ORIGINAL RESEARCH

Inhibiting the Deubiquitinase UCHL1 Reduces SARS-CoV-2 Viral Uptake by ACE2

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Abstract

Coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), remains a significant public health burden with limited treatment options. Many β-coronaviruses, including SARS-CoV-2, gain entry to host cells through the interaction of SARS-CoV-2 spike protein with membrane-bound ACE2 (angiotensin-converting enzyme 2). Given its necessity for SARS-CoV-2 infection, ACE2 represents a potential therapeutic target in COVID-19. However, early attempts focusing on ACE2 in COVID-19 have not validated it as a druggable target nor identified other ACE2-related novel proteins for therapeutic intervention. Here, we identify a mechanism for ACE2 protein modulation by the deubiquitinase (DUB) enzyme UCHL1 (ubiquitin carboxyl-terminal hydrolase isozyme L1). ACE2 is constitutively ubiquitinated and degraded by the proteasome in lung epithelia. SARS-CoV-2 spike protein cellular internalization increased ACE2 protein abundance by decreasing its degradation. Using an siRNA library targeting 96 human DUBs, we identified UCHL1 as a putative regulator of ACE2 function as a viral receptor. Overexpressed UCHL1 preserved ACE2 protein abundance, whereas silencing of the DUB in cells destabilized ACE2 through increased polyubiquitination. A commercially available small molecule inhibitor of UCHL1 DUB activity decreased ACE2 protein concentrations coupled with inhibition of SARS-CoV-2 infection in epithelial cells. These findings describe a unique pathway of ACE2 regulation uncovering UCHL1 as a potential therapeutic target to modulate COVID-19 viral entry as a platform for future small molecule design and testing.

Keywords: COVID-19; SARS-CoV-2; ACE2; deubiquitinases; ubiquitin

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the global pandemic of coronavirus disease (COVID-19) (1). SARS-CoV-2 is a member of the β-coronavirus genera (2) that includes SARS-CoV, which emerged in the early 2000s, and Middle East respiratory coronavirus. Like all β-coronaviruses, SARS-CoV-2 gains entry to mammalian host cells through interactions of viral spike glycoproteins with the host ACE2 (angiotensin-converting enzyme 2) (3). Given its necessity for viral entry and SARS-CoV-2 infection, ACE2 represents a possible therapeutic target to treat

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Clinical Relevance

COVID-19 (coronavirus disease 2019) caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) remains a significant public health burden with limited treatment options. These studies identify a novel mechanism of regulation of the SARS-CoV-2 viral receptor, ACE2 (angiotensin-converting enzyme 2), by the deubiquitinase UCHL1 (ubiquitin carboxyl-terminal hydrolase isozyme L1) and describe a potential strategy for therapeutic targeting of ACE2.

COVID-19 and has treatment potential for future novel coronaviruses.

Under physiologic conditions, ACE2 is an essential enzyme of the renin–angiotensin hormone system that regulates blood volume and systemic vascular resistance to maintain cardiovascular homeostasis. Membrane-bound ACE2 is a type I, single-pass transmembrane protein with an N-terminal zinc metalloenzyme domain on the cell surface and intracellular C-terminal collectin renal amino acid transporter domain. In COVID-19, ACE2 functions as a membrane receptor for SARS-CoV-2 entry. Structural analyses have identified 25 amino acids in hACE2 (human ACE2) necessary for binding to the receptor binding domain of the SARS-CoV-2 viral spike protein. Numerous studies have established the necessity of ACE2 for viral infection and subsequent propagation (3, 4). As ACE2 is necessary for SARS-CoV-2 infection, pharmacologic targeting of ACE2 is a logical strategy to combat viral entry into cells and pathogenesis. Although the molecular interactions and structural basis of SARS-CoV-2 binding to ACE2 are known (5, 6), novel therapeutics directly targeting SARS-CoV-2 and ACE2 interaction are lacking. Not all proteins are druggable. Alternative approaches should be explored. Hence, the identification of ACE2 upstream regulators or new binding partners may uncover alternative strategies of indirect ACE2 targeting by small molecule therapeutics.

Like most cellular proteins, ACE2 undergoes protein ubiquitination to regulate its abundance and protein stability (7). Ubiquitination is a universal modulator of cellular protein trafficking, tagging proteins for degradation or nondegradative cellular trafficking, and most proteins experience ubiquitination during their cellular lifetime (8, 9). Proteins are covalently ubiquitinated by ubiquitin ligases (E3 ligases) that attach ubiquitin and deubiquitinases (DUBs) that remove ubiquitin in the process of posttranslational modification of substrates. The dynamic balance between E3 ligases and DUBs alters the abundance and subcellular location of proteins to impact vital cellular processes such as cell cycle, apoptosis, and response to external stimuli (10). Understanding of the role of ubiquitination in infection and host response is evolving. Our laboratory has identified multiple E3 ligases (11, 12) and DUBs (13, 14) implicated in inflammatory lung disease. ACE2 ubiquitination has been previously demonstrated in lung epithelia with multiple reported putative E3 ligases. In pulmonary hypertension, ACE2 is ubiquitinated by MDM2 to regulate its abundance, which may impact pulmonary hypertension pathogenesis (7). ACE2 ubiquitination is also modified by hyperoxia in the lung (15). The E3 ligase Skp2 has also been proposed to degrade ACE2 in the setting of cigarette smoke extract (16). Recent studies have investigated ACE2 regulation by ubiquitination during SARS-CoV-2 infection identifying the ubiquitin E3 ligase UBR4 as a putative regulator (17). Although multiple E3 ligases have been identified, these enzymes have not been developed as druggable targets in SARS-CoV-2 infection.

Here we identify the DUB UCHL1 (ubiquitin carboxyl-terminal hydrolase isozyme L1) as an important modulator of SARS-CoV-2 viral entry and infection through the regulation of ACE2 protein abundance. In Calu-3 cells, ACE2 protein abundance is increased with SARS-CoV-2 spike protein ligation without an increase in ACE2 gene expression. To investigate mechanisms preserving ACE2, we developed a bronchial epithelial cell line stably expressing hACE2. Our results suggest ubiquitination of ACE2 at lysine 788 with subsequent degradation at the proteasome. Using viral particles pseudotyped with spike protein envelope, we screened a library of all human DUBs and identified UCHL1 as a putative regulator of ACE2. We demonstrate modulation of ACE2 protein ubiquitination and abundance by UCHL1, suggesting ACE2 is a new substrate for UCHL1. Targeting UCHL1 with a commercially available small molecular inhibitor decreased SARS-CoV-2 viral uptake in lung epithelia and primary human bronchial epithelial cells, underscoring the potential biological relevance of DUBs in the pathobiology of SARS-CoV-2 infection.

