



Supplementary Information for

The Mechanism of Resistance to Favipiravir in Influenza

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Supplemental Materials and Methods

Cells and cell culture

Madin-Darby canine kidney (MDCK; ATCC) and human embryonic kidney (HEK) 293T (ATCC) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; labtech.com), 1% penicillin-streptomycin (Invitrogen) and 1% non-essential amino acids (Gibco). Cell lines were maintained at 37 °C and 5% CO₂.

Virus and Drugs

The A(H1N1)pdm09 virus A/England/195/2009 (Eng195) was grown from a reverse genetic rescued virus as described previously (1). Favipiravir was kindly provided under an MTA by Toyama Chemical Co., dissolved in DMSO and stored in aliquots at -20 °C. F-RTP was synthesized by Santa-Cruz Biotechnology, dissolved in water and stored in aliquots at -20 °C.

Virus inhibition assay

Virus was added at an MOI of 0.002 to 6-well plates of confluent MDCKs and allowed to adhere. After an hour, the supernatant was removed and replaced with FBS-free media with 1 µg/ml of trypsin (Worthington) and either drug or an equivalent volume of a DMSO control. Virus infected cells were incubated at 37 °C and 5% CO₂ for 48 hours. The supernatant was then assayed for infectious virus by plaque assay. Virus inhibition was calculated as the percentage of plaques in the presence of drug compared to the absence of drug.

Experimental Evolution Protocol

Populations of Eng195 viruses were grown in 6-well plates of MDCK for 48 hours at an MOI of 0.002 in the presence of favipiravir or a DMSO control. After 48 hours, multiple aliquots

of the supernatant were frozen and stored. Supernatants were titrated by plaque assay every second passage and diluted before the next passage to achieve the same MOI. Populations were passaged 10 times with 3 μ M for passages 1-4 and 3.5 μ M for passages 5-10.

Sequencing

Passage 10 populations were sequenced at Public Health England. Viral RNA was amplified using a multisegment RT-PCR strategy (2). Amplicons were sequenced using Nextera library preparation with an Illumina MiSeq platform. Next generation data was mapped to the Eng195 reference (GQ166654-GQ166661) using BWA v.0.7.5 and variants were identified by QuasiBAM (3). Subsequently, plaques were picked and amplified in 6-well plates for Sanger sequencing. RNA was extracted from the viral supernatant using QiaAMP Viral Mini kit (Qiagen). RNA was reverse transcribed using Superscript III (Invitrogen) and random primers. Gene specific primers were used for PCR (4). Sanger Sequencing was performed by GATC Biotech and sequences analyzed manually using Geneious (v6).

Minigenome Assay

In order to measure polymerase activity in cell culture, pCAGGS plasmids containing genes encoding PB1, PB2, PA and NP from Eng195 (pCAGGS-PB1, pCAGGS-PB2, pCAGGS-PA and pCAGGS-NP, respectively, at 0.08, 0.08, 0.04, 0.12 μ g) were transfected into 293T cells using Lipofectamine 3000 (Thermo Fisher). As reporter construct, we transfected 0.08 μ g Poll-luc, which encodes a minigenome containing a firefly reporter flanked by influenza A promoter sequences. 0.1 μ g pCAGGS-*Renilla* luciferase was transfected as a transfection and toxicity control. After 21 hours, the Dual-Luciferase Reporter Assay kit (Promega) was used to lyse cells and measure luciferase activity. Polymerase activity was expressed as the ratio of

Firefly:*Renilla*. Additional experiments used pCAGGS plasmids containing genes from H3N2 (A/Victoria/3/1975) and H7N9 (A/Anhui/1/2013) the latter kindly provided by Prof. Munir Iqbal.

