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Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity

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The papain-like protease PLpro is an essential coronavirus enzyme required for processing viral polyproteins to generate a functional replicase complex and enable viral spread1,2. PLpro is also implicated in cleaving proteinaceous post-translational modifications on host proteins as an evasion mechanism against host anti-viral immune responses3–5. Here, we provide biochemical, structural and functional characterization of the SARS-CoV-2 PLpro (SCoV2-PLpro) and outline differences to SARS-CoV PLpro (SCoV-PLpro) in controlling host interferon (IFN) and NF-κB pathways. While SCoV2-PLpro and SCoV-PLpro show 83% sequence identity, they exhibit different host substrate preferences. In particular, SCoV2-PLpro preferentially cleaves the ubiquitin-like protein ISG15, whereas SCoV-PLpro predominantly targets ubiquitin chains. The crystal structure of SCoV2-PLpro in complex with ISG15 reveals distinctive interactions with the aminoterminal ubiquitin-like domain of ISG15, highlighting this high affinity and specificity. Furthermore, upon infection, SCoV2-PLpro contributes to the cleavage of ISG15 from interferon responsive factor 3 (IRF3) and attenuates type I interferon responses. Importantly, inhibition of SCoV2-PLpro with GRL-0617 impairs the virus-induced cytopathogenic effect, fosters the anti-viral interferon pathway and reduces viral replication in infected cells. These results highlight a dual therapeutic strategy in which targeting of SCoV2-PLpro can suppress SARS-CoV-2 infection and promote anti-viral immunity.

SARS-CoV-2 PLpro preferentially cleaves ISG15

While SCoV-PLpro and SCoV2-PLpro are closely related and distant from MERS-PLpro (Extended data Fig. 1a), purified SCoV-PLpro and SCoV2-PLpro show differences in their substrate preferences, as revealed by their cleavage of ubiquitin or ISG15 from substrates in interferon (IFN-α)-treated HeLa cells (Extended data Fig. 1b). SCoV-PLpro strongly reduced the smears of ubiquitinated substrates, with a lesser effect on ISGylated substrates, whereas SCoV2-PLpro preferentially reduced ISGylation of substrates (Extended data Fig. 1b).

We next employed activity-based probes (ABPs), namely a propargylamide (Prg) warhead, which forms a covalent bond with catalytic cysteines, and a 7-amido-4-methylcoumarine (AMC) probe that is cleavable and concomitant with fluorescence emission thereby enabling the monitoring of protease activity kinetics9–13. SCoV2-PLpro preferentially reacted with ISG15-Prg probe, while showing weak activity towards K48-linked di-ubiquitin (K48-UB2) and Nedd8, and no activity towards SUMO-based Prg probes (Fig. 1a, Extended data Fig. 1c). The strongest reaction for SCoV-PLpro was observed with K48-UB2 Prg-probes, with less reactivity toward the ISG15 Prg-probe (Fig. 1a, Extended data Fig. 1c). This substrate preference was further confirmed.
using competition assays with increasing doses of K48-Ub, (Extended data Fig. 1d–e). In the reaction with SCoV-PLpro, K48-Ub, effectively competed with both ISG15-Prg or ISG15-AMC, while the competitive displacement was much less effective for SCoV2-PLpro. The catalytic efficiency ($k_{cat}/K_{m}$) of both PLpros was also examined (Fig. 1b. Extended data Table 2) demonstrating that SCoV2-PLpro mediated AMC cleavage from ISG15-AMC was indeed better than from K48-Ub-AMC while SCoV-PLpro cleaved AMC from K48-Ub more efficiently. Interestingly, in these assays the apparent catalytic efficiency ($k_{cat}/K_{m}$) of both PLpros towards ISG15 was similar, yet SCoV-PLpro showed slightly higher ISG15 specificity (lower $K_{m}$, Fig. 1b, Extended data Table 2). Consistently, SCoV2-PLpro displayed a 20-fold increase in ISG15 binding compared to K48-Ub, whereas SCoV-PLpro bound to K48-Ub with a 10-fold higher affinity than ISG15 (Fig. 1c, Extended data Table 3). In fact, the delS5Glyase activity of SCoV2-PLpro toward Prg- or AMC-based substrates, was similar to or higher than that of mouse USP18 (mUSP18), a specific delS5Glyase24 (Extended data Fig. 1f, g). A weak de neddylation activity was also observed for both PLpro enzymes towards hypermed-dylated Cul1, a common feature for the bona fide de neddylation Den1 (Extended data Fig. 1c, h, i). Taken together, these results indicate that SCoV2-PLpro preferentially cleaves ISG15 from substrates in vitro over ubiquitin chains and Nedd8, whereas SCoV-PLpro targets ubiquitin chains and to a lesser extent ISG15 and Nedd8 (Fig. 1d).

SCoV2-PLpro-ISG15 structural analysis

To gain insight into the molecular basis underlying SCoV2-PLpro-ISG15 specificity, we determined the crystal structure of a SCoV2-PLpro (C111S)-murine ISG15 complex (Fig. 2a, Extended data Fig. 2a). Notably, ISG15 displays two tandem ubiquitin-like folds. The overall assembly of SCoV2-PLpro and both ISG15 domains was similar to the MERS-PLpro-human ISG15 complex (PDB ID: 6B18, Extended data Fig. 2a16). The catalytic cysteine residue is also conserved in SARS-PLpro (Extended data Fig. 2b). Compared to the crystal structure of ISG15 on its own (PDB ID: 5TLA)16, the N-terminal half of ISG15 is rotated by almost 90° and sits on the S2-helix of SCoV2-PLpro (Fig. 2b). The structure of SCoV-PLpro in complex with mISG15-C-term (PDB ID: 5TL7)16 showed that SCoV-PLpro and SCoV2-PLpro share the same binding mode to the C-lobe of mISG15 (Extended data Fig. 2c). We next compared the structure of the SCoV2-PLpro-ISG15 complex to that of SCoV-PLpro bound to K48-Ub, where the proximal ubiquitin is linked to the catalytic site. The major difference between the two complexes is an interaction remote from the catalytic site, with a protease S2 site binding the distal ubiquitin in the K48-linked chain, or the N-terminal ubiquitin-like fold in ISG15. While SCoV-PLpro Leu76 mediates a hydrophobic interaction with Ile44 on ubiquitin, the corresponding residue on SCoV2-PLpro is Thr75 (Fig. 2c, Extended data Fig. 2c). To mimic the hydrophobic interaction observed in SCoV-PLpro, we generated two variants of SCoV2-PLpro (T75A and T75L). Interestingly, the T75L mutant resulted in a K48-Ub-AMC cleavage, while T75A did not (Figure 2d). This indicates that a properly sized hydrophobic residue at this site is a critical determinant for ubiquitin binding. We also compared papain-like proteases (PLP) from other coronaviruses including common human coronaviruses OC43, 229E and NL63 (Extended data Fig. 3). Intriguingly, the S2-binding site in PLPs is the poorly conserved across coronaviruses and showed variability of hydrophobicity at the position corresponding to Thr75 of SCoV2-PLpro, which might influence substrate specificity.

Next, we examined if other SCoV2-PLpro residues contribute to its enhanced ISG15 affinity. SCoV2-PLpro Val66 faces the hydrophobic patch (Ala2, Thr20, Met23) on ISG15-N-terminal ubiquitin fold domain (Fig. 2e). Intriguingly, both PLpro enzymes share Phe (SCoV2-PLpro Phe69 and SCoV-PLpro Phe70) as the core residue mediating hydrophobic interactions with either ubiquitin or ISG15. Mutating Phe69 (F69A) or Val66 (V66A) on SCoV2-PLpro significantly decreased its enzymatic activity and showed slower reaction with ISG15-Prg compared to the wild type (Fig. 2f).

The interaction between SCoV2-PLpro and K48-Ub, and mISG15 was also examined using molecular dynamics (MD) simulations (Extended data Fig. 2d, 2e). Multi-microsecond MD simulations confirmed that SCoV2-PLpro interacts more tightly with ISG15 compared to K48-Ub, (reconfirming $k_{cat}/K_{m}$ values measured in Fig. 1e). In three independent runs of 3.2 ps each, mISG15 remained bound as in the SCoV2-PLpro:mISG15 X-ray structure. In contrast, the distal ubiquitin of K48-Ub, separated from SCoV2-PLpro in four out of six runs on a microsecond time scale. We identified L75T to be the difference between SCoV2-PLpro and SCoV-PLpro, as it weakens the hydrophobic cluster within the binding interface. We observed that water transiently enters between Ile44 and Thr75 (K48-Ub) prior to dissociation. Indeed, in simulations of the SCoV-PLpro double mutant (S67V/T75L) with K48-Ub, a similar water-mediated dissociation mechanism was observed (Extended data Fig. 2f, 2g). Together, these results suggest that the S2 region determines substrate specificity and that SCoV2-PLpro has relative preference toward ISG15.

