Supporting Information

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SI Materials and Methods

Expression Vectors. A pCMV-based mammalian expression vector for Myc-tagged rat Pragmin was kindly provided by Dr. M. Negishi, (Kyoto University, Kyoto, Japan). Myc-tagged Pragmin-Y391F, in which tyrosine-391 was substituted with phenylalanine, was generated from the rat *Pragmin* cDNA by site-directed mutagenesis. A pSP65SRα-based mammalian expression vector for the HA-tagged wild-type CagA protein has been described previously (1, 2). Rat *Csk* cDNA was FLAG-tagged and was cloned into pSP65SRα vector. A pcDNA3-derived mammalian expression vector for FLAG-tagged Grb7 was a gift from Dr. A. Villalobo (Instituto de Investigaciones Biomedicas, Madrid, Spain). To disable the SH2 domain of Csk, serine-109 of Csk was substituted with cysteine (Csk-S109C-FLAG) by site-directed mutagenesis. Duplex siRNAs for human Pragmin/SgK223 and luciferase were synthesized by Greiner Bio One. Nucleotide sequences for the human Pragmin-specific siRNA used were sense (5′-GUCACAGGCCAAGAUAGAATT-3′) and antisense (5′-UUCUAUCUUGGCCUGUGACTT-3′). Nucleotide sequences for the luciferase-specific siRNA used as a control were sense (5′-CGUACGCGGAAUACUUCGATT-3′) and antisense (5′-UCGAAGUAUUCCGCGUACGTT-3′). The siRNAs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) at a final concentration of 100 pmol/mL.

Bacteria. *Helicobacter pylori* NCTC11637 strain and its *cagA*-defective isogenic strain (*ΔcagA*) have been reported previously (3). AGS cells were infected with *H. pylori* NCTC11637 or the isogenic *ΔcagA* strain for 5 h at a multiplicity of infection (MOI) of 200 before harvest.

Antibodies and Reagents. Anti-Csk polyclonal antibody C-20 (Santa Cruz Biotechnology), anti-Myc monoclonal antibody 9E10, anti-SHP-1 polyclonal antibody C-19 (Santa Cruz Biotechnology), anti-Grb2 polyclonal antibody C-23 (Santa Cruz Biotechnology), anti-c-Abl polyclonal antibody K-12 (Santa Cruz Biotechnology), anti-CrkII monoclonal antibody (Transduction Laboratories), and anti-PI3K (subunit p85α) polyclonal antibody Z-8, anti-FLAG monoclonal antibody (M2) (Sigma-Aldrich), and anti-SHP2 polyclonal antibody C-18 (Santa Cruz Biotechnology) were used as primary antibodies for immunoblotting and immunoprecipitation. Anti-HA polyclonal antibody Y-11 (Santa Cruz Biotechnology), HA-epitope-specific monoclonal antibody 12CA5, anti-Pragmin antibody A302-675A (Bethyl Laboratories), anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology), anti-c-Src monoclonal antibody 36D10 (Cell Signaling), anti-Phospho-Src (Y416) polyclonal antibody (Cell Signaling), anti-Phospho-Src (Y530) polyclonal antibody (Santa Cruz Biotechnology), anti-RhoA antibody Sc-179 (Santa Cruz Biotechnology), anti-CagA polyclonal antibody (Austral Biologicals), and anti-Actin polyclonal antibody C-11 (Santa Cruz Biotechnology) were used as primary antibodies for immunoblotting. Normal mouse or rabbit IgG (IgG) was purchased from Santa Cruz Biotechnology. RhoA inhibitor, Clostridial C3 transferase, was purchased from Cytoskeleton. EGF was purchased from Sigma. Src family protein kinase inhibitor PP2 was purchased from Calbiochem.

Cell Culture and Transfection. AGS and MKN28 human gastric carcinoma cells were cultured in RPMI medium 1640 supplemented with 10% FCS under standard condition. Cells were transiently transfected with expression vectors using Lipofectamine 2000 reagent (Invitrogen) as previously described (1) and were harvested at 36 h after transfection. GES-1 human normal human gastric epithelial cells were cultured in DMEM supplemented with 10% FCS. To inhibit tyrosine kinase activity, cells were incubated with 10 μM PP2 for 4 h or 100 nM PP2 for 6 h. To inhibit RhoA activity, cells were treated with cell-permeable C3 transferase from *Clostridium botulinum* at the final concentration of 0.5 μg/mL for 12 h.

