Supporting Information

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SI Materials and Methods

Design and Construction of Human Norovirus Gene Expression Plasmids. The human norovirus (HuNoV) genogroup II.3 (GII.3) strain U201 (GenBank accession no. AB039782) cDNA was generated and cloned from U201 stool that was named pT7U201F clone (1). The recombination sequences attB1 and attB2 were added at the both sides of the U201 sequence of the pT7U201F clone using PCR with thermococcus kodakaraensis KOD DNA polymerase (KOD-Plus, Toyobo) with attB1 sense primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCGTGAATGAAGATGGCGTCTAAC-3′ and attB2 antisense primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTCGGGCTTTGTTAGCAG-3′. The target size 7.8-kb amplicon that contained the added attB1 and B2 sequences was purified and cloned into a pDONR221 cloning vector using the Gateway cloning system (Invitrogen); the corresponding plasmid was named pDONR-F. We constructed pKS435gateA3 Gateway destination vector from the original pKS435 vector (2) provided by Koji Sakai (National Institute of Infectious Diseases, Tokyo, Japan) that has an elongation factor-1α (EF-1α) mammalian promoter using the Gateway vector conversion system according to the manufacturer's protocols. The U201 full-length cDNA that was cloned in pDONR-F was exchanged into the Gateway cassette in pKS435gateA3 using the proprietary enzyme LR clonase provided from the Gateway cloning system. The corresponding plasmid was named $pHuNoV_{U201F}$ (Fig. S1*A*). The $pHuNoV_{U201F}$ plasmid produces primary HuNoV RNA transcripts from the human EF-1α promoter as shown in Fig. S1*A* when it is transfected into a cell. Intron 1 of EF-1α is spliced out from the primary RNA transcript, the 3′ end of the poly(A) tail is cut by an hepatitis delta virus ribozyme, and then, capped RNA transcripts are produced in the nucleus of the transfected cell. Transcribed and spliced RNA transcripts contain an additional 107-nt CTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTGTCGTGAGGAATTCAAGCTTGCCTCGAATCACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC at the 5′ end of the U201 native-sized genome RNA. Other constructs were produced from this parental vector. The $pHuNoV_{U201FproM}$ construct was produced from pT7U201F-ProM (1) using the same protocol described above (Fig. S1*C*). The $pHuNoV_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ and $pHuNoV_{U201\text{-}ORF2,3}$ were also produced from pT7U201-ORF1/IGFP and pT7U201-ORF23, respectively, using similar procedures (Fig. S1*C*). The sequences for each mature protein were cloned into pKS435gateA3 using the same protocol to produce constructs that express individual nonstructural proteins ($pHuNoV_{U201\text{-}N\text{-}term}$, $pHuNoV_{U201\text{-}NTP}$, $pHuNoV_{U201\text{-}3A\text{-}like}$, $pHuNoV_{U201\text{-}VPg}$, $pHuNoV_{U201\text{-}Pro}$, and $pHuNoV_{U201\text{-}RdRp}$) (Fig. S1*B*).

For the construction of $pHuNoV_{U201F\text{-}ORF2GFP}$ (Fig. S1*D*), an AscI site was introduced at the estimated hinge region (3) of VP1 on pDONR-F using site-directed mutagenesis with the sense primer 5′-TCAAAGACAAAGCTTTTTAggCgCgCCATTTTAACCATC-3′ (nucleotide position of U201 genome from 5,757 to 5,795) containing nucleotide changes (lowercase letters) converting the sequence into an AscI site (underlined) according to the manufacturer's protocol. In one case, an AscI site at the 5′ and PflMI site (nucleotide position 6,438) at the 3′ of a GFP were added using PCR with the sense primer 5′-CGGGTACCGGGGCGCGCCATGGTGAGCAAG-3′ and the antisense primer 5′-TCTAGAGTCGCCAGAGGATGGCTTGTACAGCTCGTCCATGCCGAGAGT-3′. Then, the amplified GFP sequence was cloned into pDONR-F using AscI and PflMI sites.