Methods

SARS-CoV-2 SPIKE Pseudotyped Viral Particles

Pseudotyped lentiviral vectors with SARS-CoV-2 spike in place of VSV-G (vesicular stomatitis virus G protein) were generated as described (18). The D614G and R682Q mutations were introduced to the spike-3×FLAG (Addgene plasmid #145780) by site-directed-mutagenesis. HEK293T cells were transfected with the third-generation lentiviral packaging plasmids pRSV-Rev (Addgene plasmid #12253), pMDLg/pRRE (Addgene plasmid #12251), and a plasmid expressing SARS-CoV-2 spike (D614G, R682Q) using the XtremeGene HP DNA transfection reagent (Roche, Applied Science). At 48 hours, the virus was then concentrated 30-fold using the Lenti-X concentrator (Takara Bio).

Luciferase Assay

For luciferase assays, cells were grown in 96-well, white tissue-culture plates (Corning). After exposure to SPIKE particles as indicated, cells were lysed with Nano-Glo Luciferase Assay reagent (Promega). Luciferase reagent was prepared according to the manufacturer's protocol. For lysis, 100 μl of a 1:1 mix of luciferase reagent and cell culture media was added to each well of the 96-well plate and incubated at room temperature for 3 minutes. The plate was read using a Biotek Synergy H1 multimode plate reader.

Stable ACE2-V5 Transgene Beas2B Cell Line

cDNA for hACE2 was a gift from Hyeryun Choe (Addgene plasmid #1786) (19). The ACE2 construct was cloned into the Sleeping Beauty backbone pSBbi-RB (Addgene plasmid #60522). The final constructs were transfected into BEAS-2B and cotransfected with the Sleeping Beauty transposase

(pCMV[CAT]T7-SB100; Addgene plasmid #34879) at a 1:5 ratio using XtremeGene HP DNA Transfection Reagent (Roche). At 4–5 days after transfection, cells were selected by puromycin A1 treatment (2 μg/ml). After selection, integration was confirmed by measuring V5 expression via immunofluorescence, flow cytometry, and Western blotting.

siRNA Transfection

All siRNA were purchased as predesigned siRNA reagents from Horizon Discovery. The DUB screen was performed using the Human ON-TARGETplus siRNA library – Deubiquitinating Enzymes – SMARTpool (G-104705) and ON-TARGETplus Nontargeting Control Pool (D-001810–10). We also used nonpooled ON-TARGETplus siRNA duplexes targeting UCHL1 (J-004309–05 and J-004309–06), UCHL3 (J-006059–05 and J-006059–06), or nontargeting control (D-001810–01 and D-001810–02). Sequences are available from the manufacturer on request. For transfections, Beas2B-*ACE2*-V5 cells were plated at 70% confluence and immediately transfected with 20 nM siRNA duplexes using GenMute siRNA Transfection Reagent (SignaGen Laboratories) for 24 hours. Cells were washed with fresh media and incubated for an additional 48 hours before assay.

DUB Library Screen

Beas2B-*ACE2*-V5 cells were plated in 96-well plates. Transfections were performed in technical duplicate. Seventy-two hours after transfection, cells were exposed to SPIKE particles for an additional 48 hours before harvesting for luciferase assay, as described above. At least six control wells were present on each plate. Within each biologic replicate, relative luciferase units (RLUs) across plates were normalized to the median RLUs of the control conditions for each individual plate. After plate normalization, the mean RLU for each experimental condition was converted to a ratio relative to the control.

