## Supplemental information:



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

HEK293-T cells were transfected with plasmids expressing His-tagged ISG15, HA-tagged NEDD8, HA-tagged ubiquitin. 24 h p.t., cells were lysed and analyzed by western blot. ISG15, NEDD8 and ubiquitin were detected using the indicated antibodies. Panels show representative blot of four independent experiments. MW, molecular weight marker.



## Supplementary Figure 2: Effects of lysine mutation to A and R on protein stability, polymerase activity and HI-titers.

**a-f** Polymerase reconstitution assay of the mutated polymerase complex with A/R-substitutions of the UB/UBL modified lysines in PB2 (a, d), PA (b, e) and PB1 (c, f). Depicted are positions showing polymerase activity within a cut-off of ± 60% wild type activity (indicated by grey boxes) upon A mutation 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 independent biological replicates. P values <0.05 compared to WT from Dunnett's multiple comparison one-way ANOVA-test are indicated. 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 h p.t., expression levels were analyzed by western blot using the indicated antibodies. Representative blots from one experiment are shown. Quantified expression levels are reported below as relative values to WT. Tubulin expression served as the housekeeping control. **g-i** Expression of mutant PB2 (g), PA (h) and PB1 (i) was assessed as described above. **j** Illustration of the localization of PA-K22 in the 3D structures of the 1918 H1N1 polymerase heterotrimer (PDB: 7NHA; Supplementary Table 1) showing the K22 containing hinge region of the PA endonuclease domain (violet) and the interacting sites in the PB1 subunit (cyan). Created with ChimeraX. **k-I** Determination of the titers of

recombinant viruses using the hemagglutination inhibition (HI) assay. 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 (k) were evaluated using the 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 (I) were determined by using 10<sup>6</sup> PFU. PB1-K578R was classified as reversion to WT based on sequencing analyses. Source data are provided as a Source data file.