GRL-0617 is an inhibitor of SCoV2-PLpro

With the pressing urgency to identify novel therapeutic strategies against COVID-19, we tested the effect of GRL-0617, a non-covalent inhibitor of SCoV-PLpro on SCoV2-PLpro (Fig. 3a)17. GRL-0617 is a naphthalene-based inhibitor developed against SCoV-PLpro and does not inhibit other host proteases25,26. Based on the binding mode of GRL-0617 and other known naphthalene inhibitors to SCoV-PLpro17-20, we postulated that the conserved Tyr268 of SCoV-PLpro could also bind GRL-0617 and block the entry of the ISG15 C-terminus toward the protease catalytic cleft (Fig. 3b, Extended data Fig. 4a–c). Indeed, the IC50 of GRL-0617 to SCoV2-PLpro was similar to that of SCoV-PLpro (Fig. 3c, Extended data Fig. 4d, e). Interestingly, this inhibitor is ineffective against MERS-PLpro20. We hypothesized that this could be due to the presence of Thr instead of Tyr at this conserved position (Extended data Fig. 3a). Accordingly, the mutation of Tyr268 to either Thr (Y269T) or Gly (Y268G) in SCoV2-PLpro strongly reduced the inhibitory effect of GRL-0617 (Fig. 3c, Extended data Fig. 4d, e), indicating the critical role of Tyr268 in this process. MD simulations of GRL-0617 with SCoV-PLpro and SCoV2-PLpro further confirmed a common binding mode between GRL-0617 and Tyr268 (Tyr269 for SARS) (Extended data Fig. 4b, c).

To assess the therapeutic value of GRL-0617 against COVID-19 we tested the effect of GRL-0617 on the SCoV2-PLpro delS4Glyase or deubiquitina- tive activities of host proteins. GRL-0617 effectively blocked SCoV-PLpro leading to increased levels of ISGylated proteins in IFN-α treated cell lysates (Extended data Fig. 4f). GRL-0617 also blocked the deubiquitination activity of SCoV-PLpro (Extended data Fig. 4f). Intrigu- ringly, the effects of GRL-0617 on the reaction between Prg-probes with SCoV2-PLpro were more prominent with ISG15 Protein (ISG15term)Prg or ubiquitin versus K48-Ub, to SCoV-PLpro (Extended data Fig. 4g, h). Consistent with the structural data indicating that the interaction between ISG15 N-terminal ubiquitin-fold domain potentiates the interaction with SCoV2-PLpro. These results showed that GRL-0617 inhibits both SCoV2-PLpro and SCoV-PLpro.

PLpro regulates IFN and NF-κB pathways

To understand the differences in the pathophysiological roles of SCoV2-PLpro and SCoV-PLpro, and to expand our knowledge of the SARS-CoV-2 protein interaction map27, we analysed the cellular interactome of both proteins. ISG15 is significantly enriched in complexes with a catalytically inactive version of SCoV2-PLpro (C111S), whereas a SCoV-PLpro mutant (C111S) predominantly associated with ubiquitin (Fig. 4a, b). In mammalian cells treated with type I IFNs (ISRE-promoter dependent, IFN-α), immunoprecipitation of unconjugated ISG15 and ISG15-positive smears, likely representing ISGylated substrates, was more pronounced with GFP-SCoV2-PLpro (C111S) than...
with GFP-SCoV-PLpro (C111S) (Fig. 4c). This association was blocked upon GRL-0617 treatment (Fig 4c). Moreover, these closely related PLpro enzymes associate with distinct and specific sets of host proteins (Fig. 4a). For SCoV2-PLpro, these include PRKDC that is related to type I interferon induction\(^1\) and heterogenous nuclear ribonucleoprotein K (HNRNPK) involved in splicing of the host RNA, a process essential for SARS-CoV-2 replication in cells\(^2\); and Galectin1, which can induce viral fusion with target cells during HIV infection\(^2\) (Fig. 4a). In contrast, SCoV-PLpro strongly associates with several serine protease inhibitors (serpins), including SERPIN B3, proposed to inhibit papain proteases\(^27\). Interestingly, expression of SERPIN B3 together with PLpro enzymes partially restored NF-κB signalling in cells expressing SCoV-PLpro, but had no effect on SCoV-PLpro regulation of the IFN pathway (Extended data Fig. 5a, b).

Consistently, expression of SCoV-PLpro and SCoV-PLpro in mammalian cells decreased ISGylation of cellular proteins following IFN-α stimulation (Extended data Fig. 5c), including that of interferon regulatory factor 3 (IRF3), a critical component in the type I interferon pathway\(^28\). Both SCoV2-PLpro and SCoV-PLpro caused the loss of IRF3 ISGylation, with SCoV2-PLpro having a much stronger effect (Fig. 4d). A decrease in phosphorylation of TBK1, IRF3 and nuclear translocation of IRF3 was detected upon SCoV-PLpro or SCoV2-PLpro expression (Extended data Fig. 5d, e). The SCoV-PLpro catalytic mutant (C111S) showed stronger dominant negative effects on IRF3 phosphorylation compared to the SCoV-PLpro catalytic mutant (C111S) (Extended data Fig. 5d, e). TBK1 phosphorylation also activates the NF-κB pathway causing upregulation of inflammatory signalling\(^29\). Although the expression of SCoV-PLpro had less impact on IRF3 ISGylation (Fig. 4d), it strongly attenuated degradation of IRF3-α (Extended data Fig. 5f, g). SCoV-PLpro also caused a severe reduction in nuclear translocation of p65 in TNF-α treated cells (Extended data fig. 5h).

Sensing of viral nucleic acids is mimicked by poly (I:C) treatment, which induces IFN-β expression\(^29\). Expression of SCoV2-PLpro more effectively decreased the level of IFN-β promoter activation compared to SCoV-PLpro following poly (I:C) treatment. This inhibitory effect of both PLpro enzymes was neutralized by GRL-0617 treatment (Extended data Fig. 6a, 6c). By contrast, expression of SCoV-PLpro predominately blocked the TNF-α-induced NF-κB p65 expression, which was also sensitive to GRL-0617 treatment (Extended data Fig. 6b, 6d). Together, we show how two closely related coronaviruses (SARS and SARS-CoV-2) differentially counteract the host immune system using their PLpro enzymes.

**PLpro inhibition affects viral spread and IFN responses**

It has been shown that GRL-0617 inhibits viral replication of SARS-CoV\(^27\). Thus, to determine whether inhibiting SCoV2-PLpro can also block SARS-CoV-2 replication, CaCo-2 cells were infected with SARS-CoV-2 and treated with GRL-0617 (Fig. 5a). The effect of GRL-0617 was measured by cytopathogenic effect (CPE) inhibition. We observed a gradual dose-dependent inhibition of SARS-CoV-2-induced CPE in the presence of GRL-0617 with 100 μM of GRL-0617 showing nearly a 100% CPE inhibition effect (Fig. 5b). In addition, GRL-0617 treatment reduced the active viral replication (SARS-CoV-2 subgenomic RNA encoding E gene), measured by genetic monitoring of the intracellular production of viral RNA (Fig. 5c). Consequently, a decrease in the release of viral particles from infected cells into the supernatant was also observed upon GRL-0617 treatment (Fig. 5d, Extended data Fig. 7a, respectively). This suggests that GRL-0617 mediated inhibition of SCoV2-PLpro impedes the viral replication thereby attenuating the ongoing viral RNA synthesis.

Having demonstrated a role for SCoV2-PLpro and SCoV-PLpro expression in dampening host anti-viral IFN pathways, we anticipated that GRL-0617 inhibition could also reverse this process. Indeed, GRL-0617 treatment of SARS-CoV-2-infected cells led to a significant increase in IRF3 ISGylation (Fig. 5e), which has previously been shown to regulate anti-viral immune response\(^30\). Moreover, phosphorylation of IRF3 and TBK1, markers for IFN pathway activation, and p65 phosphorylation, used to monitor NF-κB pathway activation, were all increased in SARS-CoV-2-infected cells upon GRL-0617 treatment (Fig 5f).

Importantly, GRL-0617 treatment significantly rescued the expression level of IFN-responsive genes (ISG15, OASL, PKR, MX1) in SARS-CoV-2 infected cells (Fig. 5g). Extended data Fig. 7b). These findings provide evidence that inhibition of SCoV2-PLpro, in addition to blocking viral RNA synthesis, can also increase anti-viral signalling via TBK1 and IRF3 (Extended data Fig. 7c). While experiments conducted with GRL-0617 provide evidence supporting the therapeutic value of pharmacologically targeting SCoV2-PLpro in patients, given its low potency, additional studies are needed in order to develop more potent and selective PLpro inhibitors.

Finally, we tested changes in CaCo-2 cells upon SARS-CoV and SARS-CoV-2 infection. GRL-0617 treatment of infected cells had an overall similar pattern in biochemical and transcriptional parameters of the type I IFN and NF-κB pathways (Fig. 5e–g). Interestingly, however, we found that GRL-0617 was consistently more effective in restoring the ISGylation and phosphorylation level of IRF3, and on the expression of IFN responsive genes upon SARS-CoV-2 infection compared to SARS-CoV (Fig. 5e–g). In contrast, transcription levels of the proinflammatory cytokines IL-6 and IL-8 appeared similar between SARS-CoV and SARS-CoV-2 in this epithelial cell culture models (Fig 5g). Consistent with these observations, a recent study has shown that SARS-CoV-2 infection, in animal models and COVID-19 patients, is correlated with low IFN type I and type III responses\(^31\). Even though preferential activity of SCoV2-PLpro towards delsGylation may contribute to decreased type I IFN signalling, more detailed studies are needed to understand key regulatory factors contributing to innate and adaptive immunity that control distinct pathologic outcomes of SARS-CoV and SARS-CoV-2 infections\(^32\).