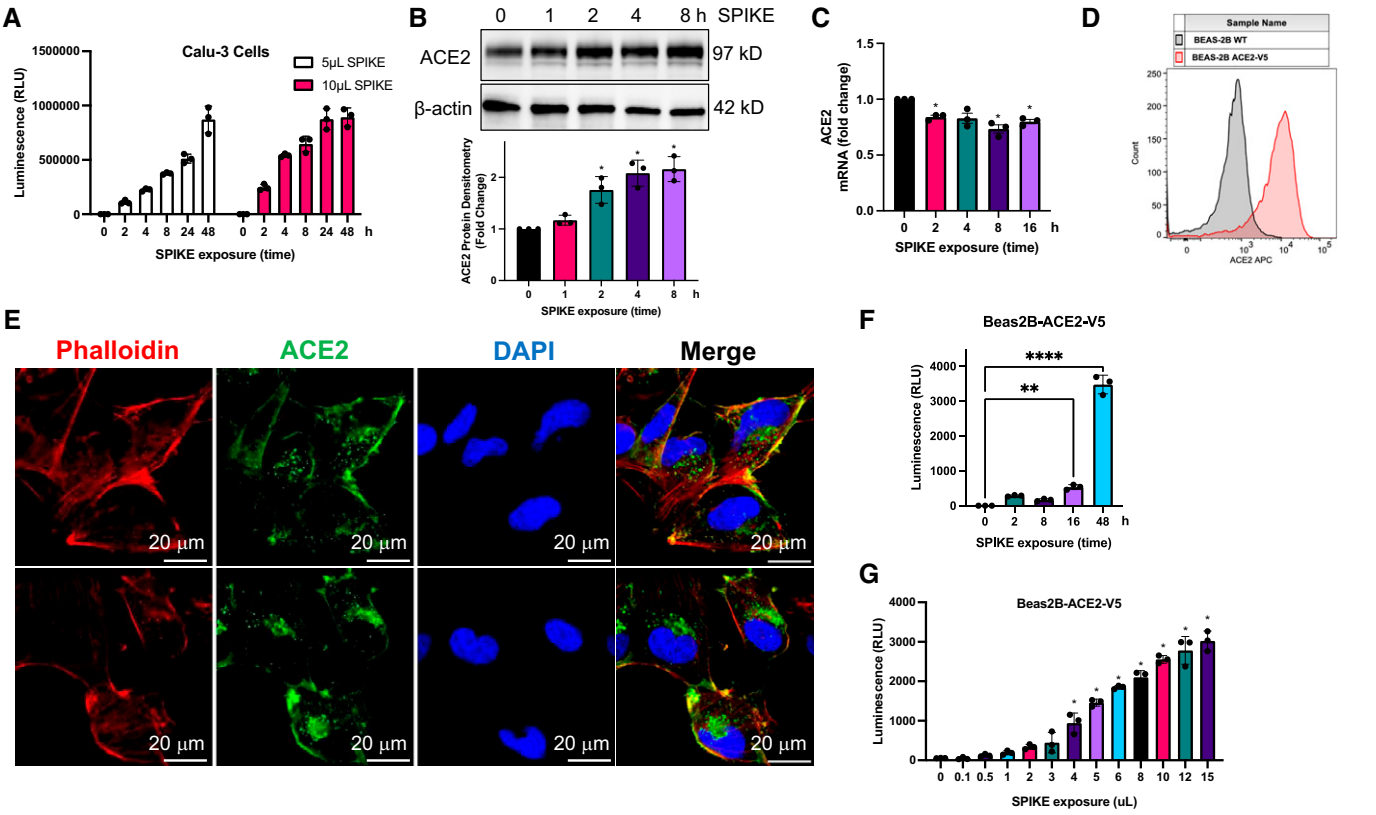

Figure 1. ACE2 (angiotensin-converting enzyme 2) protein is increased in abundance with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) ligation. (*A*) Calu-3 cells were exposed to viral particles pseudotyped with SARS-CoV-2 SPIKE with nanoluciferase for up to 48 hours, as indicated before protein harvest in nanoluciferase assay buffer. Calu-3 cells demonstrate time- and dose-dependent increases in luminescence as determined by a plate reader. Data are shown as the mean ± SEM of three separate experiments. (*B* and *C*) Calu-3 cells were exposed to SPIKE particles for the indicated times. (*B*) Cell lysates were assayed by immunoblotting, and ACE2 protein abundance increased in a time-dependent manner. Densitometric analysis of ACE2 signal over time, normalized to β-actin ($n = 3$). (*C*) Total RNA was harvested, and ACE2 gene expression decreased in a time-dependent manner by quantitative PCR (qPCR). Data are shown as mean fold change ± SEM as determined by ΔΔCq analysis ($n = 3$). (*D* and *E*) Wild-type (WT) Beas2B and stable transfected Beas2B-*ACE2-V5* cells were assayed for *ACE2* expression by (*D*) flow cytometry and (*E*) immunohistochemistry. Data are shown as representative images. Scale bars, 20 μm. (*F*) Beas2B-*ACE2-V5* cells were exposed to 10 μl SPIKE particles for the indicated times, and luminescence was determined by a plate reader. (*G*) Beas2B-*ACE2-V5* cells were exposed to SPIKE particles at the indicated doses for 48 hours, and luminescence was determined by a plate reader. (*B*, *C*, *F*, and *G*) Graphs represent the mean ± SEM of three separate experiments. *P* values determined by ANOVA with *post hoc* multiple comparisons test. $^{*}P < 0.05$ compared with the untreated control. $^{**}P < 0.005$ and $^{****}P < 0.0001$.

Results

Spike Protein Ligation Increases ACE2 Protein Abundance in Epithelial Cells

Prior *in vitro* studies have demonstrated that ACE2 protein abundance is increased by spike protein ligation (17, 20). To facilitate the mechanistic study of ACE2 handling with spike protein ligation, we developed viral particles pseudotyped with SARS-CoV-2 spike protein envelope that express nanoluciferase as a readout (referred to as SPIKE particles) to monitor viral protein cell entry. As an *in vitro* model, we first used Calu-3 cells, a human epithelial cell line derived from non-small cell lung cancer that expresses endogenous ACE2. In Calu-3 cells, we demonstrate increased luminescence over time with SPIKE exposure (Figure 1A), suggesting that Calu-3 cells can internalize SPIKE particles. Next, we exposed Calu-3 cells to SPIKE particles and observed a time-dependent increased ACE2 protein abundance (Figure 1B), which is consistent with prior published reports (17, 20). To investigate mechanisms by which ACE2 protein abundance is increased, we measured ACE2 steady-state mRNA expression by quantitative PCR and observed a modest but significant decrease in ACE2 mRNA with SPIKE exposure (Figure 1C), suggesting that increased ACE2 protein is not because of increased gene transcription.

Generation of Stable Beas2B-*ACE2-V5*–expressing Cells

Although Calu-3 cells do express endogenous ACE2, we found that transfection with both mammalian expression vectors and RNA interference led to nonspecific downregulation of ACE2 (data not shown). To further model the molecular behavior of ACE2 regulation, we also used Beas2B cells, a noncancerous human bronchial epithelial cell line used extensively to mimic lung epithelium. Beas2B cells are easily transfectable but express low concentrations of endogenous ACE2. Thus, to facilitate the turnover of ACE2 protein in Beas2B, we developed a stable *ACE2-V5* transgene Beas2B cell line

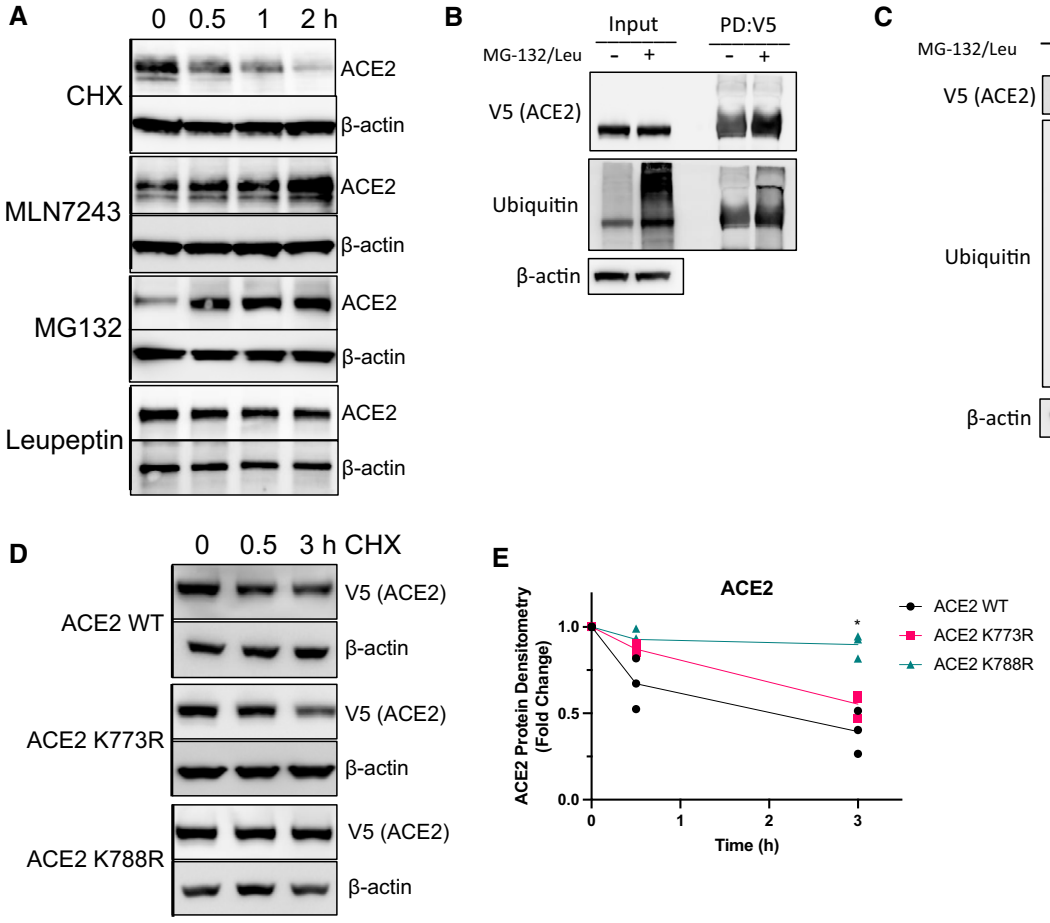

Figure 2. Ubiquitination modulates ACE2 protein stability. (*A*) Calu-3 cells were exposed to CHX (cycloheximide), MLN7243, MG-132, or leupeptin as indicated. Cell lysates were assayed by immunoblotting for ACE2 protein and β-actin as a loading control. Data are shown as representative images. (*B*) Beas2B-*ACE-V5* cells were treated with MG-132 and leupeptin for 4 hours, as indicated, before pulldown of *ACE2-V5* with V5 antibody. Cell lysates were assayed by immunoblotting for V5 (ACE2) and ubiquitin. MG-132 and leupeptin treatment resulted in increased immunoreactive Ub-*ACE2*-V5. (*C*) Beas2B-ACE-V5 cells were treated with MG-132 and leupeptin for 4 hours, as indicated, before the pulldown of ubiquitinated proteins with TUBEs and immunoblotting. (*D*) WT Beas2B cells were transiently transfected with ACE2 K→R point mutations, followed by CHX chase. ACE2 K788R mutant demonstrates increased stability compared with WT ACE2. (*E*) Densitometric analysis of V5 signal over time, normalized to β-actin. *P* values determined by ANOVA with *post hoc* multiple comparisons test. $^*P < 0.05$ compared with the untreated control subjects. TUBEs = tandem ubiquitin-binding entities.