ingrittent. FBT heix					
A/WSN/1933(H1N1)	CY034138	560-CHRGDTQIQT	RRSFEIK <b>K</b> LW	EQTHSKAGLL	VSDGGPNLYN-600
A/Puerto Rico/8/1934(H1N1)	CY121115	560-CHRGDTQIQT	RRSFEIK <b>k</b> lw	EQTRSKAGLL	VSDGGPNLYN-600
A/Panama/2007/1999(H3N2)	CY034106	560-CHRGDTQIQT	RRSFELK <b>k</b> LW	DQTQSKAGLL	VSDGGPNLYN-600
A/Thailand/1(KAN-1)/2004(H5N1)	CY111596	560-CHRGDTQVQT	RRSFELK <b>k</b> LW	EQTRSKAGLL	ISDGGPNLYN-600
A/seal/Massachusetts/1/1980(H7N7)	DQ266098	560-CHRGDTQIQT	RRSFELK <b>k</b> LW	EQTRSKAGLL	VSDGGPNLYN-600
A/flat-faced bat//2010(H18N11)	CY125943	560-CHRGDTNLET	RRT <b>K</b> SLK <b>R</b> LW	TETISKSGLL	VSDGGPNPYN-600
A/little yellow-shouldered bat//2009(H17N10)	CY103882	560-CHRGDTNLET	RRT <b>K</b> SIK <b>R</b> LW	TETISKAGLL	VADGGPNPYN-600
B/Yamagata/16/1988	CY018771	560-HRGDSKVEGK	RMKIIK <b>e</b> lwe	NTKGRDGLLV	ADGGPNIYNL-600
B/Victoria/1/2014	CY201767	560-HRGDSKVEGK	RMKIIK <b>e</b> lwe	NTKGRDGLLV	ADGGPNIYNL-600
C/Johannesburg/4/67	LC123388	562-HPWDSRVKGG	RM <b>K</b> IIN <b>E</b> FIK	TIENKDGLLI	ADGGKLMNNI-602
lignment: PB2 loop					
A/WSN/1933(H1N1)	CY034139	60-KRITEMIP <mark>E.R</mark>	NEQGQTLWSK	MNDAG.SDRVM	I VSPLAVTWWNR-101
A/Puerto Rico/8/1934(H1N1)	CY121116	60-KRITEMIP <mark>E.R</mark>	NEQGQTLWSK	MNDAG.SDRVM	I VSPLAVTWWNR-101
A/Panama/2007/1999(H3N2)	CY034107	60-KRITEMVPE.R	NEQGQTLWSK	MSDAG.SDRVM	I VSPLAVTWWNR-101
A/Thailand/1(KAN-1)/2004(H5N1)	CY111595	60-KRIIEMIPE.R	NEQGQTLWSK	TNDAG.SDRVM	I VSPLAVTWWNR-101
A/seal/Massachusetts/1/1980(H7N7)	DQ266097	60-KRIMEMIP <mark>E.R</mark>	NEQGQTLWSK	TNDAG.SDRVM	I VSPLAVTWWNR-101
A/flat-faced bat/2010(H18N11)	CY125942	60-AKIKELIPE.K	DEDGNVLWTN	TKDAG.SNRLI	VSPNAVTWWNR-101
A/little yellow-shouldered bat/2009(H17N10)	CY103882	60-SRIREMIPE.K	DEDGNTLWTN	TKDAG.SNRVI	VSPNAVTWWNR-101
B/Yamagata/16/1988	CY018771	60-LTKGDMANR.I	PLEYKGIQLK	TNAED.IGTKC	QMCSIAAVTWW-101
B/Victoria/1/2014	CY201767	60-LTKGDMANR.I	PLEYKGIQLK	TNAED.IGTKO	QMCSIAAVTWW-101
C/Johannesburg/4/67	LC123286	65-krmleeaq <mark>ipk</mark>	EHNNVALWED	TEDVSKRDHVI	ASASCINYWNF-106
	A/WSN/1933 (H1N1) A/WSN/1933 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Puama/2007/1999 (H3N2) A/Thailand/1 (KAN-1)/2004 (H5N1) A/seal/Massachusets/1/1980 (H7N7) A/flat-faced bat//2010 (H18N11) A/little yellow-shouldered bat//2009 (H17N10) B/Yamagata/16/1988 B/Victoria/1/2014 C/Johannesburg/4/67 Kgment PB2 loop A/WSN/1933 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Puerto Rico/8/1934 (H1N1) A/Faland/1 (KAN-1)/2004 (H5N1) A/Tailand/1 (KAN-1)/2004 (H5N1) A/Tailand/1 (KAN-1)/2004 (H5N1) A/flat-faced bat/2010 (H18N11) A/little yellow-shouldered bat/2009 (H17N10) B/Yamagat/16/1988 B/Victoria/1/2014 C/Johannesburg/4/67	NWSN/1933 (H1N1) CY034138   A/WSN/1933 (H1N1) CY121115   A/Puerto Rico/8/1934 (H1N1) CY121115   A/Panama/2007/1999 (H3N2) CY034106   A/Thailand/1 (KAN-1) /2004 (H5N1) CY11596   A/seal/Massachusetts/1/1980 (H7N7) DQ266098   A/flat-faced bat//2010 (H18N11) CY125943   A/little yellow-shouldered bat//2009 (H17N10) CY103882   B/Yamagata/16/1988 CY018771   B/Victoria/1/2014 CY201767   C/Johannesburg/4/67 LC123388   Kgmment: PB2 loop