Taken together, this interdisciplinary study provides mechanistic understanding of SCoV2-PLpro functions during SARS-CoV-2 infection and establishes SCoV2-PLpro as a promising target for therapeutic intervention against COVID-19. Recent reports of newly identified inhibitors of SCoV2-PLpro\(^34–36\) may lead to a rapid development of novel anti-COVID-19 therapeutics with a dual effect – blocking SARS-CoV-2 spread and promoting anti-viral immunity in the host. Also, the main protease of SARS-CoV-2 has been in the focus as a potential drug target against COVID-19 and several novel inhibitors have already been described\(^37–39\). Combining drugs targeting essential SARS-CoV-2 proteases (PLpro and/or main protease) and drugs targeting SARS-CoV-2 RNA-dependent polymerase may offer successful therapeutic options in the future\(^40\).

**Online content**

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Fig. 1 | DeISGylating and Deubiquitylating activities of SCoV-PLpro and SCoV2-PLpro. 

a, SCoV-PLpro (left) or SCoV2-PLpro (right) were incubated with indicated Prg-probes for indicated time points. Experiments were repeated three times independently with similar results. 

b, Catalytic efficiency ($k_{cat}/K_{m}$) of SCoV2-PLpro and SCoV-PLpro on K48-Ub$_2$-AMC or ISG15-AMC cleavage. 

c, Dissociation constant ($K_d$) of SCoV2-PLpro and SCoV-PLpro. Data in c, d were presented as mean ± S.D or mean ± S.E.M, respectively (n=3, independent experiments). * $p<0.05$, ** $p<0.01$, **** $p<0.0001$; two-tailed paired t-tests. 

b, Schematic representation of substrate specificity of SCoV2-PLpro and SCoV-PLpro.
Fig. 2 | Structural analysis of SARS-CoV-2 PLpro in complex with full length ISG15. a, Crystal structure of SARS-CoV-2 PLpro (3C11) in complex with murine ISG15 (mISG15). C-terminal glycine of mISG15 and catalytic triad of SCoV-PLpro are highlighted as stick model. Ubiquitin like domain (Ubl) are coloured in orange. b, Comparison of unbound form of mISG15 with mISG15 in complex with SCoV-PLpro. d, Initial velocity of AMC release from AMC-probes (K48-Ub2-AMC, ISG15-AMC) with indicated PLpro and its mutants are presented as mean ± S.D (n=3, independent experiments). ** p < 0.01; two-tailed paired t-tests. e, Comparison of K48-Ub2:SCoV-PLpro complex structure (PDB ID: 5E6J) with mISG15:SCoV-PLpro. Residues forming hydrophobic interactions are highlighted as stick model. f, ISG15-Prg were incubated with SCoV-PLpro wild type and its mutants for indicated time points. Experiments were repeated three times independently with similar results.
Fig. 3 | Effect of GRL-0617 inhibitor on SCoV2-PLpro. a. Structure of GRL-0617. b. Comparison of ISG15-bound (left) and GRL-0617 bound (right) structure. The blocking loop 2 (BL2 loop) of SCoV2-PLpro is modelled based on GRL-0617 bound SCoV-PLpro and SCoV2-PLpro structures (PDB ID: 3E9S, 6W9C). GRL-0617-interacting Tyr268 and catalytic Cys, His residues are highlighted as stick model. c. Cleavage of ISG15-AMC was measured and normalized to DMSO control. IC$_{50}$ of GRL-0617 to SCoV2-PLpro are presented. Data are presented as mean ± S.D (n=3, independent experiments).
Fig. 4 | Effect on PLpros on IFN and NF-κB pathways. 

a. Interactome analysis of SCoV2-PLpro C111S/SCoV-PLpro C111S. Statistically significant and immunity-related proteins are highlighted. 

b. Log2 fold change of ubiquitin and ISG15 from SCoV2-PLpro or SCoV-PLpro versus empty vector. Data are presented as mean ± S.D (n=3, independent experiments). 

c. ISGylated proteins were enriched from IFN-α treated A549 cells by indicated PLpro. 

d. ISGylation level of Myc-IRF3 was examined from A549 cells expressing indicated GFP-PLpros. Experiments in c, d were repeated three times independently with similar results.
Fig. 5 | Inhibitory effects of GRL-0617 on SARS-CoV2. 

a, Schematic representation of SARS-CoV-2 growth inhibition test with GRL-0617.

b, CPE inhibition rate of GRL-0617 on SARS-CoV2 infected CaCo-2 cells.

c, Intracellular active virus replication was analysed by measuring SARS-CoV-2 subgenomic RNA (subgRNA E) level and normalized to cellular actin level.

d, Release of viral particles in culture medium was analysed by PCR targeting the open reading frame for RNA dependent RNA polymerase (RdRp) of SARS-CoV-2.

e, f, The effect of GRL-0617 on type I IFN-pathway. CaCo-2 cells were infected with SARS-CoV-2 or SARS with or without GRL-0617 (50 μM).

f, Endogenous IRF3 was immunoprecipitated and analysed by immunoblotting.

f, Phosphorylation of TBK1 level were analysed by immunoblotting.

g, Relative mRNA levels of indicated genes from infected cells with or without treatment of GRL-0617 (25 μM) were analysed and normalized to 18S. Data in c, d, g are presented as mean ± S.D (n=3, independent experiments). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; two-tailed paired t-tests. Experiments in e, f were repeated three times independently with similar results.
Methods

Plasmids construction
The papain-like protease domain sequence is obtained from SARS-CoV-2 complete genome (NCBI genome databank, Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome: NC_045512). Protein sequence for CoV2 PLpro-Ubl domain (amino acids, 746-1060) of Nsp3 protein from SARS-CoV-2 (Nsp3:YP_009725299.1) was codon optimized, synthesized and cloned into pET28b with Ncol and Xhol to have C-terminal His-tag (Genescript). Protein sequences of PLpro-Ubl domain of SARS and MERS from (PDB ID: 3M5S, SWBU, respectively) were also codon optimized, synthesized and cloned into pET28b with Ncol and Xhol to have C-terminal His-tag (Genescript). Mutants were generated by PCR and verified by sequencing. For mammalian expression, PLpros are cloned into pEGFP-C1 (clontech). To produce the vector pACE-ISG15, a synthetic cDNA was used for murine ISG15 (Residues 1-155) and an N-terminal His6-Tag and the recognition site for the HRV-3C protease coded, ordered from Mr. Gene (Regensburg, Germany).

Protein purification
BL21(DE3). E. coli competent cells (NEB) were transformed with plasmids and grown in LB medium to an OD600 of 0.6-0.8 at 37 °C. Protein production was induced by addition of 0.5 mM IPTG (isopropyl D-thiogalactopyranoside) and 1 mM Zinc Chloride (ZnCl2) the cells were further grown overnight at 18 °C and harvested. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, 2 mM DTT, pH 8.5) and lysed by sonication and centrifuged at 13,000 rpm to clarify the supernatant. The supernatant was incubated 2 hours with TALON beads (Takara) pre-equilibrated with lysis buffer and non-specific proteins were cleared with washing. Proteins were eluted with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 250 mM Imidazole, 2 mM DTT, pH 8.5). Eluted proteins were buffer exchanged to storage buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM DTT, pH 8.5) and stored for biochemical analysis. For crystallization of SCoV2-PLpro-C111S, the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, 1 mM TCEP, pH 7.4) and lysed by sonication and centrifuged at 13,000 rpm to clarify the supernatant. The supernatant was incubated 2 hours with TALON beads (Takara) pre-equilibrated with lysis buffer and non-specific proteins were cleared with washing. Proteins were eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 250 mM Imidazole, 1 mM TCEP, pH 7.4) and further purified on size-exclusion column (Superdex 75 16/60, GE Healthcare) pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl, 1 mM TCEP, pH 7.4. Proteins were concentrated to 20 mg/ml and stored for crystallization. For the expression of mISG15, BL21(DE3) E. coli competent cells (NEB) were transformed with pACE-ISG15. Bacterial colony was grown for 20 h at 28 °C and incubated 10 minutes at 37 °C until reaching a OD600 grown from 0.6. The protein expression was induced by addition of 0.5 mM IPTG (isopropyl D-thiogalactopyranoside) and 1 mM Zinc Chloride (ZnCl2) the cells were further grown overnight at 18 °C and harvested. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, 2 mM DTT, pH 7.4) and lysed by sonication and centrifuged at 13,000 rpm to clarify the supernatant. The supernatant was incubated 2 hours with TALON beads (Takara) pre-equilibrated with lysis buffer and non-specific proteins were cleared with washing. Proteins were eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 250 mM Imidazole, 1 mM TCEP, pH 7.4) and further purified on size-exclusion column (Superdex 75 16/60, GE Healthcare) pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl, 1 mM TCEP, pH 7.4. Proteins were concentrated to 20 mg/ml and stored for crystallization. For the expression of mISG15, BL21(DE3) E. coli competent cells (NEB) were transformed with pACE-ISG15. Bacterial colony was grown in LB medium to an OD600 of 0.6-0.8 at 37 °C. Protein production was induced by addition of 0.5 mM IPTG (isopropyl D-thiogalactopyranoside) and 1 mM Zinc Chloride (ZnCl2) the cells were further grown overnight at 18 °C and harvested. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, 2 mM DTT, pH 8.5) and lysed by sonication and centrifuged at 13,000 rpm to clarify the supernatant. The supernatant was incubated 2 hours with TALON beads (Takara) pre-equilibrated with lysis buffer and non-specific proteins were cleared with washing. Proteins were eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 250 mM Imidazole, 2 mM DTT, pH 8.5). Eluted proteins were buffer exchanged to storage buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM DTT, pH 8.5) and stored for biochemical analysis. 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Protein production was induced by addition of 0.5 mM IPTG (isopropyl D-thiogalactopyranoside) and 1 mM Zinc Chloride (ZnCl2) the cells were further grown overnight at 18 °C and harvested. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, 2 mM DTT, pH 8.5) and lysed by sonication and centrifuged at 13,000 rpm to clarify the supernatant. The supernatant was incubated 2 hours with TALON beads (Takara) pre-equilibrated with lysis buffer and non-specific proteins were cleared with washing. Proteins were eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 250 mM Imidazole, 1 mM TCEP, pH 7.4) and further purified on size-exclusion column (Superdex 75 16/60, GE Healthcare) pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl, 1 mM TCEP, pH 7.4. Proteins were concentrated to 20 mg/ml and stored for crystallization.
refinement and manual model building were performed with Coot and Phenix. Refine (Extended Data Table 1). There are 93.26% and 6.74% of the residues in the favoured/allowed regions of the Ramachandran plot respectively, and no residues are found in disallowed regions.

