Supporting Information

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SI Text

Expression Plasmids. cDNAs for PGAM5 orthologs in human, *Drosophila* (CG14816), and *C. elegans* (R07G3.5) were cloned by reverse transcript (RT)-PCR using total RNA isolated from HEK293 cells, *Drosophila* embryos, and *C. elegans* worms, respectively. The following oligonucleotides were used for the isolation of cDNAs: *human PGAM5*, sense, 5′-gcgaattcGGCATGGCGTTCCGGCAGGCGC-3′, antisense, 5′-gcctcgagTCAGGATCGAGTGATCTTGTCG-3′; *DPGAM5* (*CG14816*), sense, 5′-gcgaattcgccATGCGAAAGTTGACTAGCTTC-3′, antisense, 5′-gctctagaCTAAACCACGTTTTTGACGTC-3′; *CePGAM5* (*R07G3.5*), sense, 5′-gcgaattcATGGTATCTAAGATCATAAAAC-3′, antisense, 5′-gcctcgagTTAGGTGAAGGAAATCTTGTTT-3′ (the nucleotides shown in the lowercase include sequences for digestion with restriction enzymes). cDNAs for human PGAM5 and CePGAM5 were digested with *Eco*RI and *Xho*I and subcloned into a mammalian expression plasmid pcDNA3 (Invitrogen) with an N-terminal or C-terminal tag. An *Eco*RI/*Xho*I fragment of human PGAM5 cDNA was also subcloned into a bacterial expression plasmid pGEX4T-1 (GE Healthcare). DPGAM5 cDNA was digested with *Eco*RI and *Xba*I and subcloned into a *Drosophila* expression plasmid pUAST. His105Ala and His105Phe mutants of PGAM5 and a His94Ala mutant of DPGAM5 were obtained using the QuikChange site-directed mutagenesis kit (Stratagene). Human PGAM1 cDNA was cloned by RT-PCR using total RNA isolated from HEK293 cells, digested with *Eco*RI and *Xho*I, and subcloned into pcDNA3 with an N-terminal Flag tag. The following oligonucleotides were used for the isolation of cDNA: sense, 5′-gcgaattcGCCATGGCCGCCTACAAACTGG-3′; antisense, 5′-gcctcgagTCACTTCTTGGCCTTGCCCTGG-3′ (the nucleotides shown in the lowercase include sequences for digestion with restriction enzymes). Expression plasmids for ASK1 and DASK1 were described in refs. 1 and 2).

Antibodies and Reagents. PGAM5 polyclonal antibody was raised against the peptide RTLGDTGFMPPDKITRS as described in ref. 3. Phospho-ASK1 antibody was described in ref. 4. Monoclonal antibodies to the hemagglutinin (HA) (clone 3F10), Flag (M2), and T7 tags were purchased from Roche, Sigma, and Novagen, respectively. Phospho-specific antibodies to JNK, p38, and ERK were purchased from Cell Signaling. Antibodies to ASK1 (H300) and p38 (C-20-G) were purchased from Santa Cruz Biotechnology. Antibody to actin (AC-40) was purchased from Sigma. Recombinant PTP1B was purchased from Biomol.