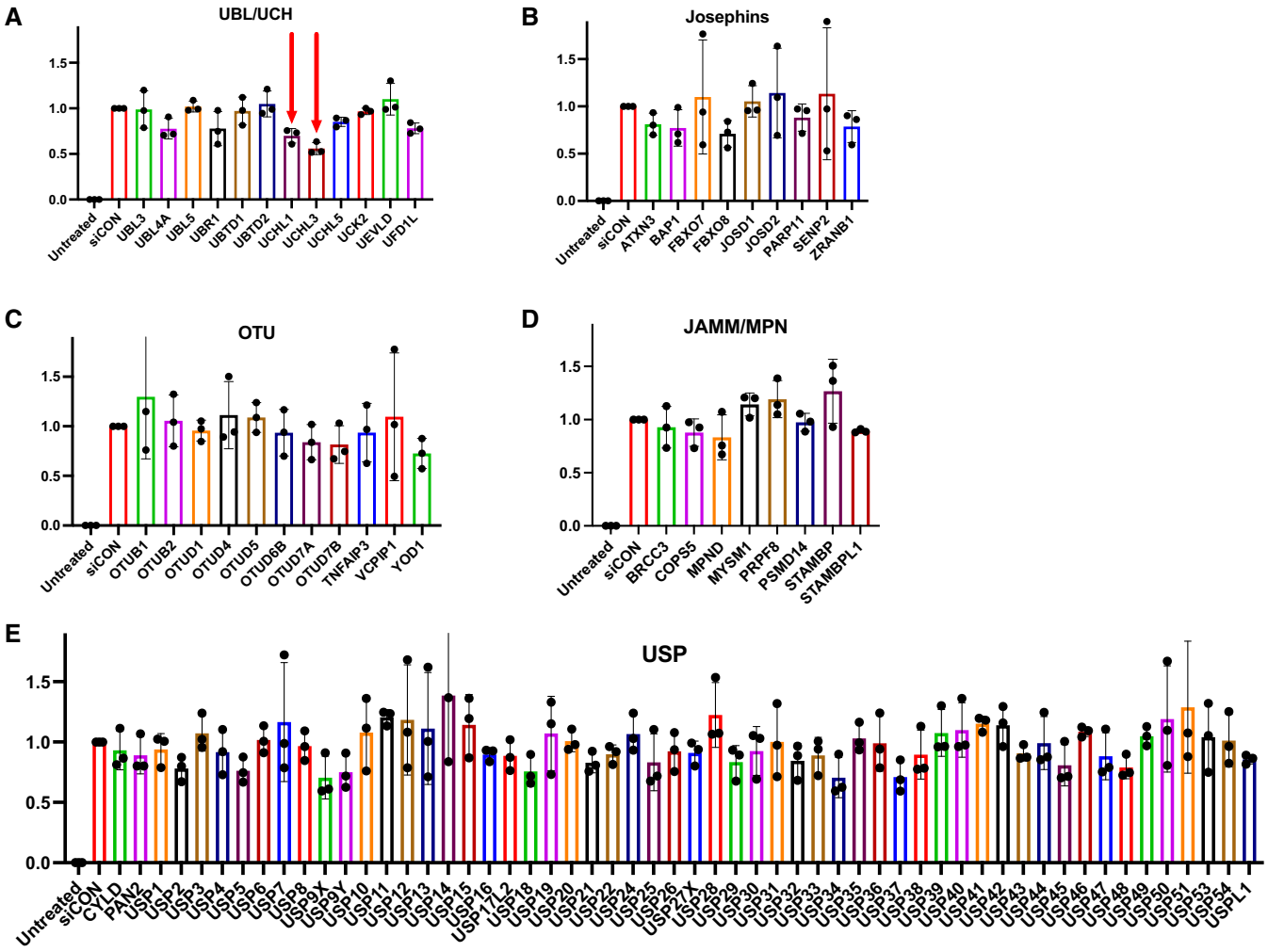

Figure 3. UCHL1 and UCHL3 siRNA decrease SPIKE particle signal in the deubiquitinase (DUB) library screen. (*A–E*) Beas2B-*ACE2-V5* cells were exposed to nontargeting control siRNA (siCON) or pools of siRNA targeting each of 96 human DUBs across the five families of DUB enzymes, including (*A*) UBL/UCH, (*B*) Josephins, (*C*) OTU, (*D*) JAMM/MPN^{+}, and (*E*) USPs. Seventy-two hours after transfection, cells were exposed to SPIKE particles for an additional 48 hours before harvest with nanoluciferase reagent and assay for luminescence via plate reader. Data are shown as signals relative to the siCON; relative luminescence units were normalized to the untreated control subject and the siCON conditions for each plate to account for plate-to-plate variability and baseline luminescence variability between replicates. Graphs represent the mean ± SEM of three separate experiments performed in technical duplicates. JAMM/MPN^{+} = JAB1/MPN/MOV34 metalloenzymes; OTU = ovarian tumor proteases; UBL/UCH = ubiquitin C-terminal hydrolases; UCHL1 = ubiquitin carboxyl-terminal hydrolase isozyme L1; USPs = ubiquitin-specific proteases.

using the Sleeping Beauty transposon system. We first confirmed *ACE2-V5* expression and localization within the plasma membrane by flow cytometry (Figure 1D) and immunofluorescence microscopy (Figure 1E). We also confirmed that Beas2B-*ACE2-V5* cells internalize SPIKE particles and display luminescence when exposed to luciferase reagent in a time- and dose-dependent manner (Figures 1F and 1G). Wild-type Beas2B cells display no luminescence when exposed to SPIKE in this assay (data not shown).