Cell lysates deubiquitination and deISGylation assay
HeLa or A549 cells were treated with IFN-α (200 U/ml) for 48 hrs to induce ISGylation. Cells were lysed with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40) and concentration was measured with BCA assay (Thermo Fisher). 10 μg of lysates were incubated with 100 nM of PLpro for indicated timepoints at 37 °C and analyzed by immunoblotting with indicated antibodies. To test inhibitory effect of GRL-0617, 40 μM of GRL-0617 was included during reaction. Images were obtained from Image lab software (Biorad).

Deneddylolation and kBα deubiquitination assay
All proteins described are of human origin. CUL1-RBX1, SKP1-β-TRCP2, UBE2M, UBE2D3, NEDD8, UB, APPBP1-UBA3, UBAI were purified as previously described. Deneddylolation assays were performed with 1 μM hyperneddylated CUL1-RBX1, and 5 μM protease (SCoV-PLpro, SCoV2-PLpro, DENI, USP2) or 20 nM CN. Reaction was performed at 37 °C in 25 mM Tris 100 mM NaCl, 5 mM DTT pH 8.5, and in the case of CN with additional 10 mM MgCl2. Samples were taken at each indicated time point and quenched with 2X SDS-PAGE sample buffer. Gels were stained by Coomassie-blue and scanned on an Amersham imager 600. kBα Deubiquitination assays were performed by first generating a ubiquitylated kBα, with 200 nM UBAI, 1 μM UBE2D3, 20 μM UB, and 500 mM neddylated CRL1180, and 5 μM fluorescently labelled kBα at 37 °C in 50 mM Tris 50 mM NaCl 10 mM MgCl2 5mM DTT pH 7.5 for 30 minutes. Reaction was quenched by adding 80 mM EDTA for 5 minutes. Deubiquitination reaction was started by mixing 3 μM of protease (SCoV-PLpro, SCoV2-PLpro, USP2) with the ubiquitylation reaction, and samples were taken at each time point and quenched with 2X SDS-PAGE sample buffer. Gels were scanned on an Amersham Typhoon (GE) detecting the fluorescently labelled kBα.

Molecular Dynamics Simulations
SCoV-PLpro (wild type and double mutant) and SCoV2-PLpro with K48-Ub2. The coordinates of SCoV-PLpro with bound K48-Ub2 were taken from PDB ID: 69WC4. For the double mutant set up, the mutations S67V and L76T were introduced using MODELLER. For SCoV2-PLpro with K48-Ub2, we extracted the minimum heavy-atom distance of the backbone carbonyl carbon atoms of Lys48 and Gly75 with a target distance of 9.5 Å and a force constant of 0.2080 kJ mol⁻¹ nm⁻². The covalent propargylamidine linker was removed.

SCoV-PLpro and SCoV2-PLpro in complex with inhibitor GRL-O617. The coordinates of the SCoV-PLpro:GRL-O617 complex were taken from PDB ID: 3E9S. The oxidized Cys112 was changed to the reduced form (SH) using MODELLER. The simulation model of the SCoV2-PLpro:GRL-O617 complex was built according to the X-ray structure of the unbound form of SCoV2-PLpro (PDB ID: 6W9C, reefined by Tristan Croll). The coordinates of the compound GRL-0617 were modeled according to PDB ID: 3E9S after PLpro alignment using PyMol. The blocking loop 2 (BL2 loop, GNYQCGH) capping the GRL-0617 binding site was remodeled according to the SCoV-PLpro X-ray crystal structure of the complex (PDB ID: 3E9S) using MODELLER. The GRL-0617 ligand was parameterized with the General Amber Force Field (GAFF)31.

SCoV2-PLpro with mISG15. The X-ray crystal structure SCoV2-PLpro:mISG15 (PDB ID: 6YVA) served as starting point. Missing residues of SCoV2-PLpro and one Zn ion were modeled according to the X-ray crystal structure (PDB ID: 6W9C, reefined by Tristan Croll).

Molecular Dynamics Simulations
All crystallographic water molecules and ions were retained, except a nickel ion in PDB ID: 5E6J. According to pKa calculations using PropKa and additional visual inspections, in all setups His17 of SCoV2-PLpro (His18 of SCoV-PLpro) and His272 of SCoV2-PLpro (His273 of SCoV-PLpro) were charged. We cannot exclude that the protonation state of the catalytic His272 of SCoV2-PLpro (His273 of SCoV-PLpro) is in equilibrium between charged and neutral forms. All other residues were simulated in their physiological protonation state. The proteins were solvated in TIP4P-D water with 150 mM NaCl. MD simulations were carried out using Gromacs2018 and the AMBER99SB-ILDN q-force field57. Each system was energy minimized, followed by five equilibration steps, in which we gradually weakened the position restraints on heavy atoms, first in an NVT ensemble (0.25 ns) and then in an NPT ensemble (4 x 0.5 ns) using a Berendsen thermostat and barostat32. Production simulations were run at a temperature of 310 K and a pressure of 1 bar in an NPT ensemble using a Nosé-Hoover thermostat33 and a Parrinello-Rahman barostat34. We set up three independent runs of the SCoV2-PLpro systems with bound substrates, starting from different MODELLER results for the apo-like model of SCoV2-PLpro:K48-Ub, and for SCoV2-PLpro:mlSISG15. For simulations with bound substrates and with bound inhibitor, we monitored the root-mean-square deviation (RMSD) of each backbone substrate (distal ubiquitin in K48-Ub and N-terminal domain of mlSISG15) and of GRL-0617 (heavy atoms) to the respective equilibrated structure after alignment on the helix backbone of PLpro (without the flexible UBL domain). From simulations of SCoV-PLpro:K48-Ub2, we extracted the minimum heavy-atom distance between F70 of SARS and I44 of ubiquitin.

Inhibitor IC₅₀ determination
For IC₅₀ value for inhibitors, ubiquitin-AMC or ISG15-AMC was used as substrate of PLpro and the release of AMC was measured by increase of fluorescence (Ex./Em. 360/487 nm) on 384-well microplate reader (PHERAstar FSX, BMG Labtech). 5ul of solution containing different concentration of GRL-0617, 40 μM of GRL-0617 was included during reaction. Images were obtained with indicated antibodies. To test inhibitory effect of inhibitor, we monitored the decrease of intensity of fluorescent signal with indicated antibodies normalized against control. IC₅₀ value is calculated by Dose-response function in Graphpad Prism with [inhibitor] vs normalized response equation. The experiment was repeated three times.

Mass-spectrometry
For interactome analysis, A549 cells were transfected with SCoV-PLpro or SCoV2-PLpro wt or mutant (CIIIS) and for comparison between SARS and SARS-CoV-2, mutant PLpro (CIIIS) versions for both proteins were transfected. Cells were stimulated with Interferon-α (200 units/ml) for 36 hours to mimic infection scenario. Cells were lysed in ice cold lysis buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Triton x-100) and equal amounts of lysates were incubated with GFP nanotrap beads in IP buffer (Lysis buffer without detergent). After incubation, IPs were washed three times with wash buffer (50 mM Tris-HCl, pH7.5; 400 mM...
NaCl; 0.5 mM EDTA) and two times with IP buffer. Then beads were incubated with 25 μl of 50 mM Tris-HCl (pH 8.5) containing 4 M urea, 1 mM TCEP, 4 mM Chloroacetamide for 1 hour in the dark at 37 °C. After overnight, samples were then diluted with 50 mM Tris-HCl pH 8.5 to final urea conc. < 2 M and digested with 0.5 μg Trypsin (Promega) at 37 °C. Peptides were labelled with TMT reagents (Thermo Fisher) as described previously. Briefly, peptides were resuspended in TMT labelling buffer (0.2 M EPPS pH 8.2, 20% Acetonitrile) and were mixed with TMT reagents in a 2:1 TMT : peptide ratio. Reaction was performed for one hour at RT and subsequently quenched by addition of hydroxyamine to a final concentration of 0.5% at RT for 15 min. Samples were pooled in equimolar ratio, acidified, and again cleaned-up using C18-stage tips. After drying, peptides were resuspended in 0.1% formic acid (FA) for LC-MS. All mass spectrometry data was acquired in centroid mode on an Orbitrap Fusion Lumos mass spectrometer hyphenated to an easy-nLC 1200 nano HPLC system with a nanoFlex ion source (ThermoFisher Scientific). A spray voltage of 2.6 kV was applied with the transfer tube heated to 300 °C and funnel RF set to 30%. Internal mass calibration was performed (lock mass 445.12003 m/z). Peptides were separated on a self-made 32 cm long, 75 μm i.d. fused-silica column, packed in house with 1.9 μm C18 particles (ReproSil-Pur, Dr. Maisch) and heated to 50 °C using an integrated column oven (Sonation). HPLC solvents consisted of 0.1% Formic acid in water (Buffer A) and 0.1% Formic acid, 80% acetonitrile in water (Buffer B). Peptides were eluted by a non-linear gradient from 7 to 40% B over 90 minutes followed by a step-wise increase to 95% B in 6 minutes which was held for another 9 minutes. Full scan MS spectra (350-1400 m/z) were acquired with a resolution of 120,000 at m/z 200, maximum injection time of 100 ms and AGC target value of 4 x 10^5. The 20 most intense precursors per full scan with a charge state between 2 and 5 were selected for fragmentation (“Top 20”). Isolated with a quadrupole isolation window of 0.7 Th and fragmented via HCD applying an NCE of 38%. MS2 scans were performed in the Orbitrap using a resolution of 30,000 at m/z 200, maximum injection time of 86 ms and AGC target value of 1 x 10^6. Repeated sequencing of already acquired precursors was limited by setting a dynamic exclusion of 60 seconds and 7 ppm and advanced peak determination was deactivated. Raw MS raw data was analyzed with Proteome Discoverer (PD, version 2.4, ThermoFisher Scientific) using Sequest HT as a search engine and performing re-calibration of precursor masses by the Spectrum Scope system. An Ar-ion laser (for excitation of GFP at 488 nm), a He-Ne laser (for excitation Alexa Fluor 546nm) were used with a 63×1.4 NA oil immersion objective. Images were analysed in Fiji to check for colocalization between DAPI and immunostained p65. Results are indicative of 50 cells taken from three independent experiments; error bars indicate standard deviation.