Immunoprecipitation and Immunoblotting. Cells were lysed as described previously (1, 2). Cell lysates were incubated with antibodies, and immune complexes were trapped on protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to SDS/PAGE, and separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membrane filters were treated with a primary antibody (1:1,000) and then with a secondary antibody (1:10,000). Proteins were visualized by using Western blot chemiluminescence reagent (PerkinElmer Life Science). Intensity of protein band was quantitated by a luminescent image analyzer (LAS-4000; FUJIFILM).

Immunofluorescence Microscopy. Cells were fixed with 3% paraformaldehyde at 20 h after transfection. Cells were then treated with the indicated antibodies and were visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen). The nuclei were stained with DAPI (Wako). Images were acquired using a confocal microscope system (TCS-SPE; Leica).

Cell Morphological Analysis. AGS cells (1.2×10^5 cells) were transiently transfected with expression vectors using Lipofectamine 2000 reagent. Morphology of AGS cells was examined at 20 h after transfection.

Data Analysis. All of the DNA and protein sequences were retrieved from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) and were analyzed using the BLAST search program. BioEdit and WebLogo were used to align and display protein sequences (http://weblogo.berkeley.edu). ClustalW and TreeView (http://align.genome.jp/) were applied to build and view phylogenic trees.

1. Higashi H, et al. (2002) Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci USA* 99:14428–14433.
2. Tsutsumi R, Takahashi A, Azuma T, Higashi H, Hatakeyama M (2006) Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with *Helicobacter pylori* CagA. *Mol Cell Biol* 26:261–276.
3. Higashi H, et al. (2004) *Helicobacter pylori* CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem* 279:17205–17216.