Ubiquitination Modulates ACE2 Stability

To investigate mechanisms modulating ACE2 protein abundance, we next investigated ACE2 protein degradation with a series of chase experiments. In Calu-3 cells, inhibition of cellular protein synthesis with the chemical inhibitor CHX (cycloheximide) led to rapid depletion of ACE2 protein with a half-life $t_{1/2}$ of 1–2 hours (Figure 2A). Exposure to MLN7243, an inhibitor of ubiquitin E1-activating enzymes, resulted in increased ACE2 protein, suggesting that ACE2 protein abundance is modulated by ubiquitination. Substrate protein ubiquitination and subsequent degradation occur via two major pathways: *1*) proteasomal degradation; or *2*) endosomal trafficking and degradation at the lysosome. ACE2 protein abundance increased with exposure to the proteasome inhibitor MG132 but not with lysosomal inhibition using leupeptin (Figure 2A), suggesting that ACE2 is degraded at the proteasome. To confirm ACE2 ubiquitination, we performed coimmunoprecipitation assays. First, we

pulled down *ACE2-V5* protein from Beas2B-*ACE2-V5* cells and demonstrated a slower migrating smear above the *ACE2* band, consistent with polyubiquitinated *ACE2* protein (Figure 2B). Next, we used tandem ubiquitin-binding entities conjugated with a biotin tag to pull down all ubiquitinated proteins from Beas2B-*ACE2-V5* cells and again observed the pulldown of *ACE2* protein (Figure 2C). *ACE2* protein ubiquitination was enhanced with pretreatment with MG-132 and leupeptin (Figures 2B and 2C). Protein ubiquitination occurs by the covalent attachment of a ubiquitin chain to a lysine acceptor site on the substrate protein. ACE2 contains only seven lysine residues on the C-terminal intracellular domain. Prior studies have demonstrated K788 as the ubiquitin acceptor site of ACE2. We generated point mutations, substituting arginine for lysine at candidate acceptor site K788 and also K773 as a control subject. With CHX treatment, the protein stability of *ACE2 K788R* mutant was significantly increased compared with the wild-type (Figure 2D and 2E), recapitulating a prior study (7).

SARS-CoV-2 SPIKE Particle Uptake Is Regulated by DUBs

ACE2 protein abundance is increased with spike protein ligation, which may function as a positive feedback loop in SARS-CoV-2 infection. Consistent with recent reports, our data demonstrate that ACE2 is regulated by ubiquitination (16, 17). Limited data is available regarding ACE2 control by DUBs. Hence, we hypothesized that deubiquitination of ACE2 decreases its degradation, thereby increasing its protein stability. To test this hypothesis, we sought to identify a DUB regulator of ACE2 using a siRNA library targeting all known human DUBs. We screened for SPIKE particle uptake to allow for higher throughput with the rationale that SPIKE particle uptake could serve as a surrogate for ACE2 abundance, acknowledging that this approach does not directly assay ACE2 protein. To perform the screen, Beas2B-*ACE2-V5* cells were transfected with pools of siRNA targeting each of 96 individual DUB enzymes or nontargeting control siRNA. Seventy-two hours after

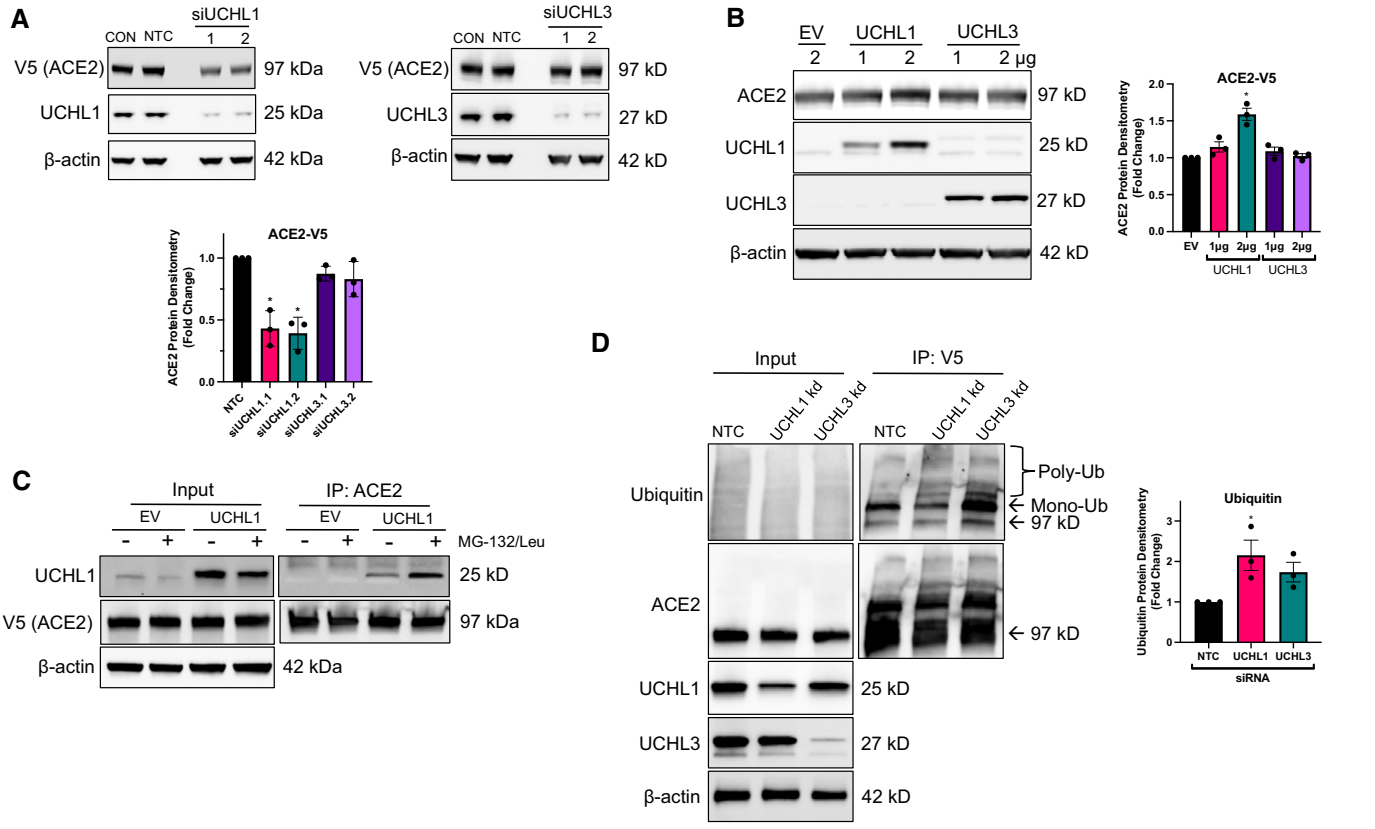

Figure 4. UCHL1 modulates ACE2 protein abundance. (*A*) Beas2B-*ACE2-V5* cells were exposed to siRNA targeting UCHL1, UCHL3, or control subject (nontargeting control [NTC]) for 72 hours before harvest and immunoblot and demonstrated decreased *ACE2-V5* signal with siRNA targeting UCHL1 compared with control subjects. Bottom, densitometric analysis of V5 signal in each condition, normalized to β-actin. (*B*) Beas2B-*ACE2-V5* cells were transiently transfected with mammalian expression vectors expressing UCHL1, UCHL3, or empty vector (EV) for 48 hours before harvest and assay with immunoblotting. *ACE2-V5* signal was increased with UCHL1 overexpression compared with EV. Right, densitometric analysis of V5 signal in each condition normalized to β-actin. (*C*) Beas2B-*ACE-V5* cells were treated with MG-132 and leupeptin for 4 hours, as indicated, before IP of *ACE2*-V5 with ACE2 antibody and immunoblotting. *UCHL1*-V5 coimmunoprecipitated with *ACE2*-V5. Concentrations of co-IP immunoreactive *UCHL1*-V5 were increased with MG-132 and leupeptin pretreatment. (*D*) Beas2B-*ACE2-V5* cells were exposed to siRNA targeting UCHL1, UCHL3, or control (NTC) for 72 hours before harvest, immunoprecipitation of *ACE2*-V5 with V5 antibody, and immunoblotting. A slower migrating ubiquitin smear signal was increased with UCHL1 siRNA compared with control subjects. Right, densitometric analysis of ubiquitin signal in each condition normalized to β-actin. (*A*, *B*, and *D*) *P* values determined by ANOVA with *post hoc* multiple comparisons test. $^*P < 0.05$ compared with the untreated control subjects. CON = untransfected control subject.