**Cell culture**

Human CaCo–2 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cells were grown at 37 °C in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and containing 100 IU/ml penicillin and 100 μg/ml streptomycin. A549 and HeLa cells were obtained from ATCC (ATCC CCL-185, ATCC CCL-2, respectively). All the cell lines used tested negative for mycoplasma.

**Antibodies**

We have used following antibodies and dilutions for this study. Ubiqutin (Cat# 3936S, Provider: Cell signaling Technology, 1:2000), ISG15 (Cat# HPA004627, Sigma Aldrich/Merck, 1:1000), GAPDH (Cat# 2118, Cell signaling Technology, 1:2000), GFP trap beads (Cat#: gta-100, Provider: ChromoTek, GFP (Cat# sc-9996, Santa Cruz Biotechnology, 1:2000), IRF3 (Cat# 4302, Cell signaling Technology, 1:2000), phospho-IRF3(Ser396) (Cat# 4947, Cell signaling Technology, 1:1000), IkBa (Cat# 4812, Cell signaling Technology, 1:2000), phospho-IkBαSer32/36 (Cat# 9246, Cell signaling Technology, 1:1000), TBK1 (Cat# 3013, Cell signaling Technology, 1:2000), pTBK1 (Cat# 3300-1 Epitomics, 1:1000), P65 (NFκB) (Cat# 8008, Santa Cruz Biotechnology, 1:2000), Lamin B1 (Cat# sc-373918, Santa Cruz Biotechnology, 1:2000).

**Virus preparation**

SARS-CoV–2/FFM1 (Accession: MT358638) was isolated from travellers returning from Wuhan (China) to Frankfurt (Germany) using CaCo–2 cells. SARS-CoV–2/FFM1 stocks used in the experiments had undergone one passage on CaCo–2 cells as described previously. Virus titers were determined as TCID_{50}/ml in confluent cells in 96-well microtiter plates.

**Antiviral and cytotoxicity assays**

Confluent layers of CaCo–2 cells in 96-well plates were infected with SARS-CoV–2/FFM1 at MOI 0.01. Virus was added simultaneously with GRL-0617 and incubated in MEM supplemented with 1% FBS with different drug dilutions. Cytopathogenic effect (CPE) was assessed visually 48 h after infection. To assess effects of GRL-0617 on CaCo–2 cell viability, confluent cell layers were treated with different drug concentration. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay modified after Mosman, as previously described. Data for each condition was collected for at least three biological replicates.

**Luciferase activity assay**

To analyze the induction of IFNβ induced genes, a luciferase reporter assay was used in A549 cells. Briefly, an expression construct containing the luciferase ORF and the IFNβ promoter (IFNβ/luciferase) was co-transfected with either a GFP control plasmid or the designated PLpro plasmid. For all transfections, 100 ng of luciferase plasmid, 400 ng of PLpro or GFP vector was used in each well of a 12 well plate. All transfections were performed in triplicate and the average of 3 experiments is shown in figures. 24h post transfection cells were treated with 500 ng poly I:C for 18h or 50 ng/ml of TNFα for 30 min. Luciferase expression was measured Luciferase Reporter assay system (Promega Inc). Fold change is calculated by taking vector treated with poly I:C or TNFα as 1.

**Immunofluorescence and confocal imaging**

HeLa cells expressing GFP tagged PLpro was treated with TNFα (50 ng/ml) for 45min. Cells were fixed with paraformaldehyde, blocked in PBS and immunostained overnight at 4 °C with antibody against p65. Confocal imaging was performed using the Zeiss LSM780 microscope system. An Ar-ion laser (for excitation of GFP at 488 nm), a He-Ne laser (for excitation Alexa Fluor 546nm) were used with a 63×1.4 NA oil immersion objective. Images were analysed in Fiji to check for colocalization between DAPI and immunostained p65. Results are indicative of 50 cells taken from 3 independent experiments; error bars indicate standard deviation.

**Nuclear Fractionation**

A549 cells from a confluent 60 mm dish were transiently transfected with GFP tagged PLpro followed by treatment with interferons (200u/ml, 36h). Cells were lysed in hypotonic buffer [10 mmol/L HEPES (pH = 7.4), 2 mmol/L MgCl₂, 25 mmol/L KCl, 1 mmol/L DTT, 1 mM PMSF, and protease

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All the cell lines used tested negative for mycoplasma.
inhibitor cocktail), kept on ice for 30 min followed by syringing lysis. 2 mol/L sucrose solution was added dropwise, followed by centrifugation at 1000g for 15 min. The supernatant was saved as the cytosolic fraction. The pellet was washed twice in wash buffer [10 mmol/L HEPES (pH = 7.4), 2 mmol/L MgCl₂, 25 mmol/L KCl, 250 mmol/L sucrose, 1 mmol/L DTT, 1 mmol/L PMSF, and protease inhibitor cocktail] and saved as the nuclear fraction.

Quantification of viral and cellular RNA
SARS-CoV-2 RNA from cell culture supernatant samples was isolated using ACL buffer and the QIAamp 96 Virus kit (Qiagen) according to the manufacturer’s instructions. RNA was subjected to OneStep qRT-PCR analysis using the LightCycler Multiplex RNA Virus Master kit (Roche). Intracellular RNA was isolated using RLT buffer and the RNeasy 96 HT Kit according to the manufacturer’s instructions. PCR was performed on a CFX96 Real-Time System, C1000 Touch Thermal Cycler. Primers and probe were adapted from the WHO protocol49 targeting the open reading frame for RNA-dependent RNA polymerase (RdRP) of both SARS-CoV-2: RdRP_SARS-f2 (GTGARATGGTCAATGTCGGCGG) and RdRP_SARS-r1 (CARATGTAAASACATCTAGTA) primers were used in a final concentration of 0.4 μM and RdRP_SARS-f2 probe (6-Fam CAGGTGGAACCTCATCAGGAGATGC BBQ1) was used with 0.2 μM, respectively. Primers for ACTB ( fwd: CATCGACGACAGGATGGCTGA; rev: TACGACGCTGATGAGACGAG )50, IGFl5 ( fwd: GAGAGCCA GCAAATCTAC )51, IL6 ( fwd: GCAGAA CTCTGAGTCA; rev: TAGCACAGCCTGGATAGCAAC )70, ISG15 ( fwd: GAGAGCA AAGGGAAAGAATC; rev: CTACATTGCCGAAGACCC )72, IL8 ( fwd: GTTT GCGAACTCATCT; rev: AGGGACACCTGGAATTCGTT )71 IL6 ( fwd: GCAGAA ATCGTCA; rev: TAGCACAGCCTGGATAGCAAC )70, ISG15 ( fwd: GAGAGCA AAGGGAAAGAATC; rev: CTACATTGCCGAAGACCC )72, IL8 ( fwd: GTTT GCGAACTCATCT; rev: AGGGACACCTGGAATTCGTT )71 IL6 ( fwd: GCAGAA ATCGTCA; rev: TAGCACAGCCTGGATAGCAAC )70, ISG15 ( fwd: GAGAGCA AAGGGAAAGAATC; rev: CTACATTGCCGAAGACCC )72, IL8 ( fwd: GTTT GCGAACTCATCT; rev: AGGGACACCTGGAATTCGTT )71 IL6 ( fwd: GCAGAA ATCGTCA; rev: TAGCACAGCCTGGATAGCAAC )70, ISG15 ( fwd: GAGAGCA AAGGGAAAGAATC; rev: CTACATTGCCGAAGACCC )72, IL8 ( fwd: GTTT GCGAACTCATCT; rev: AGGGACACCTGGAATTCGTT )71 

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Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data and code availability
The atomic coordinates of E-pro-IGFl5 (murine) have been deposited in the PDB with accession code 6YVA in the Protein Data Bank. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium58 via the PRIDE partner repository59 with the dataset identifier PXD018983. The papain-like protease domain sequence is obtained from SARS-CoV-2 complete genome (NCBI genome database, Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome; NC_045512). Proteinase sequence for CoV2 Pl pro-Ubl domain (amino acids, 746-1060) of Nsp3 protein from SARS-CoV-2 (Nsp3;YP_009725999.1).