Rescue of recombinant influenza virus by reverse genetics

The 12-plasmid viral rescue system has been previously described (5). Briefly, 8 Poll plasmids encoding the 8 genome segments of A/Eng/195/2009 virus were transfected with 'helper' plasmids pCAGGS-PB1, pCAGGS-PB2, pCAGGS-PA and pCAGGS-NP into HEK 293T cells using x-tremeGENE 9 (Sigma-Aldrich). After 16 hours, cells were detached and allowed to reattach in a co-culture with MDCK cells. Viruses were harvested after 3 days. Poll plasmids containing mutations of interest were generated using site-directed mutagenesis (QuikChange Lightning, Agilent).

Next-Generation Sequencing with Primer ID

This technique has been previously described elsewhere (3). A minigenome assay was performed with both luciferase minigenome reporter and an additional Poll H3 HA plasmid. RNA was extracted and reverse transcribed using Superscript III and a barcoded primer specific to H3 HA. qPCR was performed to quantify cDNA and ensure that equal numbers of barcoded viral RNA for each sample were sequenced. Following PCR, library preparation was performed using NebNext Ultra II (NEB). Samples were sequenced on an Illumina MiSeq machine and the data analyzed using a pipeline in Python and R as described in Goldhill *et al.* (3). A minimum cut off of 4 reads was imposed for barcode viral RNAs to be included in the analysis. The sequence data can be found at <https://www.ebi.ac.uk/ena> under project number PRJEB27963. The code that was used to run these sequence analyses can be found at <https://github.com/Flu1/Favipiravir>.

In vitro transcription assays

A synthetic 11-nt long RNA (ppGAAUACUCAAG) was capped with a radiolabeled cap-1 structure using 0.25 μM [α - ^{32}P]GTP (3,000 Ci mmole $^{-1}$, Perkin-Elmer), 2.5 U/ μl 2'-O-methyltransferase (NEB) and a vaccinia virus capping kit (NEB). The capped RNAs were purified using an Oligo Clean and Concentrator kit (Zymo Research) and eluted in 50 μl water. Recombinant polymerase preparations were prepared by transfecting 4 μg of plasmids pCAGGS-PA, pCAGGS-PB1 and pCAGGS-PB2-TAP into HEK 293T cells in a 10-cm dish using Lipofectamine 3000. After 48 hours, the expressed polymerases were extracted and purified as described previously (6). Extensions were performed as 4- μl reactions that contained: 1 mM DTT, 5 mM MgCl $_2$, 1 U/ μl RNAsin (Promega), 1500 cpm capped RNA primer, 0.7 μM 3' vRNA promoter (5'-GGCCUGCUUUCGCU (3' 4C template), 5'-GGCCUGCUUUUGCU (3' 4U template)), 0.7 μM 5' vRNA promoter (5'-AGUAGAAACAAGGCC), 5% glycerol, 0.05% NP-40, 75 mM NaCl, 10 mM HEPES pH 7.5, 500 μM UTP, 500 μM CTP, 500 μM F-RTP, and 2 μl RdRP. The ATP and GTP concentrations were varied as indicated. The reactions were incubated for 60 min at 30 °C and stopped with 4 μl formamide loading buffer. Samples were subsequently denatured and analyzed by 20% denaturing PAGE. The extended capped primers were visualized and quantified by phosphorimaging. P-values were determined using ANOVA with multiple testing or a two-sided t-test in Prism 7.

In vitro replication assays

Replication assays were performed as 4- μl reactions that contained: 1 mM DTT, 5 mM MgCl $_2$, 1 U/ μl RNAsin (Promega), 500 μM ApG (Jena Bioscience), 0.7 μM 3' vRNA promoter (5'-GGCCUGUUUUUACU (promoter up template)), 0.7 μM 5' vRNA promoter, 5% glycerol,

0.05% NP-40, 75 mM NaCl, 10 mM HEPES pH 7.5, 500 μ M UTP, 50 μ M ATP, 500 μ M CTP, 500 μ M F-RTP, 5 μ M GTP, 0.1 μ M [α - 32 P]GTP, and 2 μ l RdRP. The reactions were incubated for 60 min at 30 °C, stopped with 4 μ l formamide loading buffer, and analyzed by 20% denaturing PAGE. The replication signals were quantified by phosphorimaging and P-values were determined using ANOVA with multiple testing.