CY034107   A/Thailand/1 (KAN-1)/2004 (H5N1) CY121116   A/Puerto Rico/8/1934 (H1N1) CY034107   A/Taailand/1 (KAN-1)/2004 (H5N1) CY034107   A/Thailand/1 (KAN-1)/2004 (H5N1) CY12116   A/seal/Massachusetts/1/1980 (H7N7) DQ266097   A/little yellow-shouldered bat/2009 (H17N10) CY125942   A/little yellow-shouldered bat/2009 (H17N10) CY103882   B/Yamagata/16/1988 CY018771   B/Yamagata/16/1986 CY018771   B/Yanegata/16/1986 CY01677   C/Johannesburg/4/67 LC123	Nymetri Dirichi CY034138 560-CHRGDTQIQT   A/WSN/1933 (H1N1) CY034106 560-CHRGDTQIQT   A/Puerto Rico/8/1934 (H1N1) CY121115 560-CHRGDTQIQT   A/Panama/2007/1999 (H3N2) CY034106 560-CHRGDTQUQT   A/Thailand/1 (KAN-1)/2004 (H5N1) CY111596 560-CHRGDTQUQT   A/seal/Massachusets/1/1980 (H7N7) DQ266098 560-CHRGDTNLET   A/flat-faced bat//2010 (H18N11) CY125943 560-CHRGDTNLET   A/little yellow-shouldered bat//2009 (H17N10) CY103882 560-CHRGDTNLET   B/Yamagata/16/1988 CY018771 560-HRGDSKVEGK   C/Johannesburg/4/67 LC123388 562-HPWDSRVKGG   Igmment: PB2 loop - -   A/Thailand/1 (KAN-1) /2004 (H5N1) CY121116 60-KRITEMIPE.R   A/Puerto Rico/8/1934 (H1N1) CY121116 60-KRITEMIPE.R   A/Puerto Rico/8/1934 (H1N1) CY121116 60-KRITEMIPE.R   A/Palama/2007/1999 (H3N2) CY034107 60-KRITEMIPE.R   A/Thailand/1 (KAN-1) /2004 (H5N1) CY111595 60-KRITEMIPE.R   A/Talat-faced bat/2010 (H18N11) CY125942 60-ARITEMIPE.R	Nymetri Diricki CY034138 560-CHRGDTQIQT RRSPEIKKLW   A/WSN/1933 (H1N1) CY034138 560-CHRGDTQIQT RRSPEIKKLW   A/Puerto Rico/8/1934 (H1N1) CY121115 560-CHRGDTQIQT RRSPEIKKLW   A/Panama/2007/1999 (H3N2) CY034106 560-CHRGDTQIQT RRSPEIKKLW   A/Thailand/1 (KAN-1)/2004 (H5N1) CY111596 560-CHRGDTQIQT RRSPEIKKLW   A/flat-faced bat//2010 (H18N11) CY125943 560-CHRGDTNLET RRTKSLKRLW   A/flat-faced bat//2010 (H18N11) CY125943 560-CHRGDTNLET RRTKSLKRLW   A/flat-faced bat//2010 (H18N11) CY103882 560-CHRGDTNLET RRTKSLKRLW   B/Yamagata/16/1988 CY018771 560-HRGDSKVEGK RMKIIKELWE C/Johannesburg/4/67 LC123388 562-HPWDSRVKGG RMKIIKELWE   C/Johannesburg/4/67 LC123388 562-HPWDSRVKGG RMKIINEFIK REQGQTLWSK   A/Puerto Rico/8/1934 (H1N1) CY121116 60-KRITEMIPE.R NEQGQTLWSK   A/Panama/2007/1999 (H3N2) CY034107 60-KRITEMIPE.R NEQGQTLWSK   A/Tailand/1 (KAN-1)/2004 (H5N1) CY111595 60-KRITEMIPE.R NEQGQTLWSK	Nymetri Dirika CY034138 560-CHRGDTQIQT RRSFEIKKLW EQTHSKAGLL   A/WSN/1933 (H1N1) CY034106 560-CHRGDTQIQT RRSFEIKKLW EQTHSKAGLL   A/Panama/2007/1999 (H3N2) CY034106 560-CHRGDTQIQT RRSFEIKKLW EQTRSKAGLL   A/Thailand/1 (KAN-1)/2004 (H5N1) CY11159 560-CHRGDTQIQT RRSFEIKKLW EQTRSKAGLL   A/seal/Massachusets/1/1980 (H7N7) DQ266098 560-CHRGDTNLET RRTKSIKRLW EQTRSKAGLL   A/flat-faced bat//2010 (H18N11) CY125943 560-CHRGDTNLET RRTKSIKRLW EQTRSKAGLL   A/little yellow-shouldered bat//2009 (H17N10) CY103882 560-CHRGDTNLET RRTKSIKRLW TETISKAGLL   B/Yamagata/16/1988 CY018771 560-HRGDSKVEGK RMKIIKELWE NTKGRDGLV   C/Johannesburg/4/67 LC123388 562-HPWDSRVKGG RMKIINEFIK TIENKDGLL   Igmment PB2 loop  A/Fanama/2007/1999 (H3N2) CY034107 60-KRITEMIPE.R NEQGQTLWSK MNDAG.SDRVH   A/Paerto Rico/8/1934 (H1N1) CY121116 60-KRITEMIPE.R NEQGQTLWSK MNDAG.SDRVH   A/Panama