RNA Interference. A cDNA fragment for DPGAM5 (520 bp) corresponding to the amplicon free of off-target effects in the *Drosophila* RNAi Screening Center (DRSC) library (ID: 18551) (5) was generated by PCR using oligonucleotides with the T7 RNA polymerase sequence attached to the 5′ end and Flag-DPGAM5-pUAST plasmid as a template (*DPGAM5#1*). Another cDNA fragment for DPGAM5 (326 bp) was generated by PCR using a cDNA pool derived from S2 cells as a template (*DPGAM5#2*). A cDNA fragment for GFP was also generated for control double-stranded RNA (dsRNA) using GFP-pBluescript II SK(+) as a template. The following oligonucleotides were used: *DPGAM5#1*, sense, 5′-TAATACGACTCACTATAGGGAGAGTGCGTCCACTGCGAAA-3′, antisense, 5′-TAATACGACTCACTATAGGGAGAGGGCTCGGCAGACAAAG-3′; *DPGAM5#2*, sense, 5′-TAATACGACTCACTATAGGGAGAAGGGTTGGCTACGGATTA-3′, antisense, 5′-TAATACGACTCACTATAGGGAGACAGTCGGAAGATTCAATGA-3′; *GFP*, sense, 5′-TAATACGACTCACTATAGGGAGACTTCAGCCGCTACCCC-3′, antisense, 5′-TAATACGACTCACTATAGGGAGATGTCGGGCAGCACG-3′. Each fragment was used as a template for in vitro transcription reaction using T7 RNA polymerase (Promega). After DNase I treatment, synthesized dsRNA was purified and checked by agarose gel electrophoresis (6). S2 cells were plated into 12-well plates (4×10^5 cells per well) with 300 mL *Drosophila* SFM (Invitrogen) and 6 mg dsRNA. After a 30-min incubation at 26 °C, 700 mL Schneider's *Drosophila* Medium (Invitrogen) were added. Cells were further incubated for 72 h at 26 °C and then subjected to quantitative PCR and immunoblot analyses. For RNAi in Neuro2A cells, siRNAs targeting the 5′-AGUUGACAUCCAGGCUGGAUCACUA-3′ (#1) and 5′-UCCGACUUGCAAGCCUGGGAUUAAA-3′ (#2) sequences of mouse *PGAM5* mRNA were purchased from Invitrogen (Stealth RNAi). Stealth RNAi Negative CTL (Invitrogen) was used as a control. Neuro2A cells that were seeded in 12-well plates were transfected with 20 pmol per well siRNA using 2 μL per well Lipofectamine RNAiMAX (Invitrogen). After 48 h, cells were subjected to immunoblot analyses.

Quantitative RT-PCR. Total RNA was isolated from dsRNA-treated S2 cells using Isogen (Wako) and reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with Power SYBR Green PCR Master Mix using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The following oligonucleotides were used: *DPGAM5*, sense-1, 5′-TTATTTCCAACACTTTAACTGAGACC-3′, antisense-1, 5′-CATGGTGGTTTACACAGTCTCG-3′, sense-2, 5′-AAGCCGGAGGCATCTCAGTT-3′, antisense-2, 5′-GGTGGAAGTATCGGCGAAAG-3′; *DASK1*, sense, 5′-CCCCCTCCATAATATCACTCAC-3′, antisense, 5′-AACCCCTTTATTCTCCCTCTTAA-3′; *rp49*, sense, 5′-CGGATCGATATGCTAAGCTGT-3′, antisense, 5′-GCGCTTGTTCGATCCGTA-3′. Combinations of oligonucleotides for *DPGAM5*, sense-1/antisense-1 and sense-2/antisense-2, were used for RNAi experiments using *DPGAM5* dsRNA#1 and dsRNA#2, respectively. To normalize the relative expression of *DPGAM5* and *DASK1* to the *rp49* control, a standard curve was prepared for *rp49* and *DPGAM5* or *DASK1* in each experiment.

1. Kuranaga E, et al. (2002) Reaper-mediated inhibition of DIAP1-induced DTRAF1 degradation results in activation of JNK in *Drosophila*. *Nat Cell Biol* 4:705–710.
2. Takeda K, et al. (2007) Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1. *J Biol Chem* 282:7522–7531.
3. Saitoh M, et al. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17:2596–2606.
4. Tobiume K, Saitoh M, Ichijo H (2002) Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J Cell Physiol* 191:95–104.
5. Flockhart I, et al. (2006) FlyRNAi: The *Drosophila* RNAi screening center database. *Nucleic Acids Res* 34:D489–D494.
6. Ramadan N, Flockhart I, Booker M, Perrimon N, Mathey-Prevot B (2007) Design and implementation of high-throughput RNAi screens in cultured *Drosophila* cells. *Nat Protoc* 2:2245–2264.

