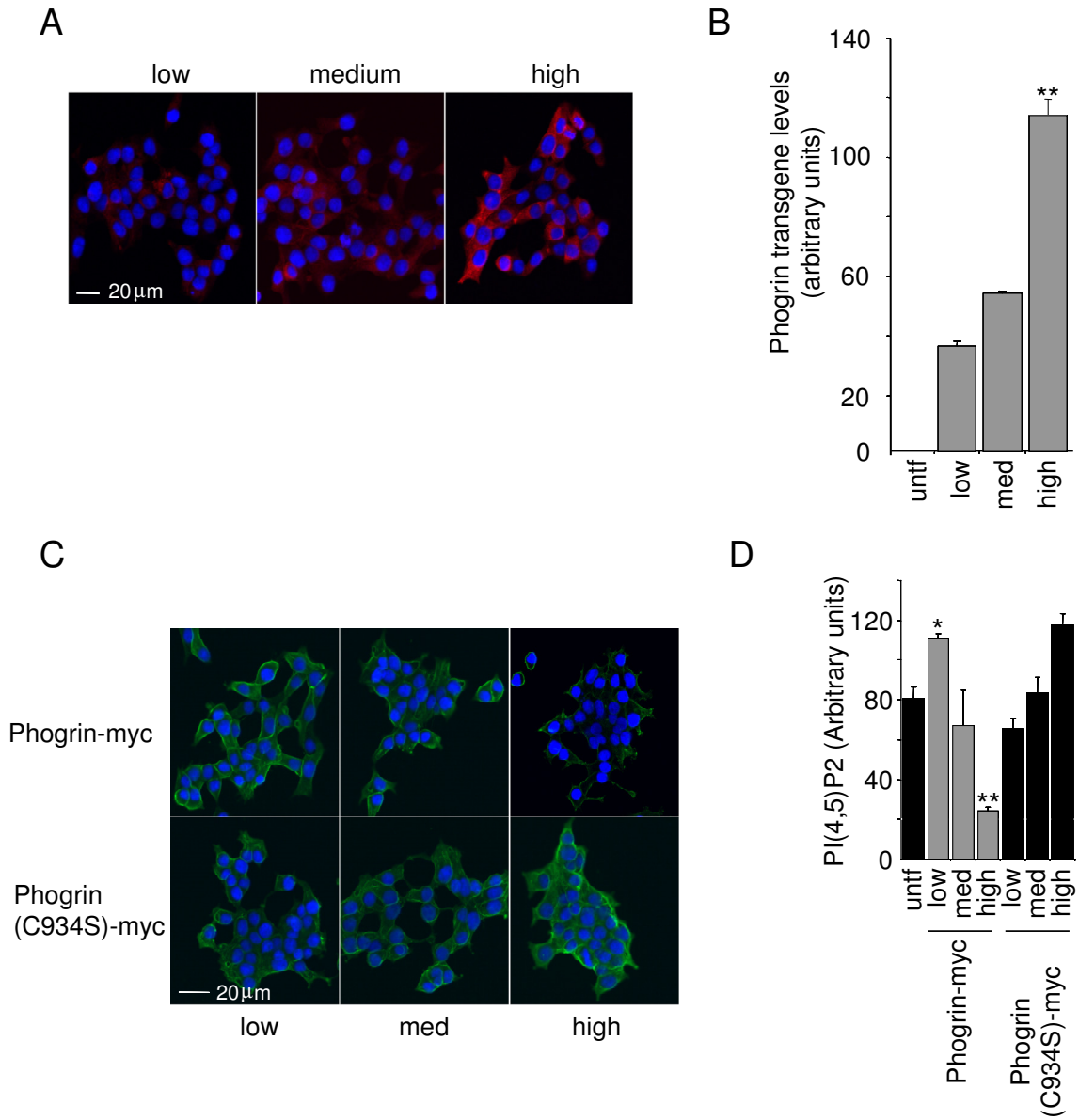
Figure S1. Reduction in PI(4,5)P2 levels in INS-1 clones is proportional to Phogrin-myc expression level. A mass culture of INS-1 cells infected with Phogrin-myc vector was plated at low density and 10 individual clones were picked and evaluated by western blot. From these 10 clones, 3 clones expressing low, medium or high levels of Phogrin-myc were chosen for further evaluation and use:
A, Confocal images of representative fields from cultures fixed with paraformaldehyde and immunostained with anti-myc and TRITC-labeled secondary antibody to detect Phogrin-myc (red). Nuclei are stained with DAPI (blue).