Structural modelling

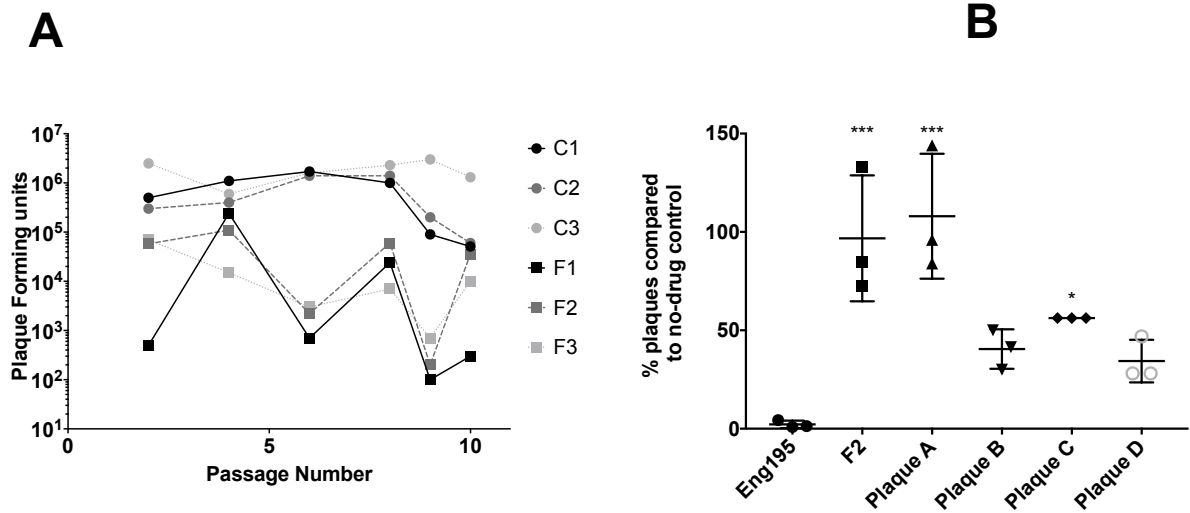
To model an F-RTP base paired with a C in the influenza A virus RdRP structure, we superposed the bat influenza A virus RdRP (PDB 4WSB) with the phi6 RdRP P2 initiation complex (PDB 1HI0) in Pymol 1.8.7 through structural alignment of motifs A and C. The F-RTP was manually inserted using the F-RMP coordinates from the hypoxanthine guanine phosphoribosyltransferase-F-RTP complex (PDB 4KN6). PB1 K229 was mutated to R using conservative backbone-dependent rotamer selection in Pymol 1.8.7.

Supplemental Figure 1. Experimental evolution leads to resistance to favipiravir following

10 passages. A) Virus titers from each passage for control populations (C1-C3) and populations exposed to drug (F1-F3). **B)** Virus inhibition assay with 5 μ M favipiravir for Eng195, population F2: passage 10 and 4 plaques taken from population F2: passage 10.

