Supporting Information

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SI Text

Cells and Viruses. ST cells were used and cultured as described previously (1). WT MEF cells and PAK1$^{-/-}$ MEF cells were described previously (2), and Pak2$^{-/-}$ MEF cells were established from 7.5- to 8.5-dpc mouse embryos and immortalized and cultured as described previously for MEF cells (2). Wild-type and isogenic US3 null PRV NIA3 viruses were used (3, 4).

Antibodies, Inhibitors, and Plasmids. FITC-labeled goat anti-mouse, Texas red-labeled phalloidin, FITC-labeled goat anti-rabbit, Cy5-labeled goat anti-mouse, and monoclonal anti-GFP (3E6) antibodies were from Invitrogen. The polyclonal anti-FLAG-tag antibody was from Sigma–Aldrich, and the monoclonal anti-E-tag antibody was from GE Healthcare. US3-specific mouse monoclonal antibodies were kindly provided by LeighAnne Olsen and Lynn Enquist (Princeton University, Princeton). Rabbit antibodies directed against PAK1, PAK2, and phosphoPAK1(T423)/PAK2(T402) were from Cell Signaling Technologies. HRP-conjugated secondary goat anti-rabbit and goat anti-mouse antibodies were purchased from Dako Cytomation. Mouse anti-actin antibodies were from Sigma Chemical. Monoclonal mouse anti-gB (1C11), anti-gE (18E8), and polyclonal porcine FITC-labeled anti-PRV antibodies were produced as described previously (5).

Inhibitors *C. difficile* toxin B, NSC23766, and NMPP1 were obtained from Calbiochem. Secramine A was a kind gift from the Kirchhausen lab (Harvard Medical School, Boston) and the Hammond lab (University of Louisville, Louisville, KY) and used as a specific inhibitor for Cdc42, as described previously (6). IPA3 was reported previously and used as described earlier (7).

The plasmids encoding PRV US3 (pKG1) and the kinase dead US3 mutant (pHF61) were described earlier (1, 8). The pTRIPΔU3-CMV-EGFP-WPRE vector (9) was a kind gift from B. Verhasselt (University of Ghent, Ghent, Belgium), whereas group A PAK inhibitor PID (10) was a kind gift from E. Manser (Institute of Molecular and Cell Biology, Singapore).

The plasmids pExpress-EGFP-rPAK2 (11), baculo His-PAK1 (7), pET-PAK2 (7), and the plasmid encoding WT PAK1 (12) were described previously. The PAK2M322G-encoding plasmid was made by mutating the pET-PAK2 vector.

Plasmids Cdc42DN(T19N) and Rac1DN(T17N) were described previously (13). Plasmid pGEX-GST-US3, used to produce recombinant US3 protein (14), was kindly provided by B. Klupp and T. Mettenleiter (Friedrich-Loeffler-Institute, Greifswald, Germany).

Transfections. Plasmids were transiently transfected in cells grown to 60–70% confluency by using Lipofectamine according to the manufacturer's instructions (Invitrogen) and were used at 24 h after transfection.

Immunohistochemistry. After being washed once in PBS, cells were fixed with 3% paraformaldehyde for 10 min, followed by permeabilization with 0.1% Triton X-100 for 2 min. Subsequently, cells were incubated with each antibody for 1 h at 37 °C. After that, cells were washed in PBS. Stainings were analyzed on a laser-scanning spectrum confocal system (TCS SP2; Leica Microsystems) linked to a microscope (DM IRBE; Leica Microsystems). Images were taken by using a 63× oil objective (N.A. 1.40–0.60) at room temperature and by using confocal acquisition software (Leica Microsystems). For triple stainings, an Olympus Cell*M system (Olympus) with Optigrid structured light illumination (Qioptic) was used.

Western Blotting. SDS/PAGE and Western blotting were performed as described previously (8). The blots were incubated for 1 h or overnight (according to the manufacturer's instructions) with primary antibodies and washed 3 times in 0.1% TBS/Tween-20 (TBS-T) buffer. Then, blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and after several washing steps they were developed with enhanced chemiluminescence (ECL; GE Healthcare). All incubation steps were done in 5% nonfat dry milk in TBS/T, except for the PAK antibodies, which were diluted in 5% BSA-TBS-T.

