Rapid parallel flow cytometry assays of active GTPases on effector beads.

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Supplemental Note 1.

Sin Nombre virus (SNV, Bunyaviridae, Hantavirus) is an enveloped virus first isolated in the Southwestern region of the U.S. and carried by the deer mouse *Peromyscus maniculatus*. Its genome is comprised of three negative-sense RNA segments, and belongs to the *Hantavirus* genus of the family *Bunyaviridae* of the New World. It is the primary causative agent of hantavirus cardiopulmonary syndrome (HCPS) in North America [1-4], where it is transmitted to humans by inhalation of virus-contaminated excreta [1-4]. HCPS is characterized by pulmonary edema due to capillary leak, followed by cardiogenic shock. The case fatality rate of HCPS is approximately 40%. No vaccine or specific therapy is widely available at present, except in specialized facilities where successful treatment of HCPS in the US relies on the use of extracorporeal membrane oxygenation (ECMO)[5]. Thus, SNV-induced HCPS is a highly fatal, relatively untreatable disease.

Because of BSL-3 limitations, little is known about the mechanism of hantavirus entry and disease pathogenesis. UV-killed, purified and well-characterized fluorescently labeled hantaviruses constitute an excellent model system to study a BSL-3 pathogen using the equipment and resources available to a BSL-2 laboratory.[6-9]

Epithelial cells present formidable barriers against pathogens, where the outward facing apical membrane of polarized epithelial cells lacks cognate receptors that are necessary for entry. The cognate cell entry receptor of pathogenic hantaviruses is $\alpha_v\beta_3$ integrin commonly expressed at basolateral domains of permissive cells.[10] Until recently it was not clear how blood-borne hantaviruses could access $\alpha_v\beta_3$ integrin in polarized microvascular endothelial cells when lateral junctions are still intact. It was recently reported that apically expressed complement-interacting proteins[11], decay-accelerating factor (DAF/CD55) and gC1qR/p32 are co-receptors for hantavirus entry. When localized in lipid rafts,[12] DAF forms complexes with Src family tyrosine kinases[13] that signal upstream of Rap, Rho and Rab GTPases and consequently regulate cytoskeletal alterations, and trafficking as needed for supporting the lifecycle of the virus.

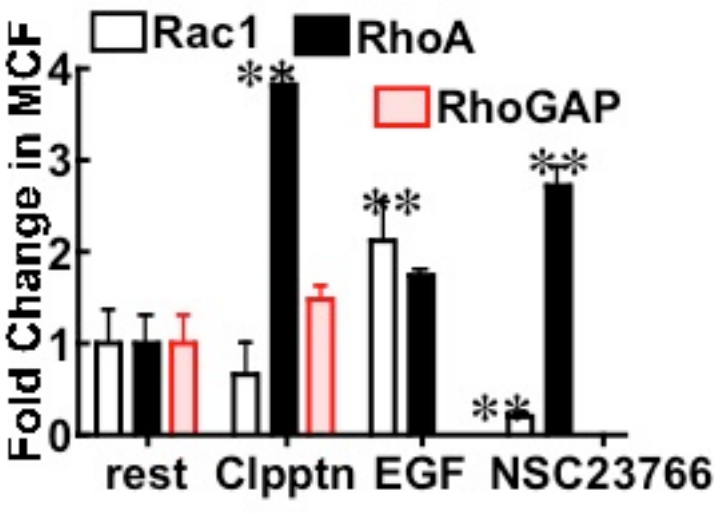

Fig S1. G-trap validation assay in Vero E6 cells. Serum starved Vero E6 cells were stimulated with: Calpeptin (clpptn) to activate RhoA, EGF to activate Rac1 and RhoA, NSC23766 to suppress Rac1 activity. The results mirror the results obtained using Hela cells described in the main text. Calpeptin-activated samples were subsequently exposed to 2µg/µl P50RhoGAP, which was used to catalyze the hydrolysis of GTP by RhoA. As shown, the level of RhoAGTP in p50RhoGAP treated samples decreased to near baseline levels after compared to the control untreated samples. Control samples (rest and non-cognate effector beads) were mock-treated with 0.1% DMSO to account for compound solvent. The errors represent standard deviation of 3 independent experiments measured in duplicate each time.