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Author contributions
BS and IC conceived the project. DS contributed protein purification, improved side-chain torsion potentials for the Amber ff99SB protein force field. Protein Struct. Funct. Bioinforma. 65, 712–725 (2006). Best, R. B., de Sarro, G. & Malinovskaya, I. Herpes simplex v...
assay. AB and GT designed, performed mass spectrometry experiments and analyzed data. LS and ARM performed MD simulations. KR contributed to qRT-PCR materials and critical advice. PPG and GJvdHvN synthesized Ub(l) probes and reagents in the lab of HO. SM and KPK provided Ub probes and reagents. BS, GH, JC, SC and ID supervised the project. DS and ID analyzed the data and wrote the manuscript with input from all the co-authors.

**Competing interests** The authors declare no competing interests.

**Additional information**

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41586-020-2601-5.

**Correspondence and requests for materials** should be addressed to I.D.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Biochemical properties of SCoV2-PLpro. a, Sequence similarity of PLpro from SARS, MERS and SARS-CoV2. b, IFN-α treated HeLa cell lysates were incubated with PLpro for indicated time points and analyzed by immunoblot. c, Propargylamide-activity based probes of ubiquitin like modifiers were reacted with (left) SCoV-PLpro (right) PLpro<sup>am</sup>. d, ISG15-Prg were incubated with SCoV-PLpro (left) or SCoV2-PLpro (right) with increasing amount of non-hydrolysable K48-Ub<sub>2</sub>. e, Initial AMC release rate from ISG15-AMC. Purified SCoV-PLpro and SCoV2-PLpro were incubated with ISG15-AMC and indicated amounts of K48-Ub<sub>2</sub>. The release of AMC was measured by increase of fluorescence at (Ex./Em. 360/487 nm). f, Purified mUSP18 (left) and SCoV2-PLpro (right) were incubated with ISG15-propargylamide activity-based probes for indicated time points. g, Catalytic efficiency (k<sub>cat</sub>/K<sub>M</sub>) of mUSP18 and SCoV2-PLpro on ISG15-AMC cleavage. h, Sequence alignment of PLpro cleavage site of Nsp1/2, Nsp2/3, Nsp3/4 from SARS-CoV2 and human ubiquitin like modifiers. i, Hyper-NEDDylated CUL1-RBX1 was incubated with purified PLpro proteins for indicated time points at 37 °C. Reactions were performed side-by-side by with well-characterized deneddylating enzymes (DEN1 with broad specificity or COP9 Signalosome CSN specific for NEDD8 linked directly to a cullin), or the broad specificity deubiquitinating enzyme USP2 as controls. Data in e, g are presented as mean ± S.D (n=3, independent experiments). ** p < 0.01, *** p < 0.001, **** p < 0.0001; two-tailed paired t-tests. Experiments in b–d, f, i were repeated three times independently with similar results.
Extended Data Fig. 2 | Complex structure of SCoV2-PLpro with mouseISG15.

a, Structural comparison of mouseISG15:SCoV2-PLpro with humanISG15:MERS-  
PLpro (PDB: 6BI8) and sequence alignment of human and mouse ISG15.

b, Activity test of wild type or catalytically inactive mutant (C111S) of SCoV-PLpro  
and SCoV2-PLpro. ISG15 Propargyl-activity based probes were mixed with  
indicated PLpro proteins. Experiments were repeated three times  
independently with similar results.

c, Structural comparison of C-terminal  
domain of ISG15 in complex with SCoV2-PLpro and SCoV-PLpro (PDB: 5TL7).

d, Snapshots from molecular dynamics simulations of SCoV2-PLpro (light pink  
cartoon) with (left) K48-Ub at 340 ns and (right) mISG15 at 3.2 µs. Key residues  
in the interface are highlighted.

e, Backbone RMSD of the N-terminal domain of  
mISG15 (green) and of the distal ubiquitin in K48-Ub, in an apo-like model  
orange, model1, SCoV2-PLpro coordinates from substrate unbound form,  
PDB: 6W9C) and in an mISG15-like model (yellow, model2, SCoV2-PLpro  
coordinates from substrate bound form, PDB: 6YVA) from their respective  
SCoV2-PLpro-bound starting structures as function of time. The RMSD was  
calculated after superimposing the helix backbone atoms of SCoV2-PLpro.  
Time points for structural snapshots in e) are marked with a cross. f, Minimum  
heavy atom distance between F70 (SARS) and I44(Ub) in wild type and double  
mutant (S67V/L76T) of SCoV-PLpro:K48-Ub in wild type and double  
mutant (S67V/L76T) of SCoV-PLpro:K48-Ub, as function of time. g, Water  
mediated dissociation pathway. (left) Initial hydrophobic interactions between  
F69(CoV2), T75(CoV2) and I44(Ub). (middle) Water wedges in between  
T75(CoV2)/F69(CoV2) and I44(Ub) leads to dissociation.
**Extended Data Fig. 3 | Sequence alignment of papain-like protease domain from coronaviruses.** The amino acid sequences of papain-like protease domain from eight different coronaviruses (SARS-CoV-2, SARS, MERS, humanCoV-OC43, humanCoV-229E, humanCoV-NL63, murineHepatitisV, bovine CoV) were aligned with Clustal Omega. Accession numbers: SARS-CoV-2 (NC_045512), SARS (PDB: 3MJ5), MERS (PDB: 5W8U), hCoV-OC43 (AY585228), hCoV-229E (X69721), hCoV-NL63 (NC_005831), murineHepatitisV (NC_001846), bCoV (NC_003045).

| SARS-CoV-2 | SARS | MERS | hCoV_OC43 | hCoV_229E | hCoV_NL63 | HepatitisV | bCoV |
|------------|-------|------|-----------|-----------|-----------|------------|------|
| **S2-binding sites** | | | | | | | |
| 57 DDTLKVLEEFLYERTDF | 57 DDTLKVLEEFLYERTDF | 60 ETAELKALEYDLGALF | 60 LSSEDLDKVKF-S | 60 LSSEDLDKVKF-S | 60 LSSEDLDKVKF-S | 60 LSSEDLDKVKF-S |
| 99 DNTLVGFDVYF | 99 DNTLVGFDVYF | 102 DNTLVGFDVYF | 102 DNTLVGFDVYF | 102 DNTLVGFDVYF | 102 DNTLVGFDVYF | 102 DNTLVGFDVYF |
| 131 DNTLVGFDVYF | 131 DNTLVGFDVYF | 134 DNTLVGFDVYF | 134 DNTLVGFDVYF | 134 DNTLVGFDVYF | 134 DNTLVGFDVYF | 134 DNTLVGFDVYF |
| 163 DNTLVGFDVYF | 163 DNTLVGFDVYF | 166 DNTLVGFDVYF | 166 DNTLVGFDVYF | 166 DNTLVGFDVYF | 166 DNTLVGFDVYF | 166 DNTLVGFDVYF |
| 264 DNTLVGFDVYF | 264 DNTLVGFDVYF | 267 DNTLVGFDVYF | 267 DNTLVGFDVYF | 267 DNTLVGFDVYF | 267 DNTLVGFDVYF | 267 DNTLVGFDVYF |

**Consensus**

| SARS-CoV-2 | SARS | MERS | hCoV_OC43 | hCoV_229E | hCoV_NL63 | HepatitisV | bCoV |
|------------|-------|------|-----------|-----------|-----------|------------|------|
| **Catalytic triad** | | | | | | | |
| 41 EYQGCHYTHITTCAETEL | 41 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL |
| 71 EYQGCHYTHITTCAETEL | 71 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL |
| 101 EYQGCHYTHITTCAETEL | 101 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL |
| 131 EYQGCHYTHITTCAETEL | 131 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL |
| 161 EYQGCHYTHITTCAETEL | 161 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL |
| 191 EYQGCHYTHITTCAETEL | 191 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL |

**Consensus**

| SARS-CoV-2 | SARS | MERS | hCoV_OC43 | hCoV_229E | hCoV_NL63 | HepatitisV | bCoV |
|------------|-------|------|-----------|-----------|-----------|------------|------|
| **Key residues on S2-binding sites** | | | | | | | |
| 41 EYQGCHYTHITTCAETEL | 41 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL | 44 EYQGCHYTHITTCAETEL |
| 71 EYQGCHYTHITTCAETEL | 71 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL | 74 EYQGCHYTHITTCAETEL |
| 101 EYQGCHYTHITTCAETEL | 101 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL | 104 EYQGCHYTHITTCAETEL |
| 131 EYQGCHYTHITTCAETEL | 131 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL | 134 EYQGCHYTHITTCAETEL |
| 161 EYQGCHYTHITTCAETEL | 161 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL | 164 EYQGCHYTHITTCAETEL |
| 191 EYQGCHYTHITTCAETEL | 191 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL | 194 EYQGCHYTHITTCAETEL |

