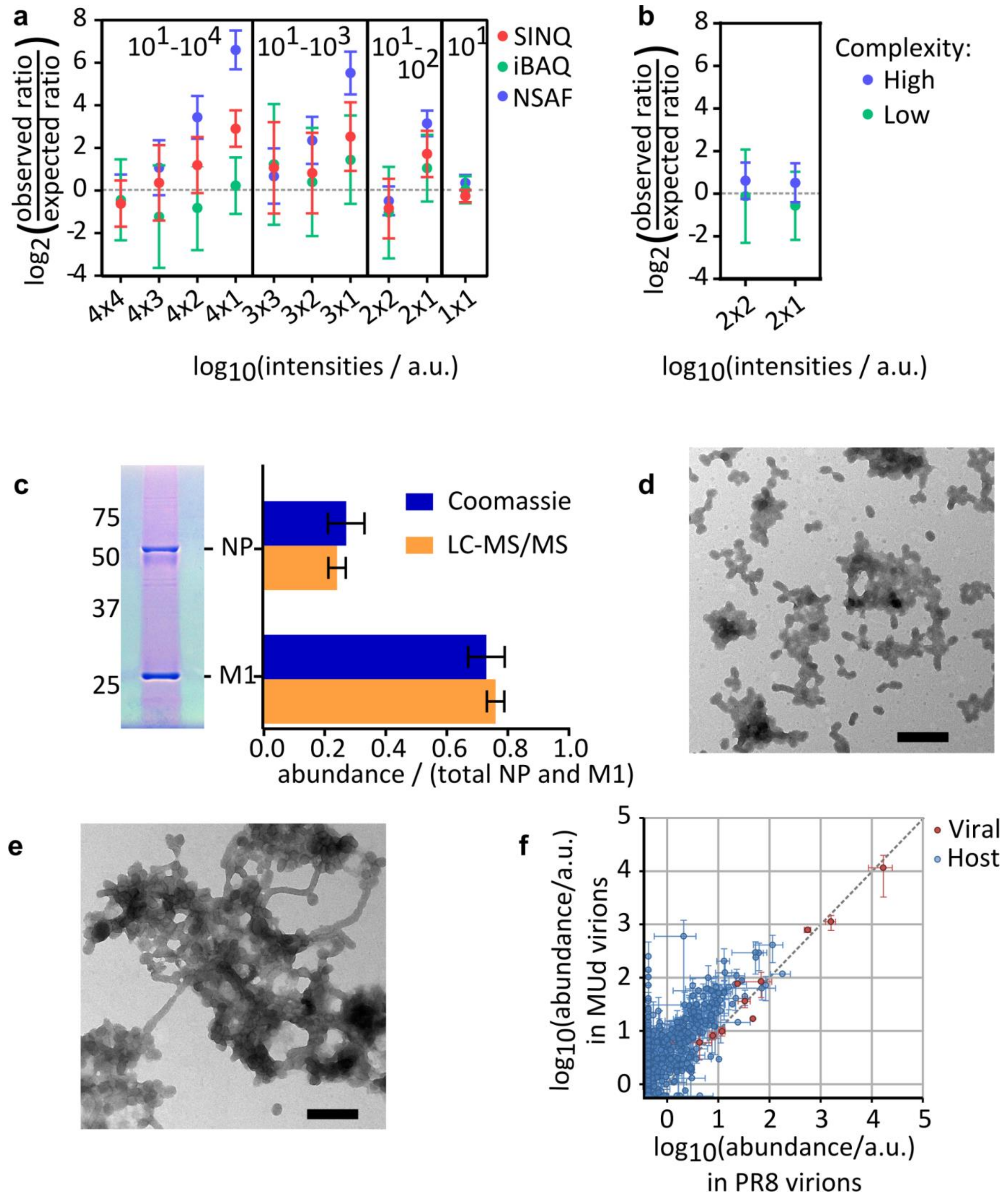
Supplementary Figure 1

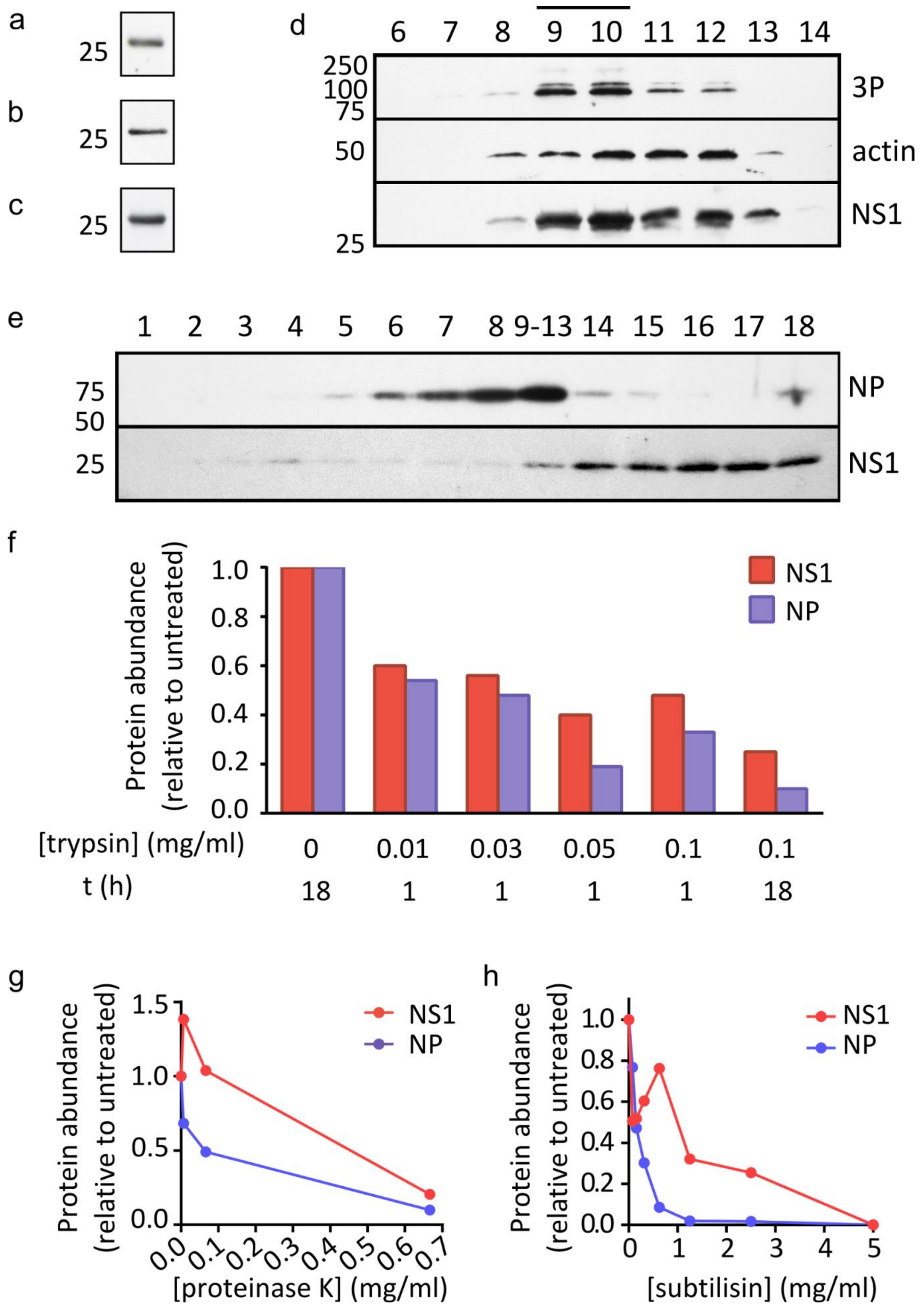


Supplementary Figure 1: Determination of pairwise protein ratios in a complex mixture by label-free absolute protein quantitation.

(**a**) A mixture of protein standards whose abundances differed by 10-fold amounts was digested with trypsin and injected three times into a Q Exactive mass spectrometer for LC-

MS/MS. The ratio of each protein detected to every other protein was calculated from mass spectra using three different methods: SINQ, iBAQ and NSAF (see Methods). For each expected ratio (1:1, 1:10, etc), the mean and s.d. of $\log_2$(observed ratio / expected ratio) is shown. (**b**) To test the accuracy of SINQ in complex mixtures, tryptic digests of material collected from the media of infected cells (a high-complexity mixture) and from uninfected cells (a low-complexity mixture) were added to the digested protein standards. The additional material had been purified by HAd and ultracentrifugation (see Fig. 3 for details), and was added such that the most abundant protein standards had a similar abundance to the viral polymerase in the infected sample. LC-MS/MS was performed using a Q Exactive mass spectrometer and protein ratios were determined by SINQ, taking the mean values from three experiments. The mean and s.d. of $\log_2$(observed ratio/expected ratio) is shown for comparisons between two levels of protein standards (10:10 and 10:1). (**c**) SINQ was compared to an orthologous method for determining protein ratios. Purified virions were lysed, separated by SDS-PAGE and stained with Coomassie Brilliant Blue. NP and M1 proteins (intense bands) were identified by electrophoretic mobility and quantified by densitometry. Separately, viruses were purified and analysed by LC-MS/MS and SINQ. The means and s.d.s of 17 (Coomassie) and 3 (LC-MS/MS) experiments are shown. When extending this analysis to other viruses it was necessary to confirm that samples consisted largely of spherical virions. To assess this, purified virions of PR8 (**d**), and MUd (**e**) were negatively-stained and visualised by EM; scale bars are 0.5 µm. (**f**) The abundance of proteins detected in purified virions of PR8 and MUd, determined by SINQ. A threshold was set at one tenth the abundance of the least abundant polymerase subunit, and any protein of lower abundance was assigned this value. The means and ranges of two experiments are shown.

