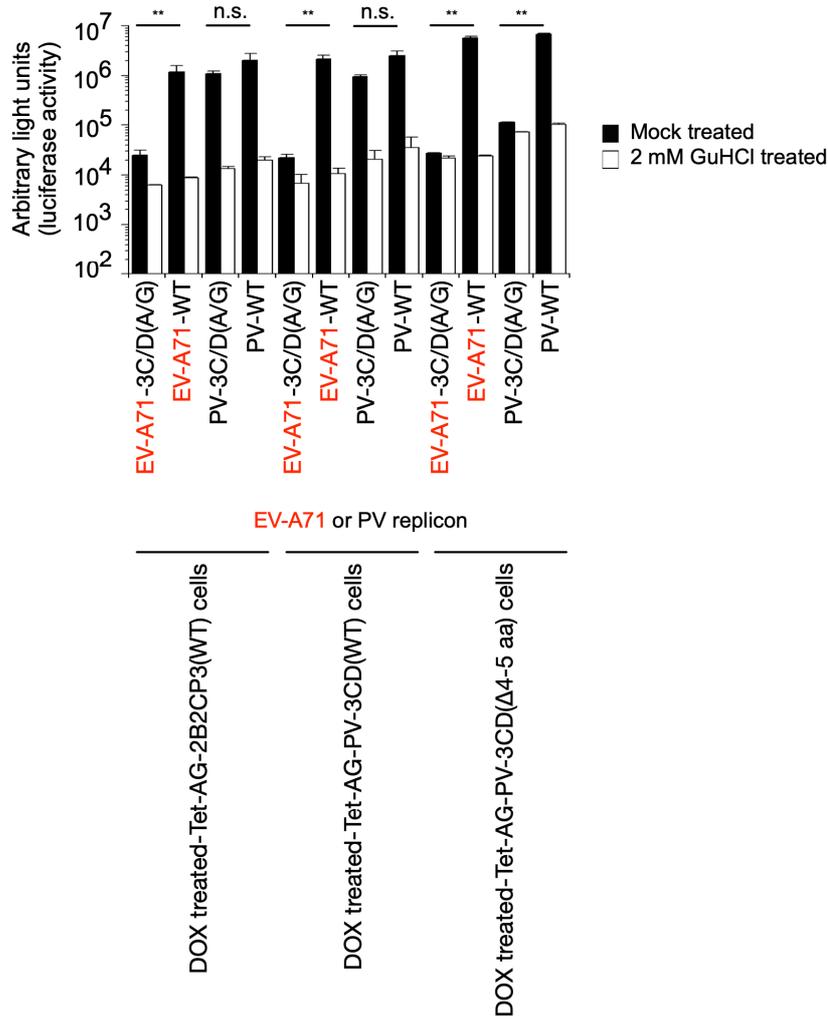


**Figure S1.** *Trans* rescue of replication of a defective PV replicon by PV 3D<sup>pol</sup> protein.

(A) Generation of DOX-inducible HEK293 cell lines (Tet-AG-3D(WT)) that expresses PV 3D<sup>pol</sup> protein (WT). Fluorescent microscope images of the cells for AG after DOX treatment (for 17 h) are shown.