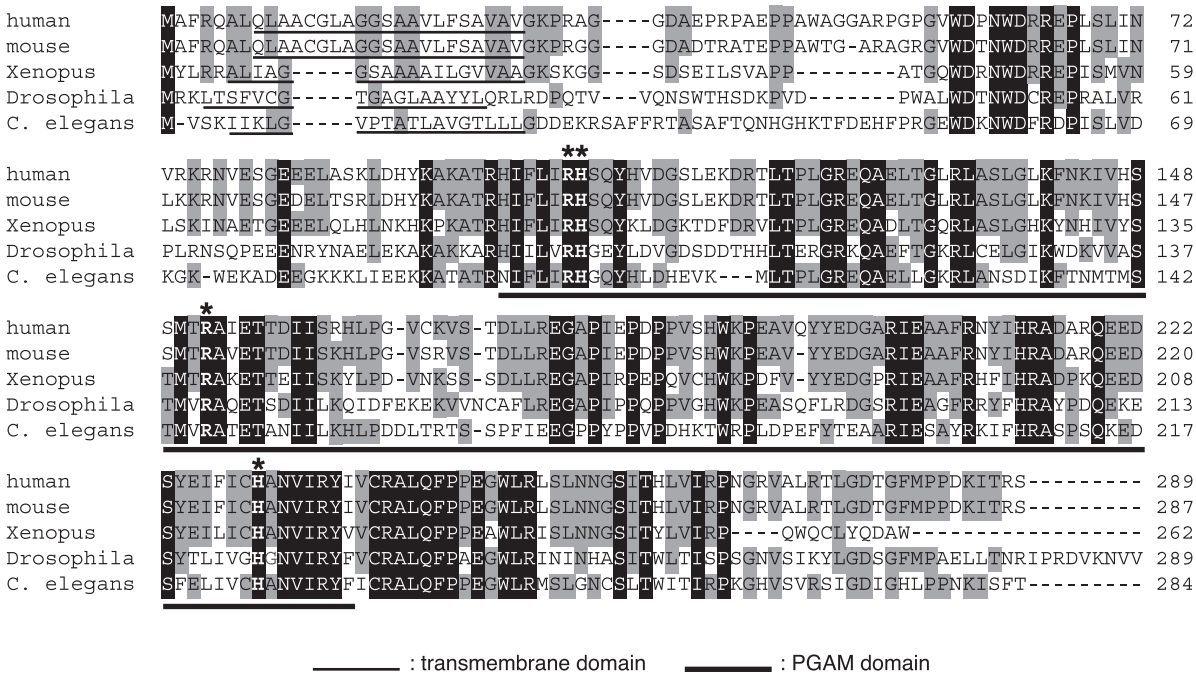

Fig. S1. ClustalW alignment of the amino acid sequences of PGAM5 orthologs. Residues identical among 3 or 4 proteins and among all 5 proteins are shaded in gray and black, respectively. The transmembrane and PGAM domains are underlined (thin and bold lines, respectively). The putative residues comprising the catalytic core are indicated by asterisks.

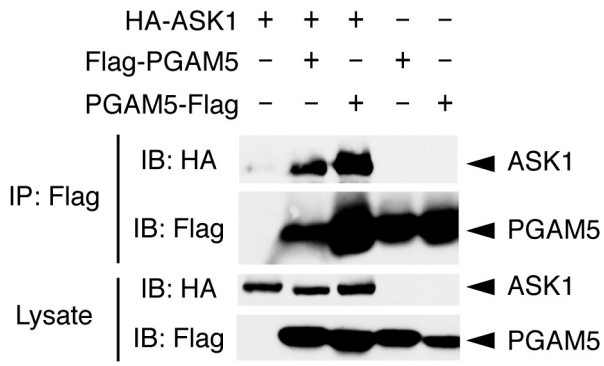

Fig. S2. Interaction between PGAM5 and ASK1 in transiently transfected HEK293 cells. N- or C-terminally Flag-tagged PGAM5 (Flag-PGAM5 or PGAM5-Flag, respectively) and HA-tagged ASK1 (HA-ASK1) were expressed in HEK293 cells. Cells were lysed with the IP lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL aprotinin). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with M2 gel (Sigma). The gels were washed 3 times with the IP lysis buffer before analysis by immunoblotting (IB).