To construct a frame shift and RNA-dependent RNA polymerase KO mutant, the 4607G residue was deleted from $pHuNoV_{U201F\text{-}ORF2GFP}$ with a site-directed mutagenesis kit (Invitrogen) according to the manufacture's protocol. Then, the $pHuNoV_{U201F\Delta4607G\text{-}ORF2GFP}$ was constructed and used as a transcription-defective control. The $pHuNoV_{U201F\text{-}ORF2Rluc}$ and $pHuNoV_{U201F\Delta4607G\text{-}ORF2Rluc}$ constructs were made from these GFP constructs by exchanging a Renilla luciferase (Rluc) gene for the GFP gene using the unique restriction sites AscI and PflMI (Fig. S1*D*).

Two protease KO reporter clones, $pHuNoV_{U201FproM\text{-}ORF2GFP}$ and $pHuNoV_{U201FproM\text{-}ORF2Rluc}$, were constructed with conventional cloning techniques that exchanged fragments between $pHuNoV_{U201FproM}$ and the KO cDNAs using the AscI and PflMI sites.

The reporter construct $pHuNoV_{U201F\text{-}NTP/GFP/3A}$ (Fig. S1*E*) was generated by PCR and the InFusion Cloning Kit (Takara Bio) according to the manufacturer's protocol. The GFP gene was amplified with 5′-GAGTTCGAGCTCCAAGGCatggtgagcaagggcgccgag-3′ and 5′- GCGGTCAAAATTGAAGGTTGGTAGATTAGGGCCTTGGAGCTCGAActtgtacagctcatccatgcc-3′ (uppercase letters indicate U201 sequences, and lowercase letters denote GFP gene sequences). The $pHuNoV_{U201F}$ or $pHuNoV_{U201FproM}$ sequences were amplified with 5′-TTGGAGCTCGAACTCATCCATTC-3′ and 5′- CCAACCTTCAATTTTGACCGC-3′. These amplicons were used for InFusion cloning (Fig. S1*E*).

The complete genomes of GI.1 NV68 strain (GenBank accession no. NC_001959), GII.p4.3 chimeric virus TCH04-577 strain (GenBank accession no. AB365435), GII.4 Saga1 strain (GenBank accession no. AB447456) (4), and GV.1 murine NoV (MNV) S7 strain (GenBank accession no. AB435515) (5) were inserted into the pKS435gateA3 vector using the InFusion cloning system according to the manufacturer's protocol. $pMNV_{S7F\Delta4572G}$, an RNA-dependent RNA polymerase KO mutant, was constructed from a pMNVS7F vector in the same manner as the $pKSNoV_{U201F\Delta4607G}$ construct (Fig. S1*F*).

All of the constructs were sequenced and confirmed before use in experiments.

Cell Lines and Antibodies. African Green Monkey SV40-transformed kidney fibroblast cells COS7 (ATCC) were maintained in DMEM supplemented with 10% (vol/vol) FBS. The HEK293T (ATCC) cell line hepatoma Huh7 (a gift from Shinji Makino, University of Texas Medical Branch, Galveston, TX) and Caco2 cell line (ATCC) were maintained in DMEM supplemented with 10% (vol/vol) FBS. Each polyclonal rabbit antiserum to U201 N-term, NTPase, 3A-like, VPg, protease, RdRp, VP1, and VP2 and an monoclonal antibody (clone 131) to VPg have been described previously (6), and these antibodies were used after purification of the IgG fraction using protein A beads according to the manufacturer's protocol before use. The polyclonal rabbit serum to U201 VLP was produced by VLP immunization of rabbits. Immunized U201 VLP included VP1 and VP2. Each polyclonal antiserum to MNV-S7 N-term and VP1 was produced by *Escherichia coli*-expressed purified N-terminal protein and VP1 immunization to rabbit. These antibodies were used after purification of the IgG described above. mAb against GFP developed in mice was purchased from Clontech.

Western Blot Detection of HuNoV Proteins. Cells washed with PBS were harvested in SDS/PAGE sample buffer with 2-mercaptoethanol,

and the proteins were separated by SDS/PAGE. The separated proteins were transferred to a PVDF membrane using the iBlot System (Invitrogen). The membranes were then incubated with primary protein-specific antibody or anti-U201 VLP antibody followed by the addition of appropriate secondary antibody, and the target protein was detected by using Supersignal Femto (Pierce) and then scanned by LAS3000 (Fujifilm).