#### C Human

Avian





# Supplementary Figure 3: Conservation and structural environment of PB1-K578 and PB2-E72 in different influenza viruses.

cRNA

mRNA

PB1 R572A

PB2 PB2 E72A Q73A

PB2 PB1 R101A K578A

a-b Sequence alignment of PB1 AA 560-600 and PB2 AA 60-101. The PB1-K578 helix is highlighted in cyan (a), the N-terminal PB2 loop is highlighted in yellow (b) in the indicated strains. c Conservation of PB1-K578 and the relative distribution of alternative amino acids in human (left panel), avian (middle panel) and swine (right panel) isolates (sequences downloaded from NCBI (PB2 and PB1: 09/15/2017, PA: 10/01/2017) presented as percentages of all analyzed sequences. d Polymerase activity of PB1-K578A/R mutants in the polymerase reconstitution assay of IAV strains SC35M and PR8. Relative FF activities are presented as the mean percentage activity relative to SC53M or PR8 WT (±SEM), n=4 independent biological replicates. P values <0.05 compared to WT from Dunnett's multiple comparison one-way ANOVA-test are indicated. e-g Zoom-in into 3D structures of bat Influenza virus A (e; PDB: 4WSB), Influenza virus B (f; PDB:4WSA) and Influenza virus C (g; PDB: 5D9A) and illustration of interacting amino acids located in the interface that covers the PB1-578 containing helix (cyan) and the N-terminal PB2 loop (yellow). Distances between electrostatically interacting amino acids that reside in the interface are indicated in Ångstrom (Å). Created with ChimeraX. h Polymerase reconstitution assay of the mutated polymerase complex with A-substitutions in the positively charged interaction surface and the PB2 loop. Relative FF activities are presented as the mean percentage activity relative to WT (±SEM), n=3 independent biological replicates. P values <0.05 compared to WT from Dunnett's multiple comparison one-way ANOVA-test are indicated. i Quantification of FF mRNA and cRNA levels from the polymerase reconstitution assay depending on the indicated mutations in PB2 and PB1 using qRT-PCR. mRNA and cRNA levels were detected using specific primers and are depicted as mean relative to the WT polymerase (±SEM), n=3 independent biological replicates. Cellular *GAPDH* mRNA levels were used as housekeeping control. P values <0.05 compared to WT from Dunnett's multiple comparison two-way ANOVA test are indicated. Source data are provided as a Source data file.



#### **Supplementary Figure 4:**

**a** 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 or without strep-tagged NP, respectively. At 24 h p.t. polymerase subunits bound to NP were strep-purified and analyzed by western blot using the indicated antibodies. A representative blot from three independent experiments is shown. **b** Assessment of NP binding to PB1 using co-affinity precipitation. HEK293-T cells were transfected with wild type PB1 (WT) or mutant PB1-K578A/R with or without strep-tagged NP, respectively. At 24 h p.t. PB1 bound to NP was strep-purified and analyzed by western blot. Quantification of the interaction is shown below. Levels of PB1 were normalized to strep-tagged NP and reported below as the mean n-fold of WT (±SEM), n=5 independent biological replicates. Dunnett's multiple comparison one-way ANOVA test showed no significant difference compared to WT (p>0.05). Source data are provided as a Source data file.