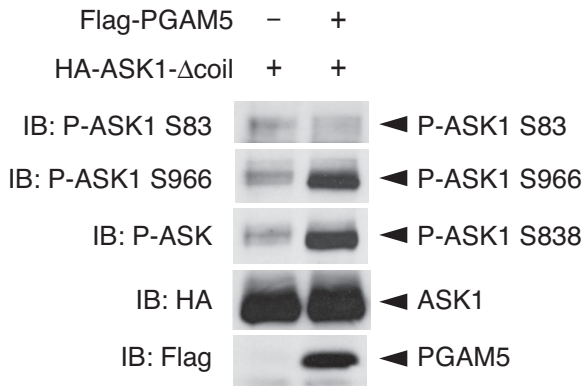

Fig. S3. Phosphorylation of neither Ser-83 nor Ser-966 in ASK1 decreases in response to overexpression of PGAM5. HA-ASK1-Δcoil was expressed with or without Flag-PGAM5 in HEK293 cells. Cell lysates were subjected to immunoblotting (IB) using P-ASK1 Ser-83 and P-ASK1 Ser-966 antibodies (purchased from Cell Signaling) and P-ASK antibody.

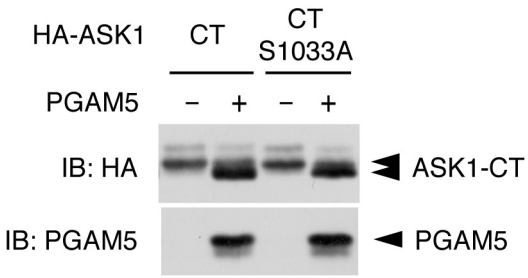

Fig. S4. PGAM5-dependent mobility shift of ASK1-CT is not affected by substitution of Ser-1033 to Ala. HA-ASK1-CT or HA-ASK1-CT Ser1033Ala was expressed with or without PGAM5 in HEK293 cells. Cell lysates were subjected to immunoblotting (IB).

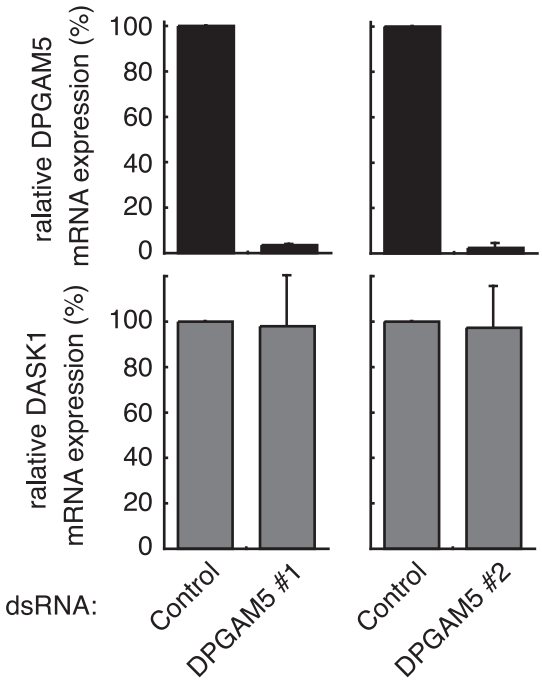

Fig. S5. Efficiency of knockdown of DPGAM5 in S2 cells. mRNA expression of *DPGAM5* and *DASK1* in S2 cells treated with control double-stranded RNA (GFP dsRNA) or dsRNA targeting *DPGAM5* was determined by quantitative RT-PCR. Combinations of oligonucleotides for *DPGAM5*, sense-1/antisense-1, and sense-2/antisense-2 were used for RNAi experiments using *DPGAM5* dsRNA#1 and dsRNA#2, respectively. Results shown are the means ± SD. of 3