Northern Blotting. RNA was extracted from cells harvested at 24 and 48 h posttransfection using Isogen RNA extraction reagents (Nippon Gene) according to the manufacturer's instructions. RNA was extracted from the original U201 stool sample with the Qiagen Viral RNA Mini Kit (Qiagen). RNA samples were loaded onto a denaturing agarose gel for Northern blotting using the NorthernMax Kit (Ambion). The RNA probe was prepared by in vitro transcription using the Riboprobe T7 Kit (Promega) in the presence of 100 μCi [α-^{33}P]UTP (20 mCi/mL). To detect positive-strand NoV RNA, the negative-sense RNA probes were generated from a 5,370–7,343-nt PCR amplicon. To detect negative-strand NoV RNA, the sense RNA probes were generated from a PCR amplicon that was produced by PCR with T7GGG-U201-7343A 5′-GATCGATCtaatacgactcactatagggTGGAGTGACAAAAGCTGTCCTGAG-3′ and U201-5370S 5′-GCTAGAATGTACAATGGTTATGCAG-3′. To detect positive-strand NoV RNA, the sense RNA probes were generated from a PCR amplicon that was produced by PCR with T7GGG-U201-5370S 5′-GATCGATC taatacgactcactatagggGCTAGAATGTACAATGGTTATGCAG-3′ and U201-7343A 5′-TGGAGTGACAAAAGCTGTCCTGAG-3′. The lowercase letters represent T7 RNA polymerase promoter sequences. The genomic and subgenomic RNA controls included RNA transcripts derived from in vitro transcription from the previously described plasmids containing genomic and subgenomic sequences under a T7 promoter (6).

Identification of 5′ and 3′ Ends of Viral Genomic and Subgenomic RNA. The nucleotide sequence of the 5′ end of the genomic and subgenomic RNA extracted from agarose gel electrophoresis and also extracted from progeny viruses was determined by 5′ rapid amplification of the cDNA end (5′ RACE) with RNA–RNA ligation (7) with purified genomic RNA and DNA–DNA ligation (8) with cDNA. The nucleotide sequence of the 3′ end of the genome was determined by 3′ RACE.

Luciferase Assays. For the measurement of luciferase activity, cells cultured in 96-well plates (2×10^4 cells/well; Falcon) were transfected with the Rluc constructs pHuNoV$_{U201F\text{-}ORF2Rluc}$ or pHuNoV$_{U201F\Delta4607G\text{-}ORF2Rluc}$. Cells in eight wells were harvested at the time indicated by adding 50 μL passive lysis buffer/well (Promega), and 5 μL lysate/well was used for the measurement of luciferase activity using the Luciferase Assay System (Promega) and a TR717 Microplate Luminometer (Applied Biosystems) according to the manufacturers' instructions.

Semiquantitative Long-Distance RT-PCR. The yield of progeny virus was determined by semiquantitative long-distance RT-PCR, because high background levels of plasmid DNA prevented performing quantitative PCR. Virus released into 1 mL culture supernatant at 48 h posttransfection was treated with RNase OUT (Invitrogen) and Turbo DNase (Ambion) for 1 h at 37 °C. The progeny virus was immunoprecipitated with 1 μg antibody against target progeny virus and 5 μg protein A magnetic beads (Invitrogen). Encapsidated RNA was extracted from the captured progeny virus and determined by long-distance nested RT-PCR. To detect NV68, Tx30SXN and COG1F primers (9) were used for first-step RT-PCR (~2.6 kbp), and then, G1SKF and G1SKR primers (10) were used for nested step PCR (~300 bp). To detect GII progeny viruses (U201, TCH, and Saga1), the Tx30SXN (6) and COG2F primers (9) were used for the first step, and then, the G2SKF and G2SKR primers (8) were used for nested step PCR. To detect MNV, Tx30SXN and MNV F1 primers (11) were used for the first step (~2.6 kbp), and MNV-S (12) and MNV-R2 (11) primers were used for the nested step (~400 bp). PCR products were subjected to agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide (Invitrogen). Band intensities were quantitated by LAS3000 (Fujifilm) with a standard curve using in vitro-transcribed complete-length U201 RNA with 10 times dilution series (10^0–10^5 copy numbers).