Binding Assay. Recombinant GST-tagged US3 and His-tagged PAK2 were produced in BL21 bacteria after 1 mM IPTG stimulation. US3 was purified with glutathione Sepharose 4 Fast Flow beads (GE Healthcare) and PAK2 by using Ni-NTA magnetic agarose beads (Qiagen) following the manufacturers' instructions. PAK2 was eluted from the beads by using elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, and 0.1% Tween-20, pH 8.0) and dialyzed overnight in interaction buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, and 0.005% Tween-20, pH 8.0). PAK2 was added to US3 bound on glutathione beads for 1 h. For PAK1 binding, recombinant GST-His-tagged PAK1 was purchased from Calbiochem. PAK1 in interaction buffer was added to the recombinant US3 protein for 1 h, and then US3 was immunoprecipitated by using monoclonal anti-US3 antibodies. After washing 3 times, samples of both assays were boiled in loading buffer and used for Western blot analysis.

Plaque Assay. Monolayers or sparsely seeded cells of the different cell lines were infected at an moi of 0.01 with either WT PRV or the US3 null mutant PRV virus. Two hours after infection, medium was replaced by 1% methylcellulose. At 24 hpi (sparsely seeded cells) or 48 hpi (monolayers), plaques were methanol-fixed and stained with anti-gB (1:30) monoclonal antibody, followed by anti-mouse FITC (1:200), and nuclei were stained by using 10 μg/mL Hoechst 33342 (Invitrogen) for 10 min before the final washing steps. Plaques were visualized by fluorescence microscopy, and the number of cells per plaque was determined.

1. Geenen K, Favoreel HW, Olsen L, Enquist LW, Nauwynck HJ (2005) The pseudorabies virus US3 protein kinase possesses anti-apoptotic activity that protects cells from apoptosis during infection and after treatment with sorbitol or staurosporine. *Virology* 331:144–150.
2. ten Klooster JP, Jaffer ZM, Chernoff J, Hordijk PL (2006) Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix. *J Cell Biol* 172:759–769.
3. Baskerville A (1973) The histopathology of experimental pneumonia in pigs produced by Aujeszky's disease virus. *Res Vet Sci* 14:223–228.
4. de Wind N, Zijderveld A, Glazenburg K, Gielkens A, Berns A (1990) Linker insertion mutagenesis of herpesviruses: Inactivation of single genes within the Us region of pseudorabies virus. *J Virol* 64:4691–4696.
5. Nauwynck HJ, Pensaert MB (1995) Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. *Arch Virol* 140:1137–1146.
6. Pelish HE, et al. (2006) Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation in vitro. *Nat Chem Biol* 2:39–46.
7. Deacon SW, et al. (2008) An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. *Chem Biol* 15:322–331.
8. Deruelle M, Geenen K, Nauwynck HJ, Favoreel HW (2007) A point mutation in the putative ATP binding site of the pseudorabies virus US3 protein kinase prevents Bad phosphorylation and cell survival following apoptosis induction. *Virus Res* 128:65–70.

9. Stove V, et al. (2005) Human immunodeficiency virus Nef induces rapid internalization of the T-cell coreceptor CD8alphabeta. *J Virol* 79:11422–11433.
10. Zhao ZS, et al. (1998) A conserved negative regulatory region in alphaPAK: Inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol Cell Biol* 18:2153–2163.
11. Jakobi R, Moertl E, Koeppel MA (2001) p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts. *J Biol Chem* 276:16624–16634.
12. Beeser A, Chernoff J (2005) Production and use of a cell permeable inhibitor of group A Paks (TAT-PID) to analyze signal transduction. *Methods* 37:203–207.
13. Schotte P, et al. (2004) Targeting Rac1 by the Yersinia effector protein YopE inhibits caspase-1-mediated maturation and release of interleukin-1beta. *J Biol Chem* 279:25134–25142.
14. Klupp BG, Granzow H, Mettenleiter TC (2001) Effect of the pseudorabies virus US3 protein on nuclear membrane localization of the UL34 protein and virus egress from the nucleus. *J Gen Virol* 82:2363–2371.