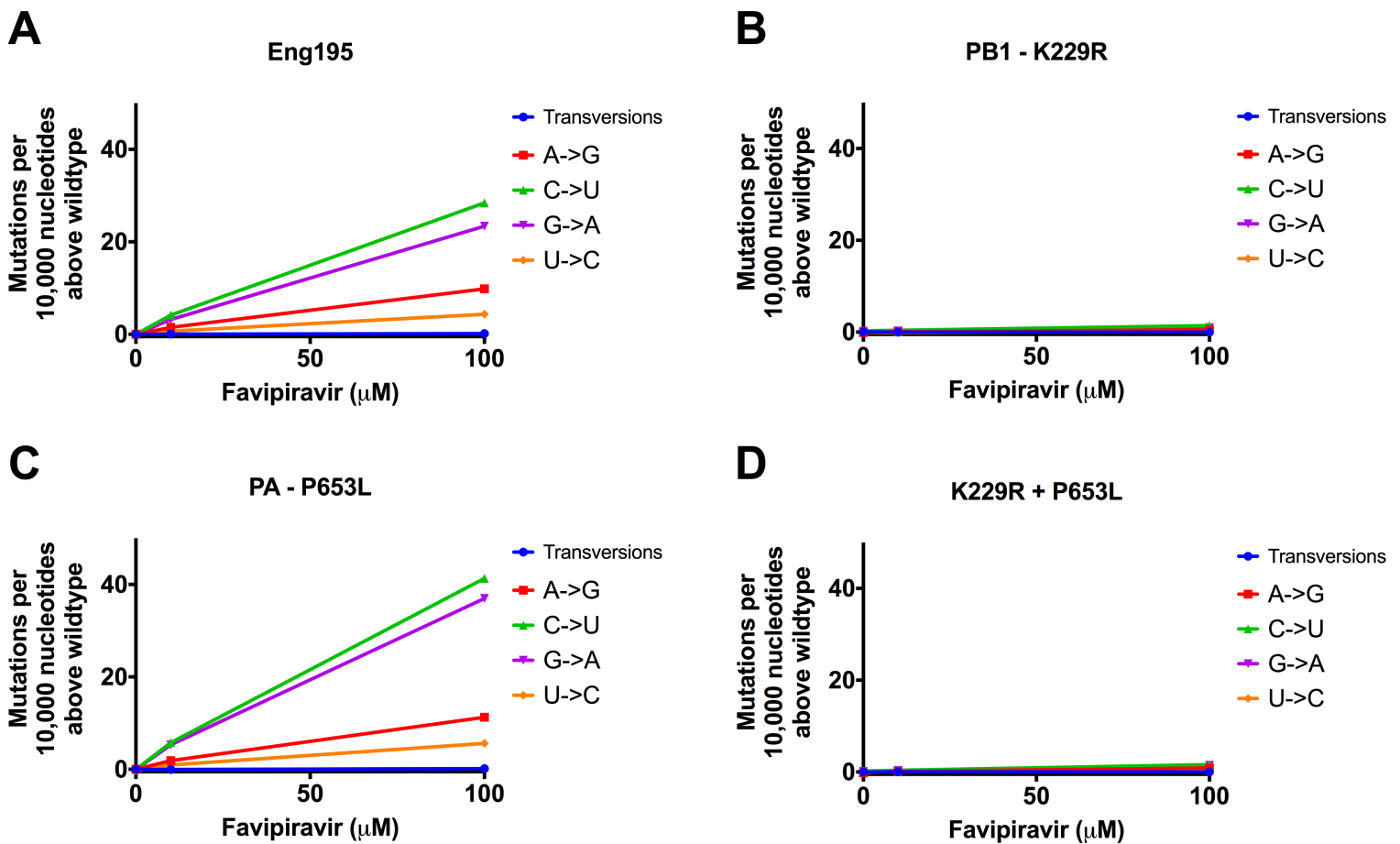
Error bars are \pm SD n=3, 1-way ANOVA with Dunnett's Multiple Comparison Test, *= P<0.05,

***= P<0.001.

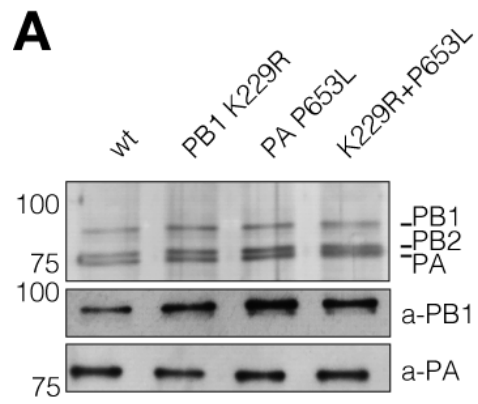


Supplemental Figure 2. PB1 K229R prevents transition mutations caused by favipiravir.