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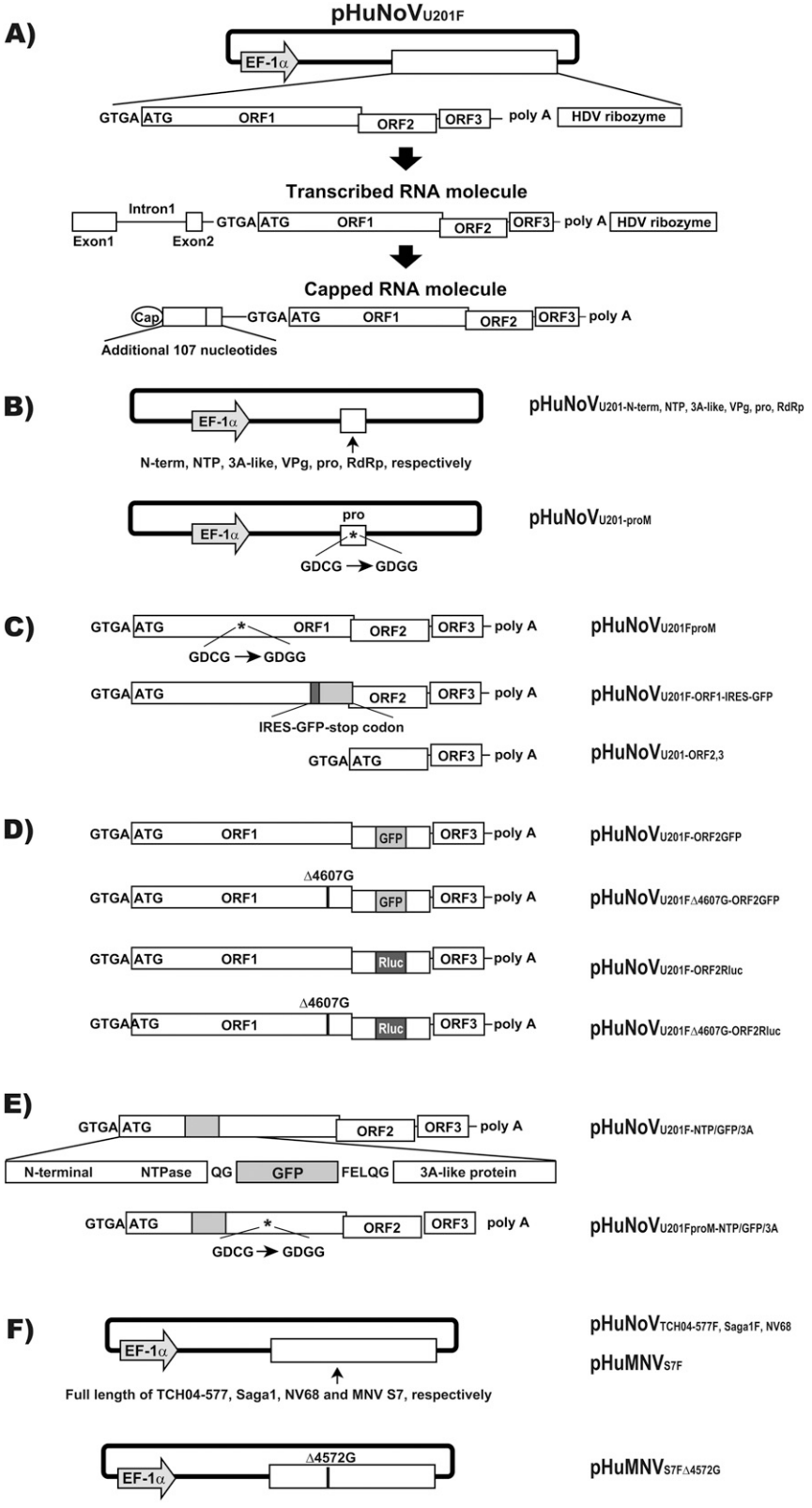