Fig. S1. Inhibition of Rho GTPases does not affect US3-mediated actin rearrangements in ST cells. (*A*) Pictures of ST monolayers (left image of each pair to visualize actin stress fibers) and sparsely seeded ST (right image of each pair, to determine cell projections), either mock-infected or at 12 h after inoculation with WT or US3 null PRV. Viral antigens are shown in green, with filamentous actin in red. Cell projections are indicated with arrowheads. (Scale bar: 10 μm.) (*B*) Effect of treatment with different Rho GTPase inhibitors or dominant-negative Cdc42 and Rac1 constructs on the percentage of PRV-infected (12 hpi) ST showing cell projections (white bars) or intact stress fibers (black bars). *, Significant differences compared with the WT PRV control at the 0.05 level. (*C*) Western blot showing expression of E-tagged dominant-negative Cdc42 and Rac1 constructs. (*D*) Pictures showing that the different Rho GTPase inhibitors used do not affect US3-mediated disassembly of actin stress fibers in ST monolayers (*Upper*) or US3-mediated formation of cell projections in sparsely seeded ST (*Lower*). (Scale bar: 10 μm.) (*E* and *F*) Pictures (*E*) and graphs (*F*) showing the ability of the different Rho GTPase inhibitors used to suppress spreading of seeded ST cells. (Scale bar: 20 μm.)

Fig. S2. Inhibition of Rho GTPases does not affect US3-mediated actin rearrangements in MEFs. (*A*) Effect of treatment with different Rho GTPase inhibitors or dominant-negative Cdc42 and Rac1 constructs on the percentage of PRV-infected (12 hpi) MEFs showing cell projections (white bars) or intact stress fibers (black bars). *, Significant differences compared with the WT PRV control at the 0.05 level. (*B*) Graphs showing the ability of the different Rho GTPase inhibitors used to suppress spreading of seeded MEF.