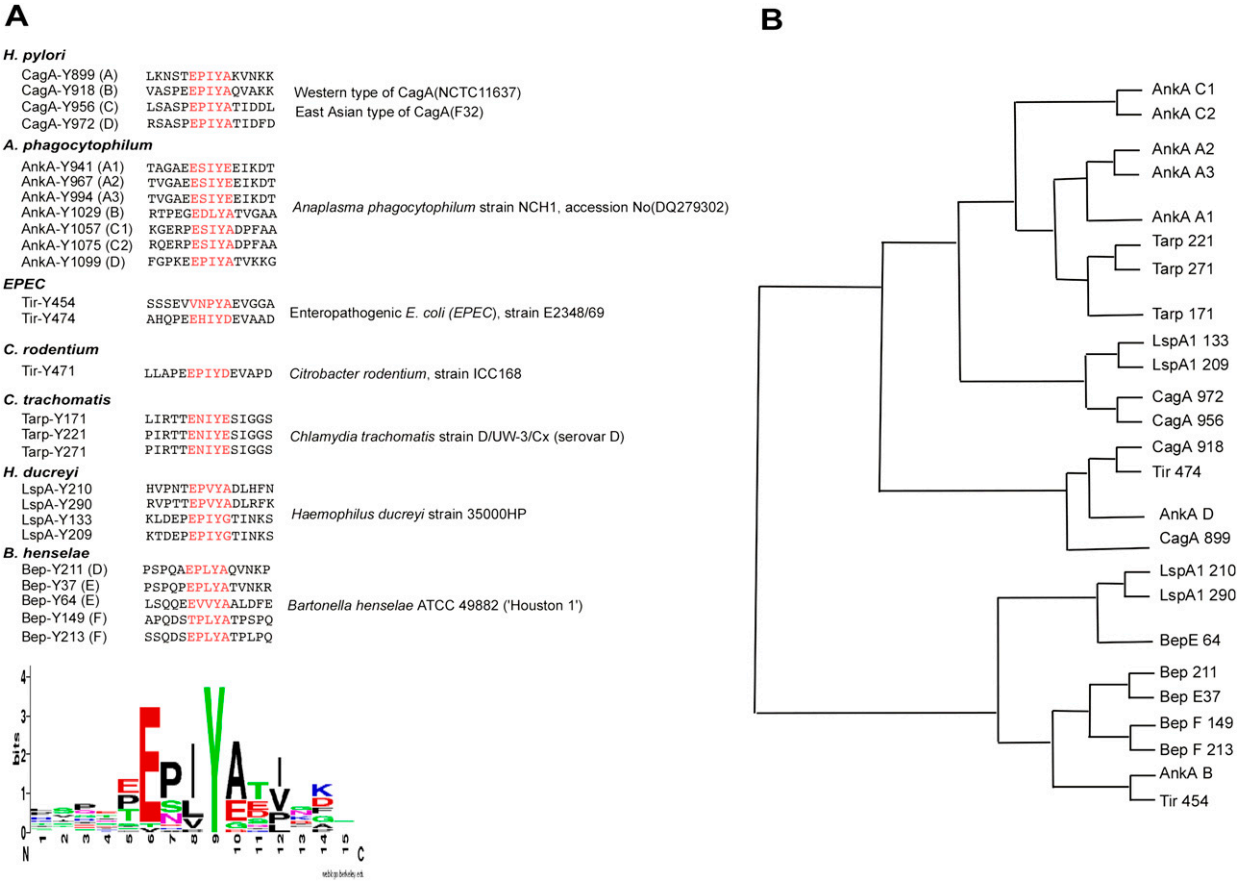

Fig. S1. Glu-Pro-Ile-Tyr-Ala (EPIYA)/EPIYA-like sequences present in bacterial EPIYA effectors. (*A*) Sequences surrounding each of the EPIYA motifs (highlighted in red) in bacterial EPIYA effectors reported to date are shown. Consensus sequence spanning the EPIYA motif is shown at bottom. (*B*) Tree of bacterial EPIYA effectors, based on the 15-aa sequences shown in *A*.

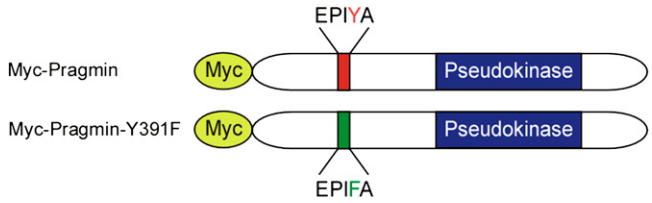

Fig. S2. Schematics of rat Pragmin and Pragmin-Y391F used in this work. Pragmin contains a pseudokinase domain, which does not have a kinase catalytic activity, in its C-terminal region. Pragmin-Y391F was made by replacing tyrosine-391, which constitutes the EPIYA motif, with nonphosphorylatable phenylalanine. Both wild-type Pragmin and Pragmin-Y391F were N-terminally tagged with the Myc epitope.

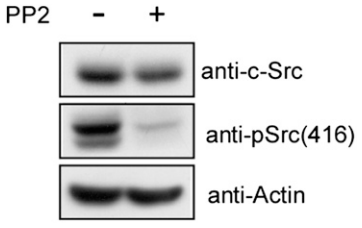

Fig. S3. Effect of 100 nM PP2 on the kinase activity of c-Src. AGS cells were treated with 100 nM PP2 for 6 h before harvest. Cell lysates were subjected to immunoblotting with an anti-c-Src antibody, anti-Phospho-Src (416) antibody [anti-pSrc(416)], which specifically reacts with the active form of c-Src, and anti-Actin antibody.