**Consensus**
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Structural analysis of GRL-0617, SCoV2-PLpro complex.
a, Structural model of GRL-0617 bound SCoV2-PLpro. The conformation of Tyr268 on SCoV2-PLpro and the coordinates of GRL-0617 is obtained from the SCoV-PLpro:GRL-0617 structure (PDB: 3E9S)^17. b, Snapshots of SCoV-PLpro (light cyan) and SCoV2-PLpro (light pink) with bound GRL-0617 (dark colors) after 1μs of molecular dynamics simulation. The protein backbones are shown in cartoon representation, and the ligand with contacting residues as sticks. c, RMSD of the GRL-0617 bound to SCoV-PLpro (light blue) and SCoV2-PLpro (light pink) as a function of time. The RMSD was calculated for non-hydrogen atoms of GRL-0617 with respect to the starting structures in the MD simulations after superimposing the helix backbone atoms of PLpro. d, In vitro PLpro inhibition assay. Initial velocity of AMC release from ubiquitin-AMC in different concentration of GRL-0617 was measured and normalized to DMSO control. IC50 of GRL-0617 to SCoV-PLpro and SCoV2-PLpro were presented. Data are presented as mean ± S.D (n=3, independent experiments). e, In vitro PLpro inhibition assay. Initial velocity of AMC release from ISG15-AMC in different concentration of GRL-0617 was measured and normalized to DMSO control. IC50 of GRL-0617 to SCoV-PLpro were presented. Data are presented as mean ± S.D (n=3, independent experiments). f, Effects of GRL-0617 on (left) deISGylase or (right) deubiquitinase activity of PLpro of SARS and SARS-CoV-2. g, Effects of GRL-0617 on SCoV-PLpro activity to (left) ubiquitin or (right) K48-Ub, propargyl activity-based probes. Inhibitory effect of GRL-0617 on ubiquitin species was tested with various concentration of GRL-0617 (0–400 μM). h, Effects of GRL-0617 on SCoV2-PLpro activity to (left) ISG15-C-term or (right) ISG15 propargylamide activity-based probes. Inhibitory effect of GRL-0617 on ISG15 was tested with various concentration of GRL-0617 (0–400 μM). Experiments in f–h were repeated three times independently with similar results.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Physiological roles of PLpro in cells. a, b, Effect of SERPIN B3 on PLpro mediated (a) IFN-β or (b) NF-κB p65 expression level. A549 Cells were co-transfected with indicated GFP-PLpro and Myc-SERPINs and treated with either poly (I:C) or TNF-α to induce IFN-β and NF-κB p65 expression, respectively. Fold changes of luciferase level are presented.
c, Effect of PLpro on IFN-induced cellular ISGylation. A549 cells were transfected with indicated PLpro plasmids and treated with IFN-α. Lysates were analysed by immune-blotting with indicated antibodies.
d, e, Effect of PLpro on IFN-signalling pathway. A549 cells were transfected with indicated PLpro plasmids and treated with IFN-α. Lysates were analysed by immune-blotting with indicated antibodies. e, Effect of PLpro on cellular localization of IRF3. Cells from (d) were fractionated into cytosol and nucleus and the level of IRF3 was analysed. Lamin B1 was used for nuclear fraction control. f, Effect of PLpro on the NF-κB pathway. IκB-α phosphorylation and degradation were examined from A549 cells expressing indicated GFP-PLpro under treatment of TNF-α.
g, In vitro IκBα deubiquitylation assay. Ubiquitinated IκBα were incubated with SCoV-PLpro or SCoV2-PLpro. USP2 were used as positive control. h, Effect of PLpro on NF-κB pathway. IκB-α phosphorylation and degradation were examined from A549 cells expressing indicated GFP-PLpro under treatment of TNF-α.
Extended Data Fig. 6 | Effect of PLpro on IFN-β or NF-κB p65 expression level. **a, b**, Effect of PLpro on (a) IFN-β or (b) NF-κB p65 expression level. A549 Cells were transfected with indicated GFP-PLpro and treated with either poly (I:C) or TNF-α to induce IFN-β and NF-κB p65 expression, respectively. **c, d**, Effect of GRL-0617 on PLpro mediated (c) IFN-β or (d) NF-κB p65 expression level. A549 Cells were transfected with indicated GFP-PLpro and treated with either poly (I:C) or TNF-α to induce IFN-β and NF-κB p65 expression, respectively. GRL-0617 is treated as indicated. All Data are presented as mean ± S.D (n=3, independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001; two-tailed paired t-tests.
Extended Data Fig. 7 Inhibitory effects of GRL-0617 on SARS-CoV2 infection. a, Intracellular virus production was analysed by PCR targeting SARS-CoV-2 RdRP mRNA. Relative expression level of SARS-CoV2-2 genomic RNA was normalized to cellular GAPDH level. b, Intracellular RNA was isolated from cells without infection or cells infected with SARS-CoV-2 with or without treatment of GRL-0617. Relative mRNA-level fold change of indicated genes were analysed in a qRT-PCR analysis and normalized to ACTB levels. Data in a, b are presented as mean ± S.D (n=3, independent experiments). * p < 0.05, ** p < 0.01; two-tailed paired t-tests. c, Schematic representation of the role of SARS-CoV2 PLpro in the viral life cycle. The physiological role of SCoV2 PLpro in both host-immune response and polypeptide processing is shown. Inhibition of PLpro by GRL-0617 is also presented.
### Extended Data Table 1 | Data collection and refinement statistics (molecular replacement)

SCoV2-PLpro (C111S): mLSG15 (PDB: 6YVA)

#### Data collection

| Space group          | P 6 2 2 |
|----------------------|---------|
| Cell dimensions      |         |
| $a$, $b$, $c$ (Å)    | 157.047, 157.047, 83.633 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 90, 120 |
| Resolution (Å)       | 45.34 – 3.185 (3.298 - 3.185) |
| $R_{sym}$ or $R_{merge}$ | 0.05751 (0.5689) |
| $I / \sigma I$       | 8.58 (1.19) |
| Completeness (%)     | 99.61 (97.77) |
| Redundancy           | 2.0 (2.0) |

#### Refinement

| Resolution (Å)       | 45.34 – 3.185 (3.298 - 3.185) |
| No. reflections      | 10590 (1008) |
| $R_{work} / R_{free}$| 0.2496 /0.2902 |
| No. atoms            | 3407 |
| Protein              | 3383 |
| Ligand/ion           | 1 |
| Water                | 23 |
| B-factors            | 87.60 |
| Protein              | 87.72 |
| Ligand/ion           | 167.02 |
| Water                | 66.67 |
| R.m.s. deviations    |       |
| Bond lengths (Å)     | 0.004 |
| Bond angles (°)      | 0.65 |

Statistics for data collection and refinement are presented. * A single crystal was used for data collection and structure determination. †Values in parentheses are for highest-resolution shell.
### Extended Data Table 2 | Kinetic parameters on AMC substrates

| Kinetic Parameter       | Triazole-linked K48-Ub2-AMC | ISG15-AMC |
|-------------------------|-----------------------------|-----------|
| **SARS-CoV-2 PLpro**    |                             |           |
| Apparent $k_{cat}/K_M$ [M$^{-1}$s$^{-1}$] | 2.41 ± 0.94 (E+05) | 5.21 ± 0.36 (E+05) |
| $k_{cat}$ [s$^{-1}$]   | 14.75 ± 3.28                | 4.43 ± 0.13 |
| $K_M$ [$\mu$M]        | 61.23 ± 19.76               | 8.50 ± 0.54 |
| Michaelis-Menten curve fit ($R^2$) | 0.9914                      | 0.9987     |
| **SARS PLpro**         |                             |           |
| Apparent $k_{cat}/K_M$ [M$^{-1}$s$^{-1}$] | 13.94 ± 3.50 (E+05) | 5.31 ± 0.56 (E+05) |
| $k_{cat}$ [s$^{-1}$]   | 62.9 ± 8.45                 | 11.89 ± 0.75 |
| $K_M$ [$\mu$M]        | 45.13 ± 9.57                | 22.41 ± 1.89 |
| Michaelis-Menten curve fit ($R^2$) | 0.9911                      | 0.9997     |
| **mUSP18**             |                             |           |
| Apparent $k_{cat}/K_M$ [M$^{-1}$s$^{-1}$] |                         | 0.68 ± 0.07 (E+05) |
| $k_{cat}$ [s$^{-1}$]   | N.D.                        | 0.06 ± 0.002 |
| $K_M$ [$\mu$M]        | 0.89 ± 0.093                |           |
| Michaelis-Menten curve fit ($R^2$) |                           | 0.9895     |

Kinetic parameters for SCoV-PLpro, SCoV2-PLpro and murineUSP18 to triazole-linked K48-Ub2-AMC or ISG15-AMC are presented. Values are presented as mean ± S.D (n=3, independent experiments).
### Extended Data Table 3 | Binding kinetics of PLpro to K48-Ub₂ or ISG15

|        | Triazole linked K48-Ub₂ | Human ISG15  | Mouse ISG15  | SARS PLpro | Triazole linked K48-Ub₂ | Human ISG15  | Mouse ISG15  |
|--------|-------------------------|--------------|--------------|------------|-------------------------|--------------|--------------|
| CoV2   |                         |              |              |            |                         |              |              |
| PLpro  |                         |              |              |            |                         |              |              |
|        | $k_{on}$ ± S.E.M<sup>a</sup> | $k_{off}$ ± S.E.M<sup>a</sup> | $K_d$ ± S.E.M<sup>a</sup> | $R^2$<sup>b</sup> | $k_{on}$ ± S.E.M<sup>a</sup> | $k_{off}$ ± S.E.M<sup>a</sup> | $K_d$ ± S.E.M<sup>a</sup> | $R^2$<sup>b</sup> |
|        | (10<sup>2</sup> M<sup>-1</sup>s<sup>-1</sup>) | (10<sup>-1</sup> s<sup>-1</sup>) | (μM)         |            | (10<sup>2</sup> M<sup>-1</sup>s<sup>-1</sup>) | (10<sup>-1</sup> s<sup>-1</sup>) | (μM)         |            |
| CoV2   |                         |              |              |            |                         |              |              |
| PLpro  |                         |              |              |            |                         |              |              |
| SARS   |                         |              |              |            |                         |              |              |
| PLpro  |                         |              |              |            |                         |              |              |