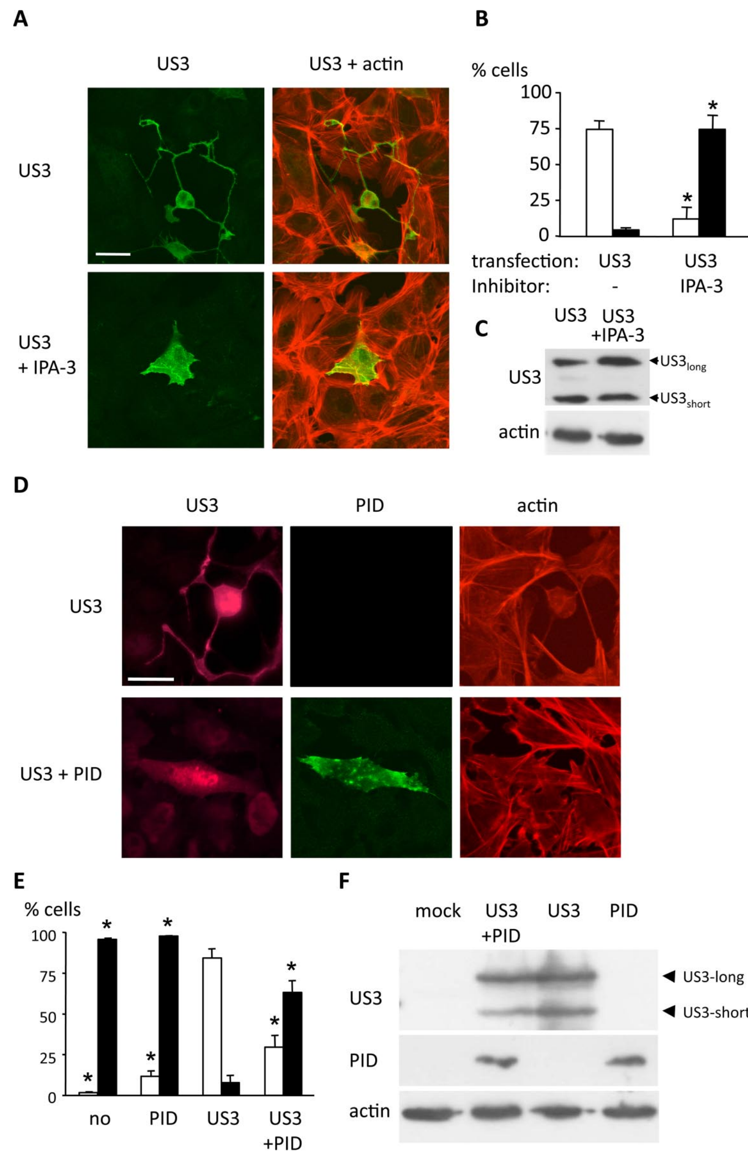

Fig. S3. Inhibition of PAKs impairs US3-induced actin rearrangements. (*A*) Pictures show actin architecture in US3-transfected ST in the presence or absence of the group A PAK inhibitor IPA-3. US3 is in green and actin in red. (Scale bar: 10 μm.) (*B*) Graphs show percentages of cells with cell projections (white bars) or intact actin stress fibers (black bars). *, Significant differences compared with the control at the 0.05 level. (*C*) Western blots show expression of US3 and actin as a loading control. Arrowheads indicate the positions of US3 long and short isoforms. (*D*) Pictures of MEFs transfected with US3 alone or cotransfected with US3 and PAK inhibitory domain (PID), and stained at 24 h after transfection for US3 (Cy5; magenta), PID (FITC; green) and filamentous actin (Texas red; red). (Scale bar: 10 μm.) (*E*) Effect of transfection of PID on the percentage of cells showing cell projections (white bars) or intact actin stress fibers (black bars). *, Significant differences compared with US3-transfected cells at the 0.05 level (*F*) Western blot showing expression of US3 and PID in mock-transfected MEFs or MEFs transfected with US3, PID, or both.

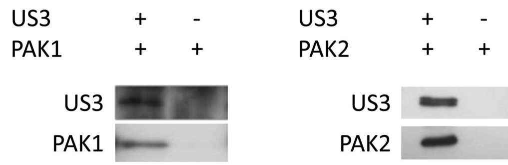

Fig. S4. US3 is able to bind PAKs 1 and 2. US3 pulldown assays using recombinant PAK1 (*Left*) or PAK2 (*Right*) with or without recombinant US3 were analyzed for the presence of US3 and PAK1 or PAK2.

Fig. S5. Lack of PAK1 or PAK2 does not affect expression of PRV structural proteins. (*A*) Western blots showing expression of PAK1 and PAK2 in MEFs, PAK1$^{-/-}$ MEFs, and PAK2$^{-/-}$ MEFs. Actin served as a loading control. (*B*) Western blots showing expression of viral envelope proteins gB, gE, and US3 in MEFs, PAK1$^{-/-}$ MEFs, and PAK2$^{-/-}$ MEFs at 0, 6, 12, and 24 h after inoculation with PRV. Actin served as a loading control. (*C*) Single-step growth curves of PRV in MEFs (solid lines), PAK1$^{-/-}$ MEFs (dotted lines), and PAK2$^{-/-}$ MEFs (dashed lines). (*Left*) Intracellular virus titers. (*Right*) Extracellular virus titers. For intracellular virus titers, no significant differences at the 0.05 level could be observed at any time point. For extracellular virus titers, no significant differences could be observed between PAK1$^{-/-}$ MEFs and wild-type MEFs, whereas significant differences could be observed between PAK2$^{-/-}$ MEFs and wild-type MEFs at 12 and 24 h after inoculation.

PRV actin merge

MEF

PAK1$^{-/-}$

PAK2$^{-/-}$

Fig. S6. Lack of group A PAKs impairs PRV-induced actin rearrangements. Pictures of low-moi experiments showing single PRV-infected cells in between noninfected cells for MEFs (*Top*), PAK1$^{-/-}$ MEFs (*Middle*), and PAK2$^{-/-}$ MEFs (*Bottom*). PRV antigens are in green and filamentous actin in red. Arrowheads indicate long cell projections in MEFs and shorter cell projections in PAK2$^{-/-}$ MEFs. (Scale bar: 10 μm.)

Fig. S7. Lack of group A PAKs results in impaired intercellular spread of PRV. (*A*) Pictures of representative plaques of sparsely seeded MEFs, PAK1$^{-/-}$ MEFs, or PAK2$^{-/-}$ MEFs at 24 h after inoculation at a low moi with WT or US3 null PRV. (Scale bar: 20 μm.) (*B*) Pictures of representative plaques of monolayers of MEFs, PAK1$^{-/-}$ MEFs, or PAK2$^{-/-}$ MEFs at 24 h after inoculation at a low moi with WT or US3 null PRV. (Scale bar: 100 μm.)