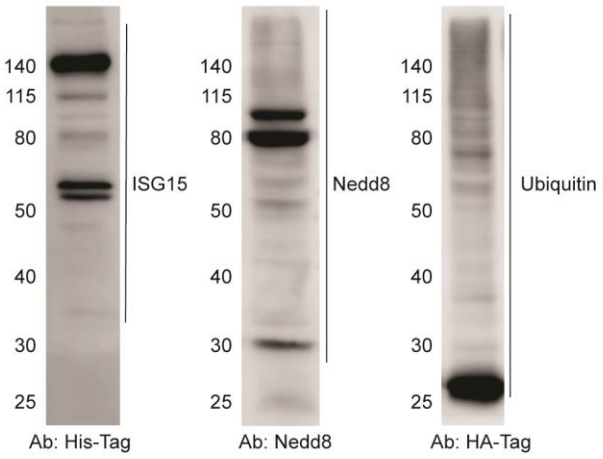
Supplemental information:

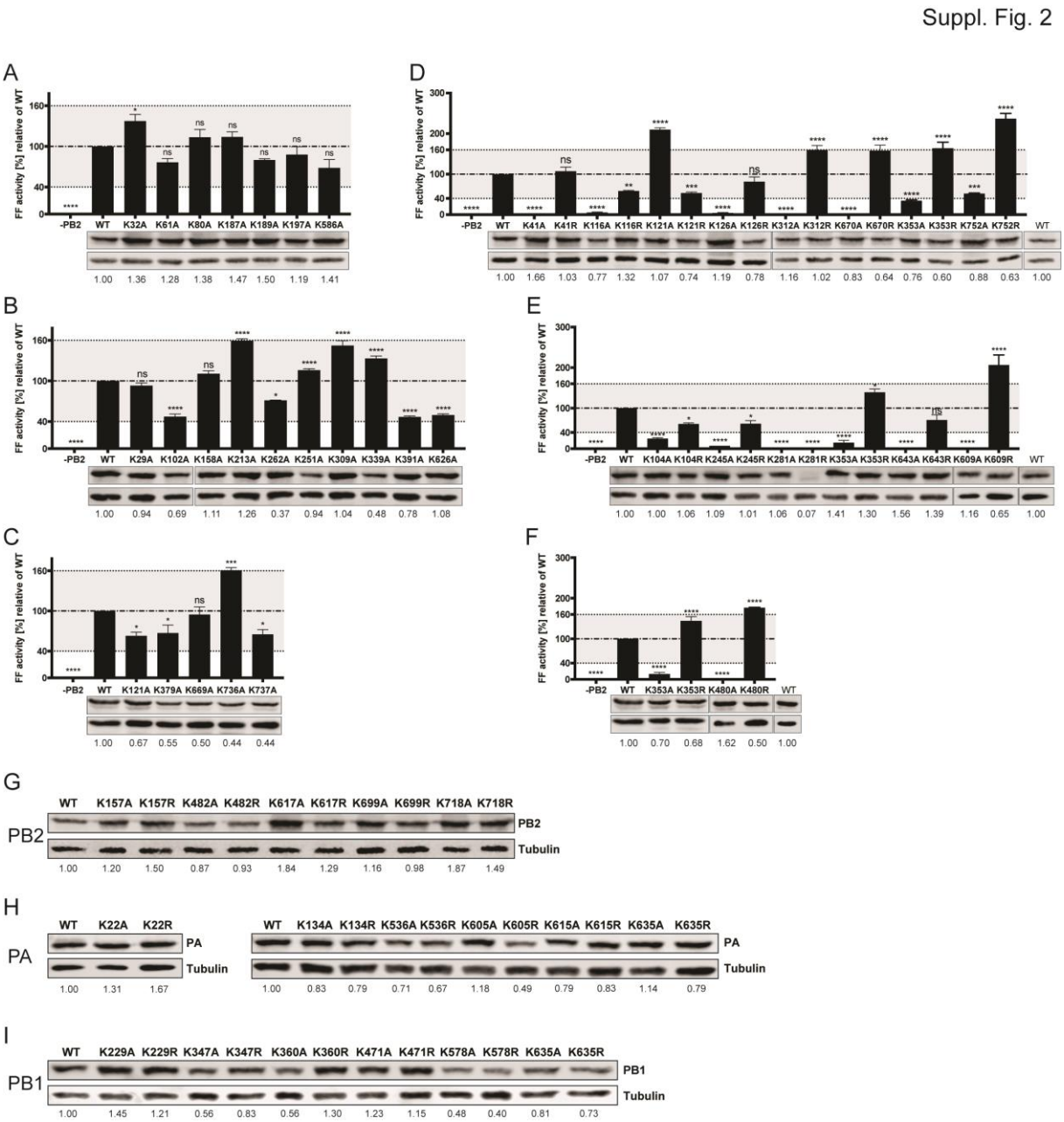
Suppl. Figure 1:



Suppl. Figure 1: Overexpression and immuno-detection of ubiquitin-like modifiers.

HEK293-T cells were transfected with plasmids expressing HA-tagged ubiquitin (**A**), HA-tagged NEDD8 (**B**) or His-tagged ISG15 (**C**). 24 h p.t., cells were lysed and analyzed by western blot. Ubiquitin, NEDD8 and ISG15 were detected using antibodies specific for HA-tag, NEDD8 or His-tag, respectively.

Suppl. Figure 2:

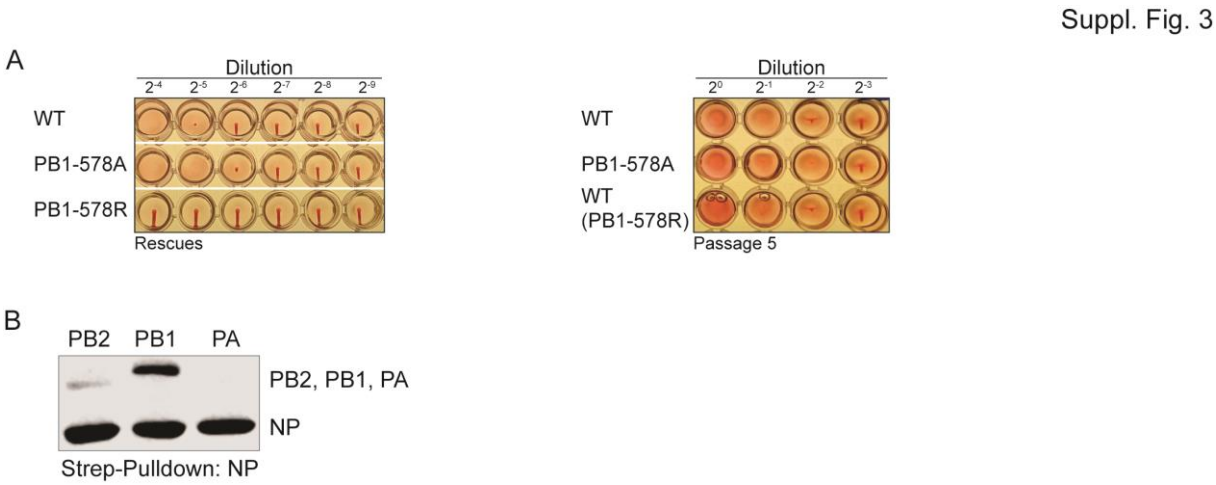


Suppl. Figure 2: Effects of lysine mutation to A and R on protein stability and viral polymerase activity.