Binding kinetic parameters for SCoV-PLpro, SCoV2-PLpro to triazole-linked K48-Ub₂, humanISG15 or mouseISG15 are presented. Values are presented as mean ± S.E.M. (n=3, independent experiments). <sup>a</sup>S.E.M., standard error of mean. <sup>b</sup>R², goodness of the curve fit between experimental data and mathematical 1:1 binding curve.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a □ □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- □ □ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- □ □ The statistical test(s) used AND whether they are one- or two-sided
- □ □ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- □ □ A description of all covariates tested
- □ □ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- □ □ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- □ □ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- □ □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ □ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | Image lab software 5.2.1
Data analysis | Image lab software 5.2.1, Prism5, MaxQuant 1.6.5, Perseus 1.6.5, Pymol (1.7.6.0), phenix (1.17.1-3660), ccp4 (7.0.078), coot (0.8.9.2), Modeller (9.24), Gromacs (2019.6)

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The atomic coordinates of PLpro-ISG15 (murine) have been deposited in the PDB with accession code 6YVA in the Protein Data Bank. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018983. The papain-like protease domain sequence is obtained from SARS-CoV-2 complete genome (NCBI genome databank, Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome; NC_045512). Protein sequence for CoV2 PLpro-UBL domain (amino acids, 746-1060) of Nsp3 protein from SARS-CoV-2 (Nsp3; YP_009725299.1). Full gel images can be found in Supplementary Figure 1 and source data that support this study and can be found in Supplementary Information. Any other relevant data are available from the corresponding authors upon reasonable request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
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- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No sample size calculation was done. Experiments were repeated three times with similar results and sample size was chosen based on the consistency and significance of measured differences between groups. We have not mentioned any differences between groups if there are differences that are not statistically significant. |
| Data exclusions | No data were excluded from analysis. |
| Replication | We have repeated each experiment in the manuscript at least three times to ensure consistent results. |
| Randomization | No randomization was necessary as various infection samples were recorded and analyzed by a computer software for extracting the significant differences. |
| Blinding | Blinding was not relevant for the experiments done as various infection samples were analyzed by a computer software for extracting the significant differences. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study |
| | □ Antibodies |
| | □ Eukaryotic cell lines |
| | □ Palaeontology |
| | □ Animals and other organisms |
| | □ Human research participants |
| | □ Clinical data |
| | □ Involved in the study |
| | □ ChIP-seq |
| | □ Flow cytometry |
| | □ MRI-based neuroimaging |

Antibodies

| Antibodies | Validation |
|-----------|------------|
| Antibodies used | Ubiquitin (Cat# 39365, Provider: Cell signaling Technology, 1:2000), ISG15 (Cat# HPA004627, Sigma Aldrich/Merck, 1:1000), GAPDH (Cat# 2118, Cell signaling Technology, 1:2000), GFP trap beads (Cat #: gta-100, Provider: ChromoTek), GFP (Cat# sc-9996, Santa Cruz Biotechnology, 1:2000), IRF3 (Cat# 4302, Cell signaling Technology, 1:2000), phospho-IkBα(Ser32/36) (Cat# 9246, Cell signaling Technology, 1:1000), Lamin B1 (Cat# sc-373918, Santa Cruz Biotechnology, 1:2000). |
| Validation | Validation statement from the manufacturer: Ubiquitin (P4D1) Mouse mAb detects ubiquitin, polyubiquitin and ubiquitinated proteins. This antibody may cross-react with recombinant NEDD8. Validation found at provider’s website: https://www.cellsignal.com/products/primary-antibodies/ubiquitin-p4d1-mouse-mab/3936 |
| | ISG15 (Cat# HPA004627, Sigma Aldrich/Merck) Validation statement from the manufacturer: species reactivity-human, validation-recombinant expression, orthogonal RNA seq Validation found at provider’s website: https://www.sigmaaldrich.com/catalog/product/sigma/hsa004627?lang=en&region=DE |
| | GAPDH (Cat# 2118, Cell signaling Technology) Validation statement from the manufacturer: GAPDH (14C10) Rabbit mAb detects endogenous levels of total GAPDH protein. Species Reactivity: Human, Mouse, Rat, Monkey, Bovine, Pig Validation found at provider’s website: https://www.cellsignal.com/products/primary-antibodies/gapdh-14c10-rabbit-mab/2118 |
GFP trap beads (Cat #: gta-100, Provider: ChromoTek)
Validation statement from the manufacturer: GFP-Trap® Agarose is an affinity resin for immunoprecipitation of GFP-fusion proteins. It consists of a GFP Nanobody/VHH coupled to agarose beads. Validation found at provider's website: https://www.chromotek.com/products/detail/product-detail/gfp-trap-agarose/

GFP (Cat#: sc-9996, Santa Cruz Biotechnology)
Validation statement from the manufacturer: Anti-GFP Antibody (B-2) is a mouse monoclonal IgG2a (kappa light chain) GFP antibody provided at 200 μg/ml, raised against amino acids 1-238 representing full length GFP (green fluorescent protein) of Aequorea victoria origin. Validation found at provider's website: https://www.scbt.com/p/gfp-antibody-b-2?productCanUrl=gfp-antibody-b-2&_requestid=272661

IRF3 (Cat#: 4302, Cell signaling Technology)
Validation statement from the manufacturer: IRF-3 (D83B9) Rabbit mAb detects endogenous levels of total IRF-3 protein. Species Reactivity: Human, Mouse, Rat, Monkey. Validation found at provider's website: https://www.cellsignal.com/products/primary-antibodies/irf-3-d83b9-rabbit-mab/4302

phospho-IRF3(Ser396) (Cat#: 4947, Cell signaling Technology)
Validation statement from the manufacturer: phospho-IRF-3 (Ser396) (4D4G) Rabbit mAb detects endogenous levels of IRF-3 when phosphorylated at Ser396. Species Reactivity: Human, Mouse. Validation found at provider's website: https://www.cellsignal.com/products/primary-antibodies/phospho-irf-3-ser396-4d4g-rabbit-mab/4947

IkBa (Cat#: 4812, Cell signaling Technology)
Validation statement from the manufacturer: IkBa (44D4) Rabbit mAb detects endogenous levels of total IkBa protein. Species Reactivity: Human, Mouse, Rat, Hamster, Monkey, Mink. Validation found at provider's website: https://www.cellsignal.com/products/primary-antibodies/ikba-44d4-rabbit-mab/4812

phospho-IkBα(Ser32/36) (Cat#: 9246, Cell signaling Technology)
Validation statement from the manufacturer: Phospho-IκBα (Ser32/36) (5A5) Mouse mAb detects endogenous levels of IkBa only when phosphorylated at Ser32/36. Species Reactivity: Human, Mouse, Rat, Monkey. Validation found at provider's website: https://www.cellsignal.com/products/primary-antibodies/phospho-ikba-ser32-36-5a5-mouse-mab/9246

TBK1 (Cat#: 3013, Cell signaling Technology)
Validation statement from the manufacturer: TBK1 Antibody detects endogenous levels of total TBK1/NAK protein. Species Reactivity: Human, Mouse, Rat, Monkey. Validation found at provider's website: https://www.cellsignal.com/products/primary-antibodies/tbk1-NAK-antibody/3013

pTBK1 (Cat #: ab109272, abcam)
Validation statement from the manufacturer: This antibody only detects NAK/TBK1 phosphorylated at serine 172. Validation found at provider's website: https://www.abcam.com/naktbk1-phospho-s172-antibody-epr28672-ab109272.html

P65(NFkB) (Cat#: 8008, Santa Cruz Biotechnology)
Validation statement from the manufacturer: Anti-NFkB p65 Antibody (F-6) is a mouse monoclonal IgG1 (kappa light chain) NFkB p65 antibody provided at 200 μg/ml, raised against amino acids 1-286 mapping at the N-terminus of NFkB p65 of human origin. Validation found at provider's website: https://www.scbt.com/p/nfkappab-p65-antibody-f-6?productCanUrl=nfkappab-p65-antibody-f-6&_requestid=285577

Lamin B1 (Cat#: sc-373918, Santa Cruz Biotechnology)
Validation statement from the manufacturer: Lamin B1 Antibody (G-1) is a mouse monoclonal IgG3 (kappa light chain) provided at 200 μg/ml, specific for an epitope mapping between amino acids 559-586 at the C-terminus of Lamin B1 of mouse origin. Validation found at provider’s website: https://www.scbt.com/p/lamin-b1-antibody-g-1?requestFrom=search

**Eukaryotic cell lines**

**Policy information about cell lines**

| Cell line source(s) | AS49 cells (ATCC® CCL-185™), HeLa (ATCC® CCL-2™), CaCo-2 (DSMZ, ACC 169) |
|---------------------|--------------------------------------------------------------------------|
| **Authentication**   | Cell lines were authenticated using STR DNA profiling.                   |
| **Mycoplasma contamination** | All the cell lines tested negative for mycoplasma.                      |
| **Commonly misidentified lines** | (See ICLAC register) The cell lines used in the study are not in the commonly misidentified lines list. |