transfection, cells were exposed to SPIKE particles for 48 hours and assayed for luminescence, indicative of viral uptake and replication. After normalization, we calculated mean effects and 95% confidence intervals of the mean for each DUB. Six DUBs had a 95% confidence interval upper limit of less than one (Table E1 in the data supplement). Of those six DUBs, we observed the greatest decrease in luciferase signal in those cells transfected with siRNA targeting UCHL1 and UCHL3 (Figure 3A, arrows) compared with the control siRNA. DUBs were screened across all five DUB families (Figures 3A–3E). Luciferase was not significantly increased in any condition. These findings demonstrate that the knockdown of UCHL1 or UCHL3 reduces SPIKE particle uptake and/or replication. The results suggest that inhibition of these DUBs might destabilize ACE2 through selective increases in the receptor's polyubiquitination.

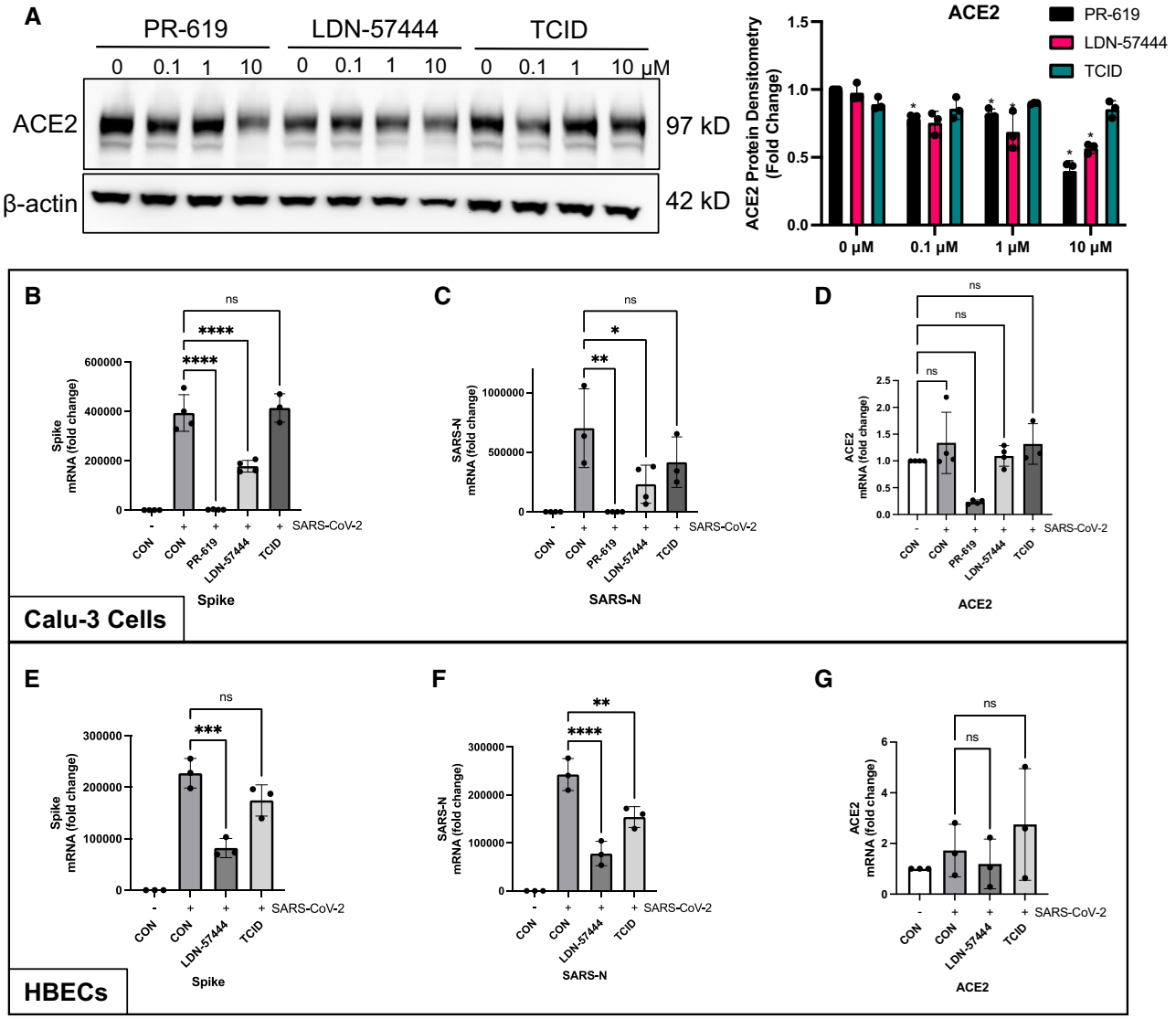

Figure 5. DUB inhibitors decrease SARS-CoV-2 viral mRNA. (*A*) Calu-3 cells were exposed to various concentrations of DUB inhibitors, PR-619 (broad-spectrum DUB inhibitor), LDN-57444 (UCHL1 inhibitor), or TCID (UCHL3 inhibitor), for 16 hours before harvest and assay by immunoblotting. Immunoreactive ACE2 decreased with PR-619 and LDN-57444 exposure compared with untreated control subjects. Right, densitometric analysis of ubiquitin signal in each condition normalized to β-actin. (*B–D*) Calu-3 cells were exposed to DUB inhibitors, PR-619 (broad-spectrum DUB inhibitor), LDN-57444 (UCHL1 inhibitor), or TCID (UCHL3 inhibitor) for 16 hours before exposure to SARS-CoV-2 WA1 (original 2020 strain) live virus for 24 hours. Total RNA was harvested before the assay of the viral spike, viral SARS-N (SARS-CoV-2 nucleocapsid protein) expression, and human ACE2 expression by qPCR. Data are shown as mean fold change ± SEM as determined by ΔΔCq analysis ($n = 4$). (*E–G*) HBECs were exposed to DUB inhibitors, LDN-57444, or TCID for 16 hours before exposure to the SARS-CoV-2 WA1 live virus for 24 hours. Total RNA was harvested before the assay of the viral spike, viral SARS-N expression, and human ACE2 expression by qPCR. Data are shown as mean fold change ± SEM as determined by ΔΔCq analysis ($n = 3$). (*A–G*) *P* values determined by ANOVA with *post hoc* multiple comparisons test. $^{*}P < 0.05$ compared with the untreated control subject. HBECs = human bronchial epithelial cells; ns = not significant. $^{**}P < 0.005$, $^{***}P < 0.0005$, and $^{****}P < 0.0001$.