A-F: Polymerase reconstitution assay of the mutated polymerase complex with A/R-substitutions of the UBL modified lysines in PB2 (**B, E**), PA (**C, F**) and PB1 (**D, G**). Depicted are positions showing polymerase activity within a cut-off of ± 60% wild type activity (indicated by grey boxes) upon A mutation (B-D) and positions for which introduction of an arginine reverted the significant change in polymerase activity upon A mutation. Relative FF activities are reported as the mean percentage activity relative to WT (±SEM), n=3. Statistical significance was assessed using a multiple comparison one-way ANOVA-test (*p ≤ 0.05; **p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). For evaluating the expression levels of PB2, PB1 and PA HEK293-T cells were transfected with plasmids encoding either wild type or mutant PB2,

PB1 or PA. At 24 hp.t., expression levels were analyzed by western blot. Quantified expression levels are reported below as relative values to the wild type protein. Tubulin expression served as the housekeeping control. **G-H:** Expression of mutant PB2 (G), PA (I) and H (PB1) was assessed as described above.

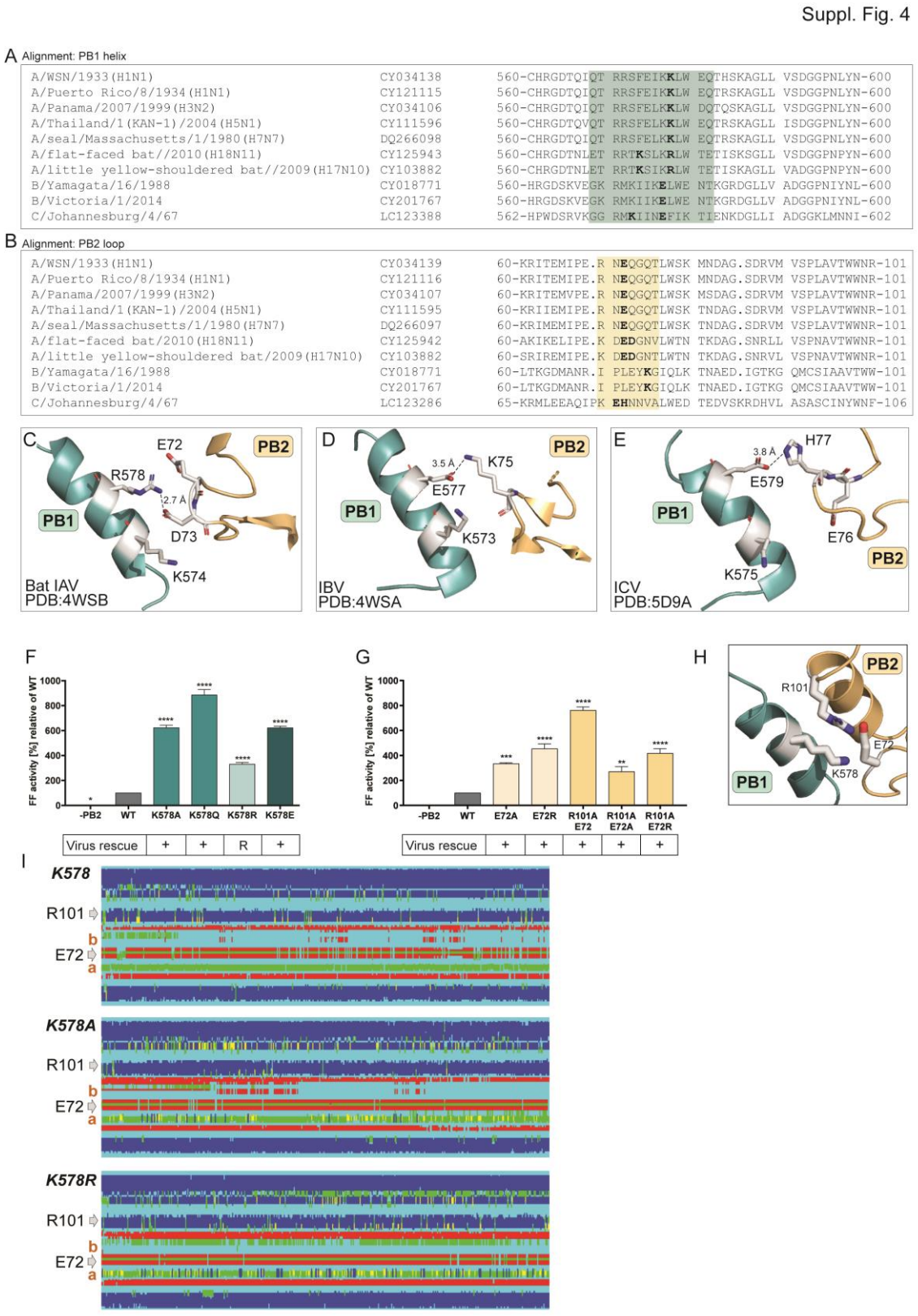
Suppl. Figure 3:



Suppl. Figure 3: Effect of PB1-K578A/R mutations on HI titers and viral replication.

A: Determination of the HI titers of recombinant viruses. Virus rescue was carried out using the pHW2000 plasmid system with either wild type or mutant PB1. 48 h p.t., HI titers in the supernatants after rescue were evaluated using same volume of undiluted supernatant. Viruses were propagated on A549 cells for 5 passages (MOI: 0.01, except for the first passage of PB1-K578R: MOI=0.001) and viral titers were examined 48 h. p.i. HI titers at passage 5 were determined by using 10^6 PFU. PB1-K578R was classified as reversion to WT based on sequencing analyses. **B:** Assessment of NP binding to the individual polymerase subunits PB2, PB1 and PA using co-affinity precipitation. HEK293-T cells were transfected with wild type PB1, PB2, PA together with strep-tagged NP, respectively. At 24 h p.t. polymerase subunits bound to NP were strep-purified and analyzed by western blot.

Suppl. Figure 4:



Suppl. Figure 4: Conservation and structural environment of PB1-K578 and PB2-E72 in different influenza viruses

A-B: Sequence alignment of PB1 AA 560-600. The PB1-K578 helix is highlighted in cyan (**A**) and PB2 AA 60-101. The N-terminal PB2 loop is highlighted in yellow (**B**) in the indicated strains. **C-E:** Zoom-in into 3D structures of bat influenza virus A (**C**; PDB: 4WSB; vRNA-promoter bound structure), influenza virus B (**D**; PDB:4WSA; vRNA-promoter bound structure) and Influenza virus C (**E**; PDB: 5D9A) and illustration of interacting amino acids located in the interface that covers the PB1-578-helix (cyan) and the N-terminal PB2 loop (yellow). Distances between electrostatically interacting amino acids, that reside in the PB1-helix or the PB2 loop were measured using PYMOL and are indicated in Ångstrom. **F-G:** Polymerase reconstitution assay of the mutated polymerase complex with indicated substitutions PB1 (**F**) and PB2 (**G**). Relative FF activities are presented as the mean percentage activity relative to WT (±SEM), n=3. Statistical significance was assessed using a multiple comparison one-way ANOVA-test (*** $p \leq 0.001$; **** $p \leq 0.0001$). Success (+) or reversion to WT (R) to generate recombinant viruses is depicted below. **H:** Illustration of the localization of PB1-K578, PB2-E72 and PB2-R72 in the WSN 3D structure (vRNA-bound conformation). **I:** Illustration of the dynamics of secondary structures in the PB2 N-terminal domain depending on the amino acid at position PB1-578 (K578: upper panel, K578A: middle panel, K578R: lower panel). Positions PB2-E72 and R101 as well as distal regions of the PB2 loop (region a: aa 63-67; region b: aa 78-82) are indicated. The types of secondary structure were analyzed YASARA following molecular dynamic simulations for 100 ns and are illustrated using a color scheme (dark blue: helix, yellow: 3^{10} helices, red: beta sheet, green: turn, lighter blue: coil).