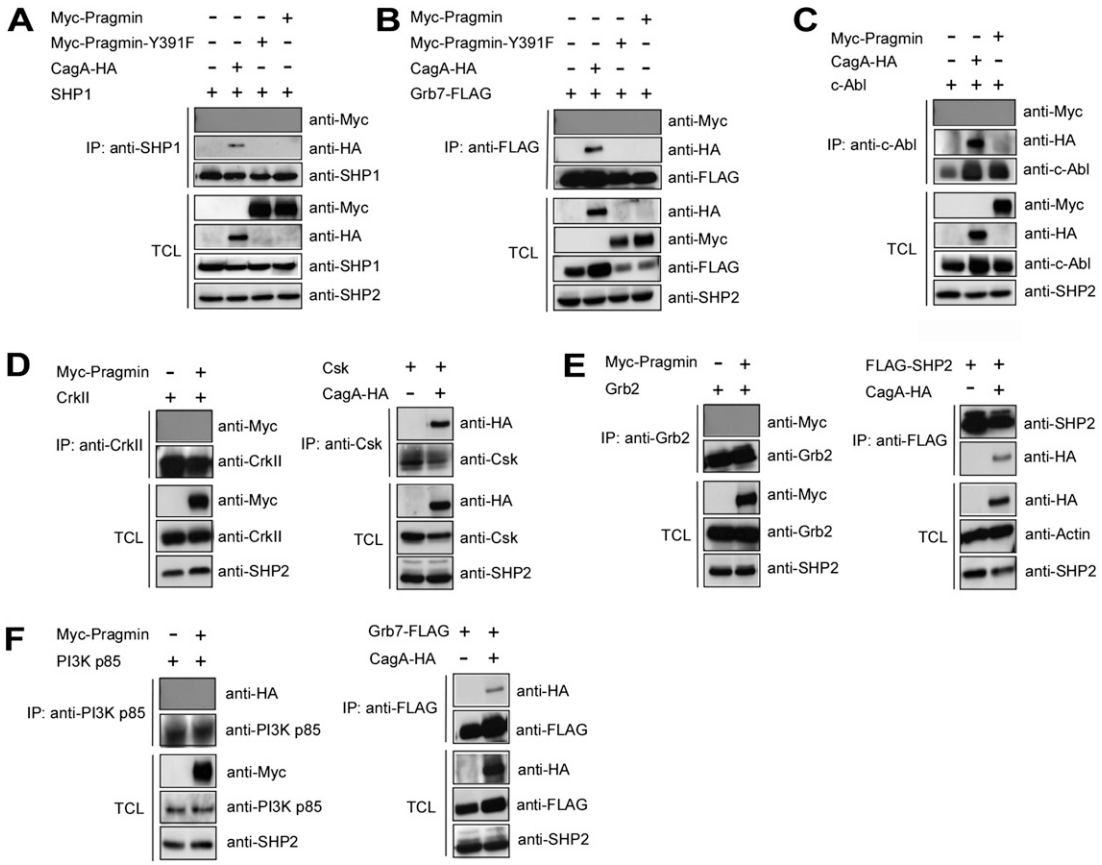

Fig. S4. SH2-binding specificity of Pragmin. (*A*) AGS cells were transfected with the indicated vectors. Total cell lysates (TCLs) were immunoprecipitated with an anti-SHP1 antibody, and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and SHP1. The CagA-SHP1 interaction is shown as a positive control of the experiment. (*B*) AGS cells were transfected with the indicated vectors. Total cell lysates were immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and Grb7-FLAG. The CagA-Grb7 interaction is shown as a positive control of the experiment. (*C*) AGS cells were transfected with the indicated vectors. Total cell lysates were immunoprecipitated with an anti-c-Abl antibody, and the immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and c-Abl. The CagA-c-Abl interaction is shown as a positive control of the experiment. (*D*–*F*) AGS cells were transfected with a Myc-Pragmin vector together with a CrkII vector (*D*), Grb2 vector (*E*), or PI3-kinase (PI3K) p85 vector (*F*). Total cell lysates were immunoprecipitated with an anti-CrkII antibody (*D*), anti-Grb2 antibody (*E*), or anti-PI3K p85 antibody (*F*). The immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and CrkII (*D*), between Myc-Pragmin and Grb2 (*E*), or between Myc-Pragmin and PI3K p85 (*F*). The CagA–Csk interaction (*D*), the CagA–SHP2 interaction (*E*), and the CagA–Grb7 interaction (*F*) are shown as positive controls of the transfected protein–protein interaction, which were simultaneously performed in each of the transfection experiments (*D*–*F*).