UCHL1 DUB Activity Modulates ACE2 Protein Abundance

As knockdown of UCHL1 or UCHL3 led to decreased SPIKE particle uptake, we hypothesized that these DUBs may regulate viral kinetics through modulation of ACE2 protein abundance. To test this hypothesis, we performed DUB genetic knockdown and overexpression studies in Beas2B-*ACE2-V5* cells. Using siRNA targeting UCHL1, UCHL3, or nontargeting control (NTC), we observed a decrease in ACE2 abundance with UCHL1 knockdown but not UCHL3 (Figure 4A). We next overexpressed UCHL family DUBs using a mammalian expression vector pLX304 that transiently expresses *UCHL1-V5*, *UCHL3-V5*. or *UCHL5-V5*. We observed modest increases in ACE2 abundance selectively with UCHL1 overexpression (Figure 4B). To determine if UCHL1 and ACE2 interact in the cell, we next performed coimmunoprecipitation studies. We transfected mammalian expression vectors expressing *UCHL1-V5* or an empty vector into Beas2B-*ACE2-V5* cells. To enrich for ubiquitinated substrate proteins, cells were treated with or without proteasome and lysosome inhibitors. We used ACE2 antibody to immunoprecipitate endogenous and overexpressed *ACE2-V5* and demonstrated molecular interaction of *UCHL1-V5* with coimmunoprecipitated *UCHL1-V5* with proteasome and lysosome inhibitors (Figure 4C). This suggests that ACE2 and UCHL1 interact in the cell, and this interaction is enhanced with the preservation of ubiquitinated ACE2. We next hypothesized that UCHL1 modulates ACE2 protein abundance through UCHL1 DUB activity. To test this hypothesis, we performed knockdown in Beas2B-*ACE2*-V5 cells using siRNA targeting UCHL1, UCHL3, or NTC (control subject), before V5 immunoprecipitation and immunoblotting for ACE2 ubiquitination. We observe an increase in the slower migrating polyubiquitin smear with UCHL1 knockdown compared with the NTC or UCHL3 conditions (Figure 4D). Of note, we observed an increase in a slightly slower migrating band in the UCHL3 knockdown condition (Figure 4D, labeled mono-Ub), suggesting increased mono-ubiquitination of ACE2 with UCHL3 knockdown. As UCHL3 did not modulate ACE2 protein abundance (Figures 4A and 4B), this may represent nondegradative ubiquitination. These findings suggest that ACE2 is a substrate for UCHL1 DUB activity.

Small Molecule UCHL1 Inhibition Limits Live SARS-CoV-2 Infection in Epithelia

Our findings demonstrate that UCHL1 DUB activity modulates ACE2 protein stability in Beas2B-*ACE2*-V5 cells. We aimed to recapitulate these findings in Calu-3 cells, which express endogenous ACE2. However, because of limitations of manipulating Calu-3 cells with expression vectors or siRNA, we used chemical inhibitors of DUBs, specifically PR-619 (an inhibitor of all cysteine isopeptidase DUBs), LDN-57444 (UCHL1 inhibitor), and TCID (UCHL3 inhibitor). Exposure of cells to these compounds led to an overall decrease in ACE2 abundance at 24 hours with PR-619 and LDN-57444 but not TCID (Figure 5A). LDN-57444 inhibits UCHL1 DUB activity, suggesting that ACE2 stability is modulated by UCHL1. We next hypothesized that UCHL1 inhibition may provide an

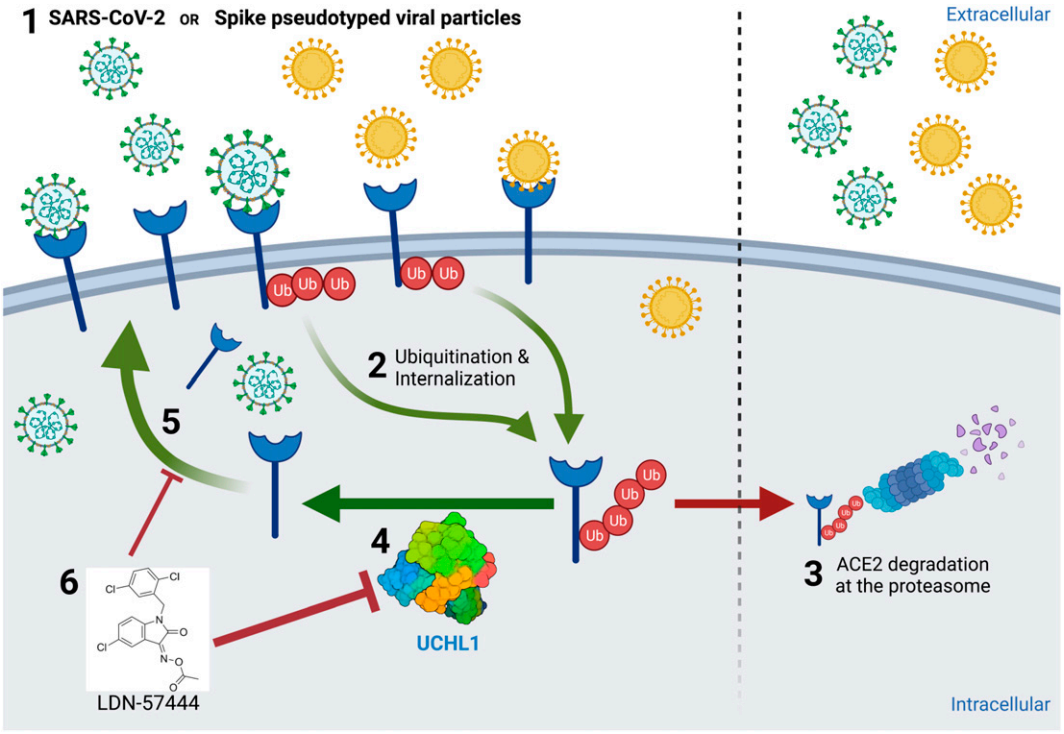

Figure 6. ACE2 protein is stabilized by UCHL1 DUB activity that is antagonized by LDN-57444. (1) SARS-CoV-2 or spike pseudotyped viral particles require membrane-bound ACE2 for entry into epithelial cells. (2) ACE2 is a ubiquitinated protein that (3) is degraded at the proteasome. (4) Deubiquitination by UCHL1 rescues ACE2 protein from degradation, which (5) preserves ACE2 for recycling to the cell membrane. (6) LDN-57444 antagonizes UCHL1 DUB activity, inhibiting ACE2 rescue and SARS-CoV-2 viral entry.

opportunity to therapeutically target ACE2 and limit ACE2 function as a SARS-CoV-2 viral receptor. To test this hypothesis, we pretreated Calu-3 cells with DUB inhibitors before exposing the cells to live SARS-CoV-2 virus for 24 hours. By quantitative PCR, we measured the abundance of spike and SARS-N (SARS-CoV-2 nucleocapsid protein) viral mRNA to determine viral infection and hACE2 as a control subject.