Fig. S1. Schematic diagrams of pHuNoV$_{U201F}$- and pHuNoV$_{U201F}$-based constructs. (*A*) Transcribed RNA molecules and spliced RNA molecules that contain a 5 prime cap (Cap) are represented. ORFs are indicated by white boxes. (*B*) Constructs to express individual nonstructural proteins and a mutant protease protein with a point mutation (GDCG to GDGG) that knocks out protease activity. *Point mutation site. (*C*) Construct expressing the full-length genome (pHuNoV$_{U201FproM}$) with a point mutation in the protease motif, knocking out protease activity. Construct pHuNoV$_{U201F-ORF1-IRES-GFP}$ has an encephalomyocarditis virus, internal ribosomal entry site, and GFP (EMCV-IRES-GFP) gene (1,373 nt) with a stop codon that was cut from the pIRES2-EGFP vector using restriction enzymes XhoI and XbaI and the U201-ORF1 region from 2,518 to 3,859 nt (3A-like protein to protease coding region; 1,341 nt) using unique restriction sites XhoI and XbaI. This construct

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was used as a transfection control to monitor transcription from the EF-1α promoter in Northern blotting. Construct pHuNoV$_{U201\text{-}ORF2,3}$ lacks ORF1 and expresses the capsid proteins. *Point mutation site. (*D*) Constructs where GFP (light gray boxes) or Rluc (dark gray boxes) is inserted into ORF2 in the full-length genome. Two additional constructs contain a point mutation at nucleotide position 4607G in the RdRp gene, resulting in a frame shift in the U201 genome sequence; these mutants with the 4607G deletion do not produce RdRp. (*E*) Construct with GFP (light gray box) inserted between the NTPase and the 3A-like protein surrounded by protease cleavage sites. A derivative construct contains the point mutation in the protease. *Point mutation site. (*F*) The full-length genomes of TCH04-577 (GII.P4-GII.3 chimeric virus), Saga1 (GII.4), NV68 (GI.1), and MNV S7 (GV.1) were inserted into the pKS435 vector at the same position as pHuNoV$_{U201F}$ using the InFusion cloning system. The pMNV$_{S7F\Delta4572G}$ was constructed from pMNV$_{S7F}$ as a negative control similar to pKSF$_{U201\Delta4607G\text{-}ORF2GFP}$ and pKSF$_{U201\Delta4607G\text{-}ORF2Rluc}$.

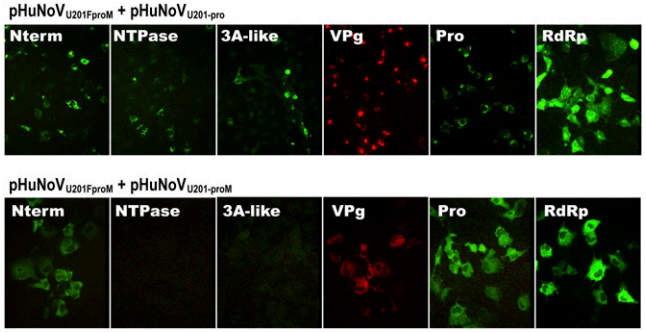

Fig. S2. Detection of pHuNoV$_{U201FproM}$- and pHuNoV$_{U201\text{-}pro}$- or pHuNoV$_{U201\text{-}proM}$–transfected COS7 cells at 24 h posttransfection by immunofluorescence staining for each nonstructural protein using antiserum against the N-term, NTPase, 3A-like, protease, and RdRp (from left to right, respectively) followed by incubation with Alexa Fluor 488-labeled anti-rabbit IgG. VPg protein was detected with the mAb U201 VPg and Alexa Fluor 594-labeled anti-mouse IgG.