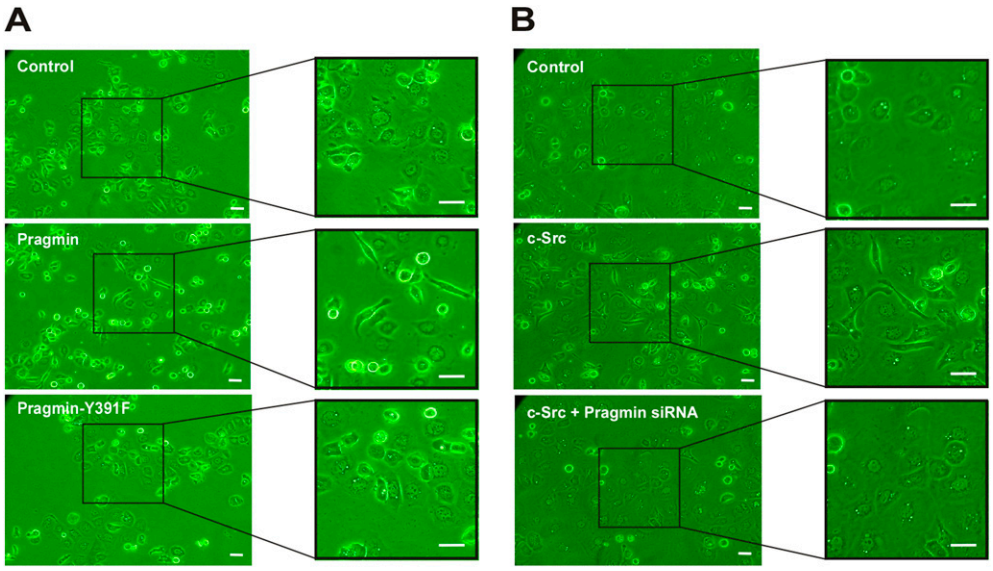

Fig. S5. Magnification of cell morphology shown in Fig. 3 (main text). (*A*) AGS cells were transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector. Morphology of transfected cells was analyzed by microscope. (*B*) AGS cells treated with or without the Pragmin-specific siRNA were transfected with a c-Src vector. Cells were subjected to morphological investigation using microscopy. (Scale bars, 10 μm.)

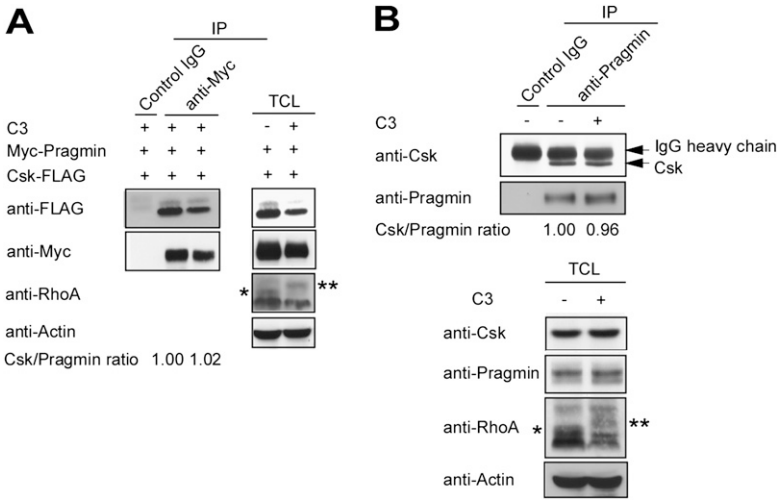

Fig. S6. Effect of RhoA inhibition on complex formation of Pragmin with Csk. (*A*) AGS cells transfected with Myc-Pragmin and Csk-FLAG vectors were treated with Clostridial C3 transferase, a specific inhibitor of Rho GTPases, for 12 h before harvest. Total cell lysates (TCLs) were immunoprecipitated with an anti-Myc antibody. Total cell lysates and immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. Values shown are relative ratios of Csk that bound to Pragmin (Csk/Pragmin ratios), which were calculated from the quantitation data of protein bands using a luminescent image analyzer. The positions of RhoA (*) and ADP ribosylated (i.e., inactivated) RhoA (**) are indicated. (*B*) AGS cell lysates were immunoprecipitated with an anti-Pragmin antibody. Total cell lysates and immunoprecipitates were subjected to immunoblotting with the indicated antibodies. Values shown are relative ratios of Csk that bound to Pragmin (Csk/Pragmin ratios), which were calculated from the quantitation data of protein bands using a luminescent image analyzer. The positions of RhoA (*) and ADP ribosylated (i.e., inactivated) RhoA (**) are indicated.