The mutations from Figure 5B were subdivided into transversions and each individual transition. Individual mutations per 10,000 sequenced nucleotides above the control (0 μM , Eng195) were measured by primer ID at 0, 10 and 100 μM favipiravir from RNA expressed in a minigenome assay with reconstituted polymerases. A) Eng195, B) PB1 K229R, C) PA P653L and D) PB1 K229R + PA P653L. Each point is an average of 2 replicates.

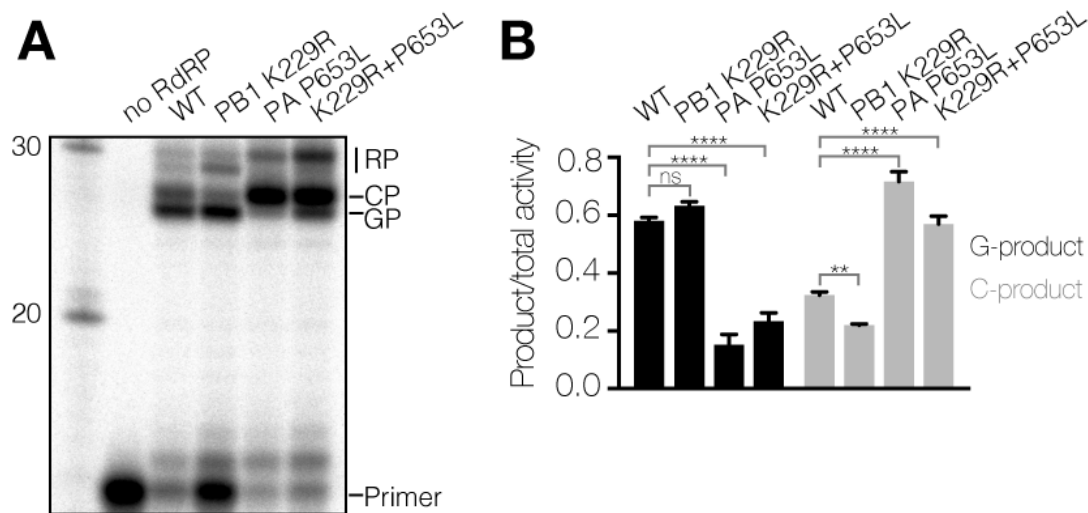


Supplemental Figure 3. Expression of wild type and mutant polymerases. Silverstain (top)
and Western blot analysis of purified influenza A virus RNA polymerase complexes.



Supplemental Figure 4 The P653L mutation favours the C product during transcription *in*

vitro. A) PAGE analysis of influenza A virus transcription initiation on a 4U 3' promoter using a radiolabeled capped primer. The C-product (CP) and G-product (GP) are indicated. In addition, the realignment products (RP) are marked, which are generated when the polymerase realigns a partially extended elongation product from 3' 4U to 1U (6), thereby extending CP and GP by 3 nt. **B)** Quantitation of CP and GP formation normalized by the total RNA polymerase activity. Error bars are \pm SD, n=3, 1-way ANOVA with Dunnett's Multiple Comparison Test, *=P<0.5, **= P<0.01, ***= P<0.001, ****=P<0.0001, and ns= not significant.



Supplemental References

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2. Zhou B, *et al.* (2009) Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza A viruses. *J Virol* 83(19):10309-10313.
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4. Hoffmann E, Stech J, Guan Y, Webster R, & Perez D (2001) Universal primer set for the full-length amplification of all influenza A viruses. *Archives of virology* 146(12):2275-2289.
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