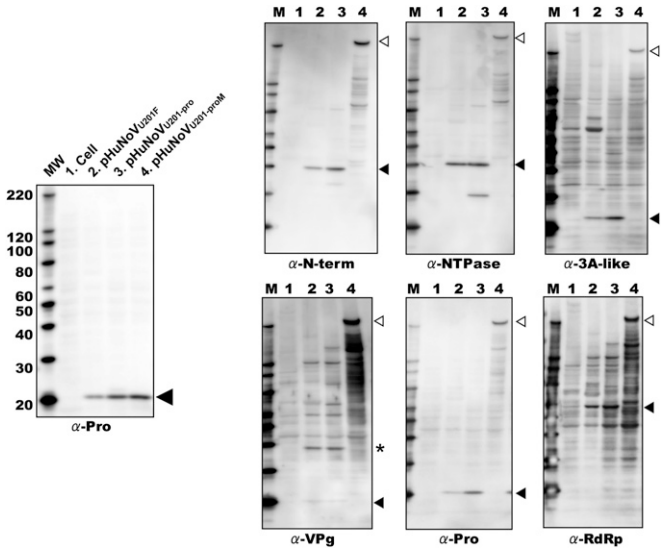

Fig. S3. The ORF1 polyprotein is cleaved by the HuNoV protease provided *in trans*. (*Left*) Protease expression was confirmed by Western blot after expression of the full-length pHuNoV$_{U201F}$ or individual protein-expressing pHuNoV$_{U201\text{-}pro}$ and pHuNoV$_{U201\text{-}proM}$ constructs. Although a mutant protease was expressed from the pHuNoV$_{U201\text{-}proM}$ construct, this mutant protease was nonfunctional; however, cleavage of the ORF1 protein occurred if a functional protease was cotransfected into cells, which is shown in lane 3 in *Right*. *Right* shows Western blotting performed using the rabbit purified IgG against the individual proteins (N-term, NTPase, 3A-like, VPg, protease, and RdRp). M represents molecular mass markers, lane 1 shows mock-transfected cells, lane 2 shows pHuNoV$_{U201F}$-transfected cells, lane 3 shows pHuNoV$_{U201FproM}$ cotransfected with pHuNoV$_{U201\text{-}pro}$, and lane 4 shows pHuNoV$_{U201FproM}$ cotransfected with pHuNoV$_{U201\text{-}proM}$. Black arrowheads indicate each mature protein. White arrowheads indicate the noncleaved ORF1 polyprotein. MW, molecular mass.