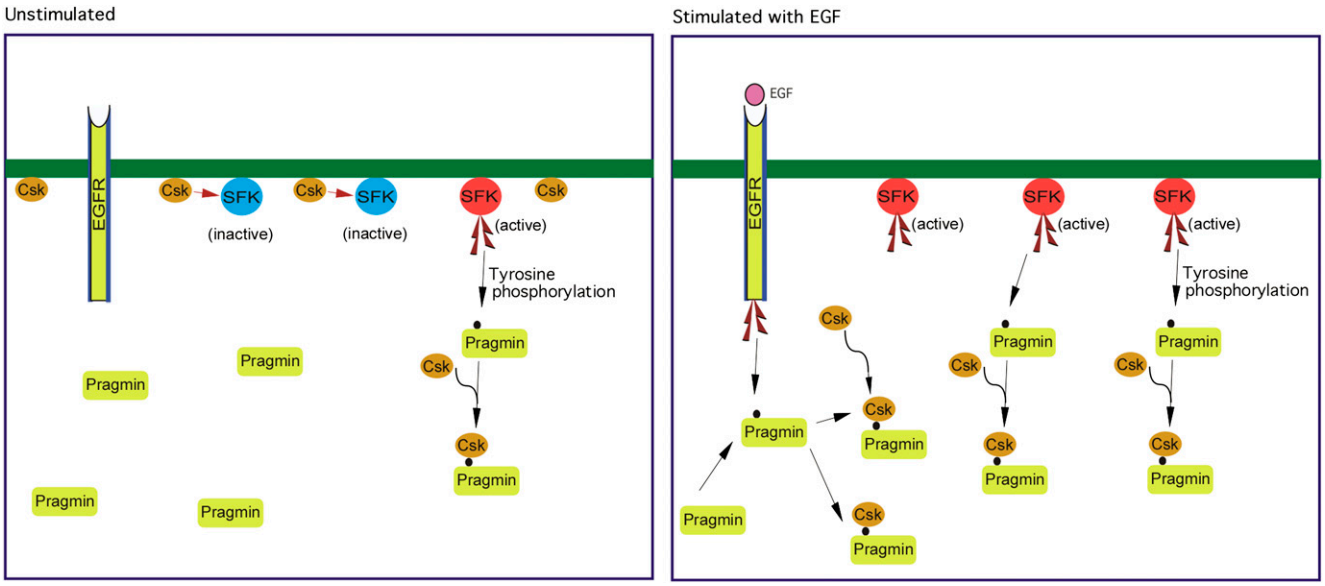

Fig. S7. A proposed model for the positive feedback regulation of SFKs by Pragmin. Basally activated SFKs in unstimulated cells phosphorylate a small fraction of Pragmin molecules, which reduce the amount of membrane-associated Csk below a certain level to keep the basal SFK activity. Stimulation of cells with EGF increases the amount of Pragmin that is phosphorylated at the EPIYA motif, which sequestrates Csk in the cytoplasm and thereby prevents translocalization of Csk to the membrane. As a result, SFKs are kept in their active forms and further phosphorylate Pragmin at the EPIYA motif, giving rise to the formation of a positive feedback loop that ensures sustained SFK activation, which promotes cell proliferation as well as cell motility.

Table S1. List of human EPIYA-containing proteins

Ig heavy chain variable region (anti-vaccinia virus)	VDPEDGEPIYAEKFGGR
General transcription factor TFIIE, α subunit	RFNEQIEPIYALLRETE
Solute carrier family 2/facilitated glucose transporter member 3	KDAGVQEPIYATIGAGV
Transmembrane protein 218	LIHYVLEPIYAKPLHSY
Pragmin/SgK223	REATQPEPIYAESTKRK
Coiled-coil domain-containing protein 146	KDEKDQEPIYAIVPTIN