Site	Polar interaction	Structure	PDB	
		PB2		
K41	vRNA	Transcription (Bat IAV):		
		Pre-initiation complex	6T0N	
		Termination complex	6TW1	
		Stuttering complex	6T0S	
	PB1-Y30	Elongation complex	6T0V	
K116	PB2-E120	Model: WSN polymerase (vRNA bound)		
		Transcription (Bat IAV):		
		Pre-initiation complex	6T0N	
		Model: WSN polymerase (cRNA bound)		
K121	PB1-H605	Model: WSN polymerase (vRNA-bound)		
		Transcription (Bat IAV):		
		Pre-initiation complex	6T0N	
		Elongation complex	6T0V	
		Pre-Termination complex	6SZU	
		Termination complex	6TW1	
		Recycling complex	6T2C	
	PB1-E656	Pre-Initiation complex	6T0N	
		Asymmetric dimer (both polymerases) <sup>9</sup>		
K126	PB1-E614	Symmetric dimer	6QNW	
	PA-D431	Asymmetric dimer (both polymerases) <sup>9</sup>		
	PB1-R602	Asymmetric dimer (both polymerases) <sup>9</sup>		
K312	PB2-E304	Model: WSN polymerase (cRNA bound)		
	PB2-L298			
	PB2-N301	Model: WSN polymerase (vRNA bound)		
	PB2-T303			
K353	PB2-E341	Cap-binding domain with cap	2VQZ	
K670	PB2-Q136	Model: WSN polymerase (vRNA bound)		
		Transcription (Bat IAV):		
		Elongation complex	6T0V	
		Termination complex	6TW1	
	PB2-E681 Model: WSN polymerase (cRNA bound)			
1/050				
K353	VRNA	I ranscription (Bat IAV):		
		Elongation complex	6100	
		Pre-I ermination complex	65ZU	
			01001	
1/00	DD4 T450		751115	
KZZ	PB1-1150	1918 H1N1 polymerase neterotrimer	/NHA	
	PD1-E109 DB1 9160			
K104	mRNA2	Model: PAL-RNA complex 50		
K2/5	PR1_081 and	Model: WSN polymerase (VRNA bound)		
IX24J		Transcription (Rat $ \Delta \rangle$ ).		
		Pre-initiation complex	6TON	
		Flongation complex	6T0\/	
		Pre-Termination complex	6570	
		Stuttering complex	6T0S	
		Dissociation complex	6T0U	
		Recycling complex	6T2C	
K353	PA-K353	Symmetric dimer interface	60NW	
K600	PA-D383	Asymmetric dimer interface <sup>9</sup>		
K643	PR1_T20	Model: WSN polymerase (vRNA bound)		
110-10		Symmetric dimer	60NW	
		Asymmetric dimer: encansidating Pol <sup>9</sup>		
	PB1-F491	Asymmetric dimer: replicating Pol <sup>9</sup>		

Supplementary Table 1: Modified sites with charge-dependent effects on the RdRP activity: List of interacting residues

	PB2 residue	WT / K578R	WT / K578A	K578R / K578A
61	K	0.8316	0.0011	0.0003
62	R	0.7189	0.0042	0.0009
63	I	0.5804	0.0091	0.0012
64	Т	0.4036	0.0242	0.0014
65	E	0.4231	0.0659	0.0066
66	М	0.2344	0.2657	0.0165
67		0.1385	0.3526	0.0109
68	Р	0.2646	0.4987	0.0475
69	E	0.3717	0.4090	0.0558
70	R	0.4684	0.5967	0.1590
71	Ν	0.3053	0.5821	0.0740
72	E	0.2752	0.7741	0.1233
73	Q	0.2785	0.9215	0.2695
74	G	0.3277	0.6267	0.5316
75	Q	0.3282	0.4267	0.8021
76	Т	0.4744	0.5124	0.9243
77	L	0.5049	0.4525	0.9313
78	W	0.5201	0.3883	0.7857
79	S	0.5539	0.3923	0.7229
80	K	0.7218	0.4878	0.5444
81	М	0.7957	0.7996	0.3868
82	N	0.2275	0.6958	0.0346

Supplementary Table 2: p values generated by Welch corrected t-test for RMSF values

Uncropped Blots from supplementary data:

### Supplementary Figure 2

II Part 1 - Staining: PA/Tubulin Part 2 - Staining: PA/Tubulin



## Staining: PB1/Tubulin



### **Supplementary Figure 4**





### **b** Pulldown - Staining: PB2/NP



Input - Staining: PB2/PB1/PA/NP/Tubulin



#### Input - Staining: PB1





#### Tubulin