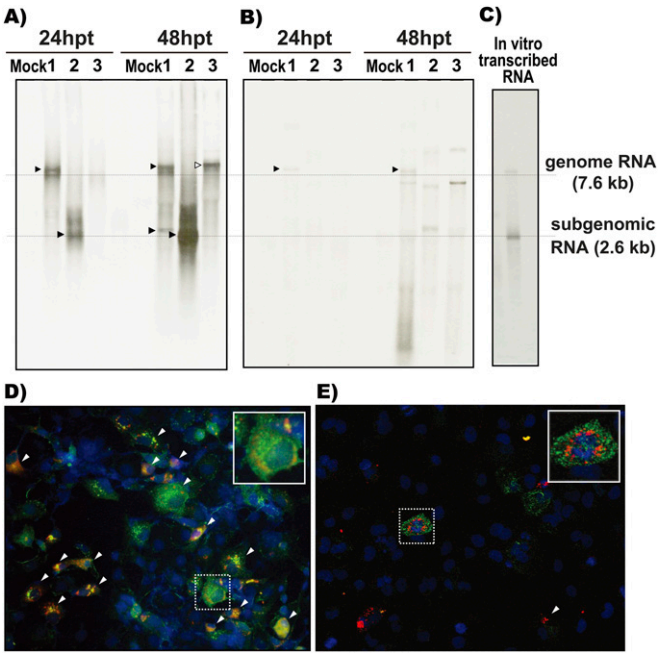

Fig. S4. Detection of U201 RNA transcription and replication in COS7 plasmid construct-transfected cells at 24 and 48 h posttransfection (hpt) using Northern blotting. Each lane contains 1 μg total RNA; lane 1 is from pHuNoV$_{U201F}$-transfected COS7 cells, lane 2 is from pHuNoV$_{U201\text{-}ORF2,3}$ cells, and lane 3 is from pHuNoV$_{U201F\text{-}ORF1/IRES\text{-}GFP}$ cells as an RdRp KO negative control. (*A*) Positive-sense HuNoV RNA was detected by a ^{33}P-labeled negative-strand RNA probe. (*B*) Negative-strand HuNoV RNA was detected by a ^{33}P-labeled positive-strand RNA probe. (*C*) In vitro-transcribed U201 genome-sized RNA and subgenomic-sized RNA were detected by a negative-strand RNA probe. Black arrowheads denote genome- and subgenomic-sized bands. White arrowhead denotes transcribed RNA from the pHuNoV$_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ that is 39 nt longer than pHuNoV$_{U201F}$-transcribed genome-sized RNA. Dotted lines across *A*–*C* are included for alignment for genome RNA and subgenomic RNA. (*D*) The nonstructural protein VPg labeled by Alexa Fluor 594 (red) and structural protein VP1 labeled by Alexa Fluor 488 (green) were detected with immunofluorescent staining of pHuNoV$_{U201F}$-transfected COS7 cells at 24 hpt. Arrowheads represent VPg- and VP1-colocalized yellow signal. (*E*) Expressed VPg and VP2 were detected with immunofluorescent staining of pHuNoV$_{U201F}$-transfected COS7 cells at 24 hpt. Arrowhead shows VPg (red) and VP2 (green) colocalized cells. Nuclei were counterstained with DAPI. *Insets* show high-magnification views.