With PR-619 (broad DUB inhibitor and positive control) and LDN-57444 (UCHL1 inhibitor) treatment, we observed a significant decrease in SARS-CoV-2 spike and SARS-N RNA at 24 hours (Figures 5B and 5C). TCID (UCHL3 inhibitor) pretreatment did not significantly impact viral RNA (Figures 5B and 5C). Of note, broad DUB inhibition with PR-619 significantly decreased ACE2 mRNA, whereas LDN-57444 and TCID did not (Figure 5D). The effect of PR-619 on ACE2 mRNA is likely through an indirect mechanism but suggests that other DUBs may play a role in ACE2 modulation. To further investigate UCHL1 inhibition as an antiviral approach in SARS-CoV-2 infection, we pretreated primary human bronchial epithelial cells from two different donor lots with DUB inhibitors before exposure to live SARS-CoV-2 virus. Recapitulating our findings in Calu-3 cells, we observed a significant decrease in SARS-CoV-2 spike and SARS-N RNA at 24 hours in human bronchial epithelial cells pretreated with the UCHL1 inhibitor, LDN-57444, but not the UCHL3 inhibitor, TCID (Figures 5E and 5F). Neither UCHL1 nor UCHL3 inhibition significantly altered ACE2 mRNA (Figure 5G). This data suggests that specific UCHL1 inhibition with LDN-57444 represents one approach for decreasing SARS-CoV-2 infection through therapeutic targeting of ACE2 (Figure 6).

Discussion

These studies demonstrate the unique ability of the DUB UCHL1 to regulate SARS-CoV-2 viral uptake and infection by modulation of ACE2 ubiquitination. Thus, UCHL1 inhibition may represent a potential strategy for ACE2 therapeutic targeting in COVID-19. The new findings of this study are that *1*) ACE2 protein is stabilized by UCHL1 and represents a new substrate for the DUB; *2*) UCHL1 inhibition decreases SARS-CoV-2 viral uptake and infection in epithelial cells; and *3*) pharmacologic targeting of UCHL1 DUB activity with a commercially available small molecule decreases ACE2 protein abundance and inhibits SARS-CoV-2 viral infection *in vitro*. These findings demonstrate the feasibility of indirect targeting of ACE2 as a strategy to combat SARS-CoV-2 infection. This work uncovering UCHL1 regulation of ACE2 may provide a mechanistic platform for additional virtual or high-throughput screening to interrogate newer chemical entities that could enter the drug discovery pipeline to attenuate SARS-CoV-2 viral cell entry.

Despite the incredible effort and a rapidly evolving understanding of SARS-CoV-2 infection and COVID-19, highly effective therapeutic options remain limited. ACE2 is an attractive target because of its central role in SARS-CoV-2 infection. Coronaviruses infect host cells through two mechanisms: entry of free virus into host cells and transmission of virus through cell-to-cell contact (21, 22). ACE2 is necessary for cell-free SARS-CoV-2 entry into cells (3, 4). Although ACE2 is not required for cell-to-cell spread, ACE2 does enhance SARS-CoV-2 cell-to-cell transmission (23). Consistent with prior reports (20), our data demonstrate increased ACE2 with spike protein ligation, which may help to propagate infection within the host. Endogenous expression and physiologic cellular handling of ACE2 are variable among human cell types and experimental cell lines (24). Prior reports demonstrate modest transcriptional upregulation of ACE2 protein with spike protein transfection (20) or exposure to interferons (24). We demonstrate posttranslational regulation of ACE2 in response to spike ligation. Physiologically, ACE2 is a component of the renin–angiotensin system but also plays a role in the regulation of inflammatory responses (25). In the kidney, ACE2 deletion or inhibition promotes hypertension and renal inflammation (26). In a mouse model of *Pseudomonas aeruginosa* pneumonia, ACE2 reduction was crucial for neutrophil recruitment to the lung (27). Early targeting of ACE2 may represent a viable treatment strategy to reduce viral infection. However, in later-stage SARS-CoV-2 infection, this strategy may be injurious for patients with ongoing, unresolved inflammation. Thus, the emergence of other molecular targets that may impact ACE2 activity indirectly, allowing more calibrated expression of this key homeostatic enzyme, may be important in designing therapeutic strategies.

To date, the primary treatment strategies for COVID-19 are reducing viral replication (28, 29) and limiting injurious inflammation (30). Prior studies have investigated the structural relationship between ACE2 and SARS-CoV-2 spike protein (31) and identified multiple repurposed pharmaceuticals (32–35) and natural compounds (36–38) as potential therapeutics. However, compounds targeting ACE2 have not progressed to clinical use. Here we investigated ACE2 ubiquitination as a strategy to identify potential upstream regulators of ACE2 protein abundance. Most cellular proteins undergo posttranslational modification with ubiquitin; ACE2 is no exception (7, 15). More recently, studies have demonstrated ACE2 ubiquitination in SARS-CoV-2 infection and identified E3 ligases Skp2 (16) and UBR4 (17) as putative regulators. However, strategies for therapeutic targeting of these ubiquitin E3 ligases or DUBs were not investigated. For ACE2, DUB targeting is an attractive therapeutic strategy. With the goal of destabilizing ACE2 to reduce SARS-CoV-2 viral entry, DUB inhibition is one strategy to increase ACE2 ubiquitination and subsequent degradation. Prior work from our group has demonstrated the rationale for targeting DUBs (14). To date, there are no DUB inhibitors that have reached the point of testing in clinical trials. Here our data demonstrate an indirect approach of ACE2 targeting through inhibition of the DUB UCHL1. UCHL1 DUB activity is necessary to stabilize ACE2 protein in cellular models. UCHL1 has previously been demonstrated to regulate protein trafficking to the cell membrane and degradation of proteins by promoting trafficking to the proteasome (39). Loss or inhibition of UCHL1 DUB activity is associated with neurofibrillary tangles in patients with Alzheimer's disease and Parkinson's disease (40), neural recovery after cerebral injury (41, 42), and multiple cancers (43, 44). Although a UCHL1 inhibitor is commercially available, it has not been developed through investigational new drug-enabling studies to advance to the clinic. Indirect targeting of ACE2 through UCHL1 inhibition may provide the tools to test the feasibility of ACE2 inhibition as a therapeutic strategy. Future studies will need to assess additional target validation of UCHL1, including DUB genetics and off-targets of lead compounds that might emerge

from high-throughput screening or other approaches that could then enter the drug development pipeline.

Conclusions

As the SARS-CoV-2 virus continues to evolve and mutate, newer strains demonstrate greater evasiveness against the protections offered by vaccination and prior infection (45). As all β-coronaviruses require ACE2 for viral entry and infection, targeting ACE2 or related regulators such as DUBs may serve as an attractive and generalizable approach for current and future viral strains. That is, the successful development of an inhibitor that decreases ACE2 function as a viral receptor could provide a broad-spectrum tool against all coronaviridae that use ACE2 for host infection. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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