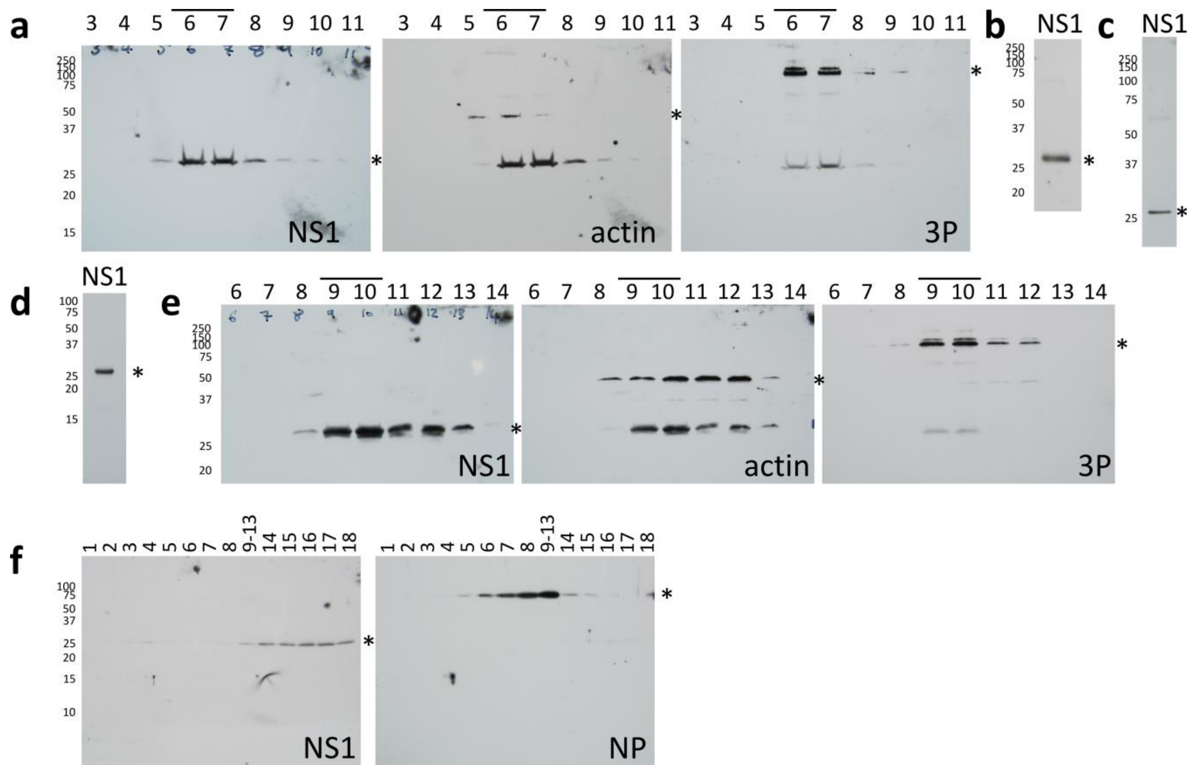
Supplementary Figure 2



Supplementary Figure 2: Evidence for NS1 in influenza virions.

Virions were purified without HAd from the media of bovine epithelial (MDBK) cells infected with WSN. Their protein content was analysed by SDS-PAGE and Western blotting using (**a**) the rabbit anti-NS1 antibody used in Fig. 1e, (**b**) a different rabbit anti-NS1 antibody and (**c**) a sheep anti-NS1 antibody. All antibodies gave a clear band close to the position of a 25 kDa molecular weight marker (indicated). (**d**) Virions were typically purified using a sucrose density gradient (Fig 1e). As an alternative method, material purified using a 10 – 40 % gradient of OptiPrep™ and harvested in 15 fractions from the top. Concentrated virions were visible between fractions 9 and 10 (line). The indicated fractions were analysed by Western blotting using, for NS1, the same antibody as in (**a**); the positions of molecular weight markers are indicated in kDa. (**e**) Virions (purified using a sucrose density gradient) were lysed and then separated though a 33 – 70 % glycerol gradient, which was harvested as 18 fractions from the bottom. Fractions were analysed by Western blotting; the position of molecular weight markers is shown in kDa. Fractions 9-13, which contain the majority of the viral ribonucleoproteins, were pooled. (**f**-**h**) Virions were purified using a sucrose density gradient and then subjected to proteolytic digest. Purified virions were either (**f**) stored overnight at 4 °C then digested with trypsin for either 1 h at room temperature or 18 h at 10 °C; (**g**) stored at -80 °C then digested with proteinase K for 1h at 37 °C; or (**h**) digested, immediately after purification, with subtilisin A for 16 h at 37 °C. Undigested proteins were quantified by Western blotting and densitometry.

Supplementary Figure 3



Supplementary Figure 3: Uncropped scans of Western Blots

Uncropped images of Western blots shown in other figures. The proteins bound by primary antibodies are named and their positions are indicated with asterisks. Where multiple antibodies were used, images obtained with each antibody are shown in sequence. For panels (**a**), (**e**) and (**f**) the lane numbers are those of the corresponding figures. Panels correspond to (**a**) Fig. 1e, (**b**) Supplementary Fig. 2a, (**c**) Supplementary Fig. 2b, (**d**) Supplementary Fig. 2c, (**e**) Supplementary Fig. 2d and (**f**) Supplementary Fig. 2e.