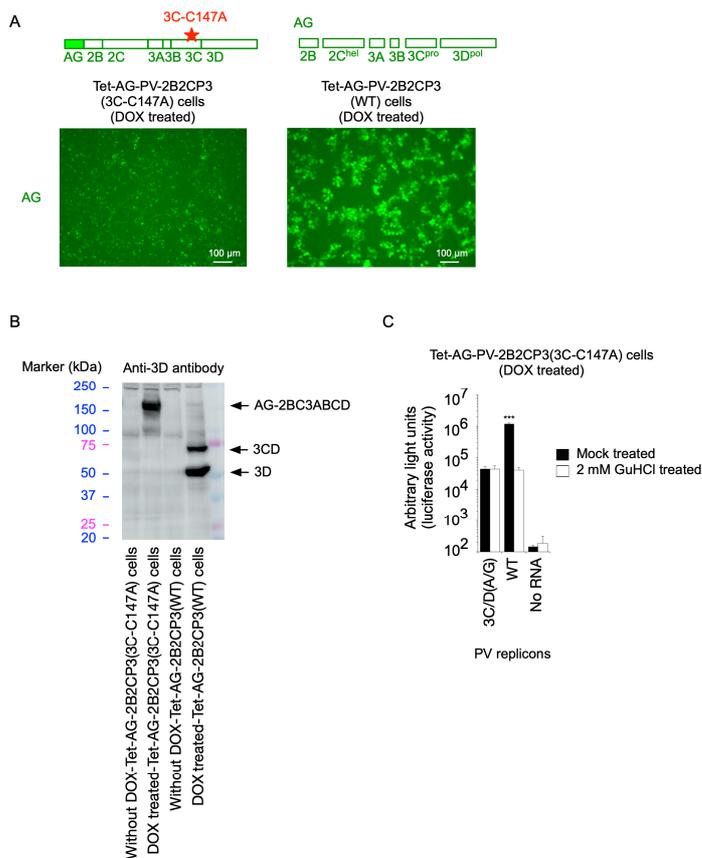
(B) SDS-PAGE analysis of the expressed proteins in the cells. The cells were treated with or without DOX for 17 h. Fluorescence of AG on the gel was shown.

(C) *Trans* rescue of replication of a defective PV replicon in Tet-AG-3D(WT) cells. After the DOX treatment, the cells were transfected with each RNA transcript of the replicons (WT or 3C/D[A/G] mutant) that has firefly luciferase reporter in the presence of absence of GuHCl. The luciferase signals measured at 7 h post RNA are shown.



**Figure S2.** *Trans* rescue of replication of a defective EV-A71 or PV replicon.

After the DOX and GC376 treatment of indicated cells, the cells were transfected with RNA transcript of each replicon that has firefly luciferase reporter in the presence of absence of GuHCl (a 2C inhibitor). The luciferase signals measured at 7 h pt RNA are shown.



**Figure S3.** Effect of the 3C<sup>Pro</sup> activity on *trans* rescue of replication of a defective PV replicon.

(A) Generation of a HEK293 cell line (Tet-AG-PV-2B2CP3[3C-C147A]) that expresses a PV polyprotein without the 3C protease activity conferred by a 3C-C147A substitution in the presence of DOX. Fluorescent microscope images of the cells for AG after DOX treatment (for 17 h) are shown.

(B) Western blot analysis of the expressed proteins in the cells. The cells were treated with or without DOX for 17 h. Processing intermediates of the polyprotein were detected by anti-3D antibody.

(C) *Trans* rescue of replication of a defective PV replicon in Tet-AG-PV-2B2CP3(3C-C147A) cells. After DOX treatment for 17 h, the cells were transfected with each RNA transcript of the replicon that has firefly luciferase reporter in the presence of absence of GuHCl. The luciferase signals measured at 7 h p.t. of the RNA transcripts are shown.

A

3C<sup>pro</sup> aa (1-20 aa)

EV-C: PV1(Mahoney)-V01149	<b>GPGFD</b> YAVAMAKRNIVTATT...
EV-C: CVA13(Nancy)-AF499637	GPGFDYAVAMAKRNITTATT...
EV-C: CVA17(G12)-AF499639	GPGFDYAVAMAKRNIVTATT...
EV-C: CVA20(IH35)-AF499642	GPGFDYAVAMAKRNIVTATT...
EV-D: EV-D68(Fermon)-NC_038308	GPGFD <b>FAQAIMKKN</b> TVIART...
EV-B: CVB3(Nancy)-M16572	GP <b>AFF</b> AVAM <b>MKRNS</b> STVKT...
EV-A: EV-A71(BrCr)-U22521	GP <b>SLDF</b> AL <b>LSLLR</b> RNIR <b>QAQ</b> T...

B



**Figure S4.** Comparison of primary and tertiary structures of 3C<sup>pro</sup>

(A) Sequence of aa 1-20 of 3C<sup>pro</sup> of enterovirus is shown. Difference of aa from that of PV1(Mahoney) is highlighted in red. The aa 1-5 of PV1(Mahoney) is highlighted in green.

(B) Tertiary structure of PV 3C<sup>pro</sup> (PDB: 4dcd, Kim Y. et al., 2012, JVI, DOI: <https://doi.org/10.1128/jvi.01348-12>) is shown. The aa 1-5 is highlighted in green.