Supplemental Note 2. Integrins are allosterically flexible adhesion molecules that operate by means of conformational changes.[14] Conformational changes are associated with the response to cell stimulation through cellular receptors (inside-out signal) or from direct engagement of ligands or cations such as Mn^{2+} (outside in). Activated integrins undergo affinity related conformational changes, which expose neo-epitopes known as ligand-induced binding-sites (LIBS).[15, 16] Certain antibodies preferentially bind to the neo-epitopes. Here we have used AP-5 antibodies, which recognize the epitopes presented in extended conformation $\alpha_v\beta_3$ integrins.[15]

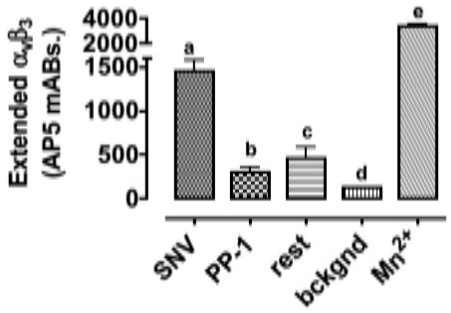

Fig. S2. To establish quantitative limits on our observations on integrin activation we used a plate reader to obtain average intensity readings of 20,000 cells exposed to virus. We used a selective Src-family kinase inhibitor ($p56^{lck}$ and $p59^{fynT}$) PP1 to connect Src kinase activity upstream of integrin activation[17, 18], and Mn^{2+} as a positive control for integrin activation. Virus exposure resulted in integrin activation 3-fold above that seen in resting cells, which was abolished by PP-1 treatment, demonstrating the specificity of the response. At the dose of virus used, integrin activation was 2-fold less than maximal activation induced by manganese treatment. The inhibition of integrin activation by PP1 suggests that integrin activation is mediated by a Src family kinase downstream of DAF ligation.

Supplemental Note 3. To visualize Rab7 dependant endocytic traffic of virus particles by microscopy, low temperature incubation (15°C for 30 min) was used to arrest the virus in early endosomes. Synchronized trafficking of SNV^{R18} was then initiated by rapidly raising the temperature of the cells to 37°C and the progress of itinerant SNV^{R18} was monitored by fixing and staining cell monolayers with anti Rab 7 antibodies at selected time intervals. Colocalization of SNV^{R18} with immunostained endogenous Rab7 GTPase was *qualitatively* analyzed at each time point. At 15°C, (0' in Figure S3) a significant fraction of the Rab7 proteins was notably distributed at the perinuclear region. After 3 min at 37°C the Rab7 positive endosomes redistribute from perinuclear space and move towards the cell periphery and begin to colocalize with cargo. Increased colocalization was accompanied by the onset of fusion of viral envelope membranes with endosomes as evident from the dequenching of R18 in maturing early endosomes. [22-24] After 10 min increasing fluorescence intensity co-localized with Rab7-positive endosomes. After 30 min at 30°C Rab7 appeared to disengage from R18 stained organelles, as shown by the change in color from yellow to red.

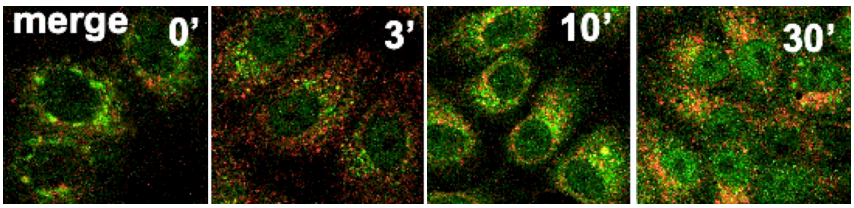

Fig. S3. Endocytosed SNV^{R18} (orange) partially colocalizes with Rab7 (green). The progress of itinerant SNV^{R18} (SNVR18 becomes brighter after membrane fusion) was monitored by fixing and staining cell monolayers with anti-Rab 7 antibodies at selected time intervals and confocal imaging. After 3 min the Rab7 redistributed from random distribution around perinuclear space (**0'**) to polarized morphology. We used Slidebook software to quantify colocalization. Colocalization of SNV^{R18} and Rab7 positive endosomes peaked at 10min and declined thereafter. After 10 min SNV^{R18} was in bright punctate structures that co-localized with the distinctly perinuclear Rab7. After 30 min at 30°C, the R18 stained organelles were largely negative for Rab7. Suggesting the maturing of Rab7 positive late endosomes into some other unlabelled organelle (e.g. lysosomes).

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