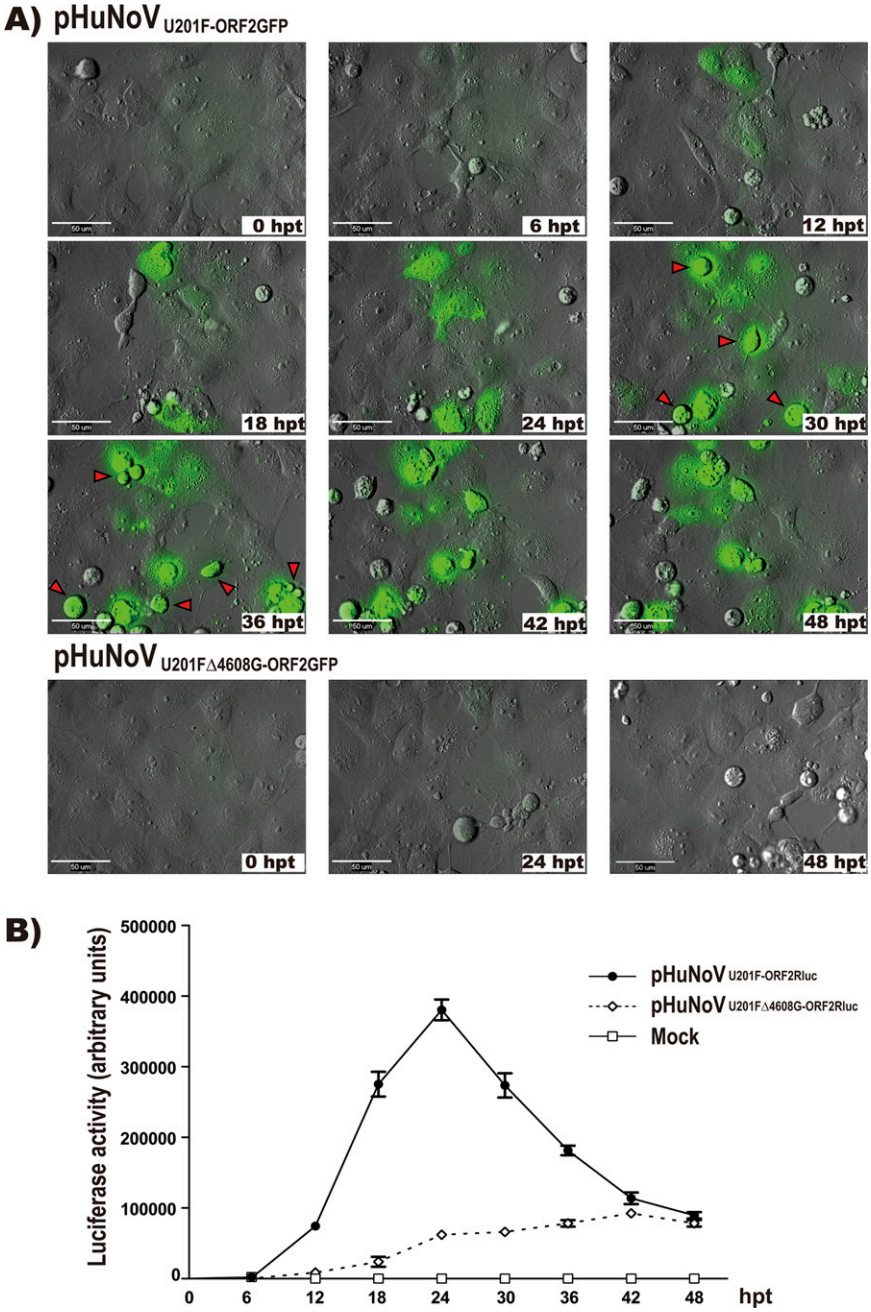

Fig. S5. Reporter expression observed in live cells using time-lapse microscopy. (*A*) GFP expression was observed over time in COS7 cells transfected with the pHuNoV$_{U201F\text{-}ORF2GFP}$. Results with pHuNoV$_{U201FproM\text{-}ORF2GFP}$–transfected COS7 cells only show 0, 24, and 48 h posttransfection (hpt). Red arrowheads show rounded dead cells with cytopathic effects. (*B*) Rluc assay of plasmid-transfected COS7 cells at different times posttransfection; *x* axis shows luciferase activity in arbitrary units, and *y* axis shows time posttransfection. Cells were transfected with pHuNoV$_{U201F\text{-}ORF2Rluc}$ (black dots) or pHuNoV$_{U201F\Delta4607G\text{-}ORF2Rluc}$ (empty circles) or mock-transfected (empty squares). The errors bars represent the SD from eight wells for each time point.

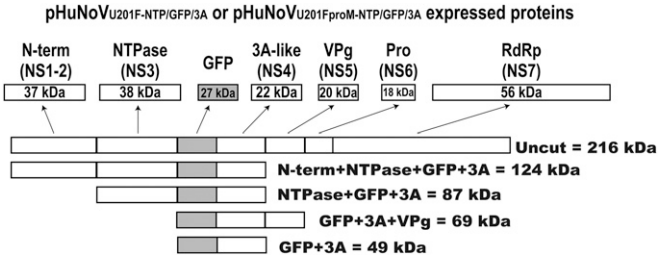

Fig. S6. Schematic of estimated intermediate precursor proteins, mature proteins, and uncut polyprotein. The alternative nonstructural protein nomenclature is shown in parentheses. Molecular masses of each protein were predicted from amino acid composition. The shaded box represents GFP; white boxes show nonstructural proteins.

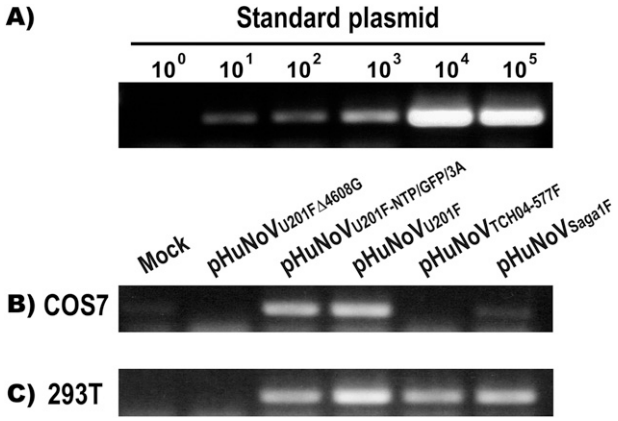

Fig. S7. Semiquantification of progeny virus RNA using RT-PCR. (*A*) Agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide of PCR products produced from known amounts of plasmid DNA (10^0–10^5 copy numbers) as a standard. (*B* and *C*) The amount of progeny virus was determined by detection of RNA from 1 mL supernatant harvested at 48 h posttransfection from either COS7 or 293T cells, respectively, transfected with the indicated plasmid constructs for GII strains using conventional RT-PCR. The $pHuNoV_{U201F\Delta4607G}$ RdRp deletion mutant was used as a negative control.