B, The level of Phogrin-myc expression in the confocal images in panel *A* was calculated by image analysis as described in Experimental Procedures.
C, INS-1 clones expressing low, medium, and high levels of Phogrin-myc or Phogrin(C934S)-myc were fixed with paraformaldehyde. PI(4,5)P2 was detected by incubation with a GST-PLCδ1 fusion protein visualized with mouse monoclonal anti-GST and FITC-labeled goat anti-mouse IgG green). Nuclei were stained with DAPI (blue).
D, Levels of PI(4,5)P2 from panel C were quantitated as described in Experimental Procedures. * $p<0.05$, ** $p<0.01$ compared to untransfected.

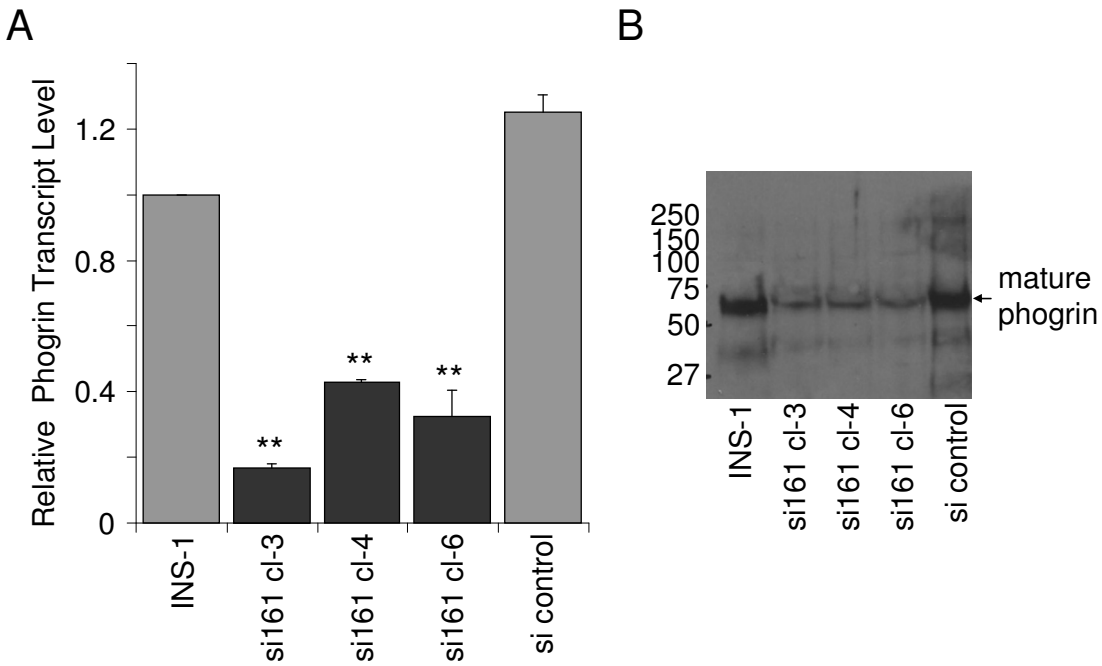
Figure S2. Expression of si construct targeting phogrin reduces phogrin transcript and protein levels in INS-1 cells. Anti-phogrin construct si161, or a control sequence, were transfected into INS-1 cells and 3 clones picked and expanded for evaluation. Panel A shows phogrin transcript levels evaluated by reverse transcription and real-time PCR normalized to 18S transcript levels. Panel B shows reduction of phogrin protein level evaluated by Western blot using rabbit IgG against the phogrin catalytic domain.

Figure S3. Phogrin expression level affects the second phase of glucose-stimulated insulin secretion. INS-1 cells (open diamonds), or INS-1 cells transfected with Phogrin-myc (open squares), Phogrin(C934S)-myc (filled circles), si161 antisense phogrin (filled diamonds) or si control construct (filled triangles) were pre-incubated for 30 min in KRBH with 5 mM glucose, then in KRBH 3 or 22.2 mM glucose for 60 min. Aliquots of medium were collected every 3 minutes for the first 15 minutes then at 30 and 60 minutes. Insulin present in the incubation buffer was determined by ELISA and the rate of accumulation during each interval calculated as pg/ml/minute.

Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3