p53 down-regulates SARS coronavirus replication and is targeted by the SARS-unique domain and PL\textsuperscript{pro} via E3 ubiquitin ligase RCHY1

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Highly pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV) has developed strategies to inhibit host immune recognition. We identify cellular E3 ubiquitin ligase ring-finger and CHY zinc-finger domain-containing 1 (RCHY1) as an interacting partner of the viral SARS-unique domain (SUD) and papain-like protease (PL\textsuperscript{pro}), and, as a consequence, the involvement of cellular p53 as antigen of coronaviral replication. Residues 95–144 of RCHY1 and 389–652 of SUD (SUD-NM) subdomains are crucial for interaction. Association with SUD increases the stability of RCHY1 and augments RCHY1-mediated ubiquitination as well as degradation of p53. The SUD/calmodulin-dependent protein kinase II delta (CAMK2D), which normally influences RCHY1 stability by phosphorylation, also binds to SUD. In vivo phosphorylation shows that SUD does not regulate phosphorylation of RCHY1 via CAMK2D. Similarly to SUD, the PL\textsuperscript{pro}s from SARS-CoV, MERS-CoV, and HCoV-NL63 physically interact with and stabilize RCHY1, and thus trigger degradation of endogenous p53. The SARS-CoV papain-like protease is encoded next to SUD within nonstructural protein 3. A SUD–PL\textsuperscript{pro} fusion interacts with RCHY1 more intensively and causes stronger p53 degradation than SARS-CoV PL\textsuperscript{pro} alone. We show that p53 inhibits replication of infectious SARS-CoV as well as of replicons and human coronavirus NL63. Hence, human coronaviruses antagonize the viral inhibitor p53 via stabilizing RCHY1 and promoting RCHY1-mediated p53 degradation. SUD functions as an enhancer to strengthen interaction between RCHY1 and nonstructural protein 3, leading to a further increase in in p53 degradation. The significance of these findings is that down-regulation of p53 as a major player in antiviral innate immunity provides a long-sought explanation for delayed activities of respective genes.

Significance

Severe acute respiratory syndrome coronavirus (SARS-CoV) is one of the most pathogenic human coronaviruses. Virulence is reflected in the molecular interplay between virus and host cells. Here we show a strategy of how SARS-CoV antagonizes the host antiviral factor p53, which impairs viral replication. The papain-like protease of the nonstructural protein 3 of SARS-CoV and other coronaviruses physically interact with and stabilize E3 ubiquitin ligase ring-finger and CHY zinc-finger domain-containing 1 (RCHY1), thereby augmenting RCHY1-mediated degradation of p53. The SARS-unique domain (SUD) enhances these effects. Knockout of p53 promotes replication of SARS-CoV replicons and of infectious virus. Taken together we identify cellular p53 as antiviral measure of coronavirus-infected cells, which is counteracted via the stabilization of RCHY1 by viral SUD and papain-like protease (PL\textsuperscript{pro}) proteins and via ubiquitination of p53.

p53 antiviral activity | SARS-CoV SUD | papain-like protease | E3 ubiquitin ligase RCHY1 | coronavirus replication

The global outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 caused 774 deaths among 8,098 infected individuals, although human coronaviruses (HCoVs) had always been regarded relatively harmless until then (1, 2). To date, six coronaviruses (CoVs) are known to infect humans; these are HCoV-229E and HCoV-NL63 belonging to the genus Alphacoronavirus as well as SARS-CoV, Middle East respiratory syndrome CoV (MERS-CoV), HCoV-HKU1, and HCoV-OC43 of the Betacoronavirus genus. MERS-CoV is another highly pathogenic coronavirus connected with an even higher case/fatality rate. Despite a decade of research efforts, there are neither approved antiviral treatments either specific for SARS-CoV or with a broad-spectrum profile for all human coronaviruses, nor any vaccine available (2–4). Therefore, it is necessary to further study coronavirus-host relations to discover new targets and signaling pathways for antiviral intervention. Applying high-throughput yeast-2-hybrid (Y2H) methodologies to screen for important virus-host protein–protein interactions (PPIs), we identified ring-finger and CHY zinc-finger domain-containing 1 (RCHY1) and calcium/calmodulin-dependent protein kinase II delta (CAMK2D) as two interacting partners of the SARS-unique domain (SUD), which is part of SARS-CoV nonstructural protein 3 (Nsp3). Containing various subdomains [ubiquitin-like (Ubl) globular fold, acidic domain, catalytically active ADP ribose-1′′-phosphatase (X-domain), SUD, catalytically active papain-like protease (PL\textsuperscript{pro}) domain, nucleic acid-binding domain, G2M marker domain, two predicted double-pass transmembrane domains, a putative metal binding region, Y domain of unknown function], Nsp3 protein represents the largest Nsp of SARS-CoV (5) and plays an essential role for the formation of viral replication complexes. Two macrodomains of SUD (SUD-N and, in particular, SUD-M) have been shown to bind oligo(G) nucleotides (both deoxynucleotides and ribonucleotides) that are able to form G-quadruplexes (6). Interestingly,}

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amino acid residues of SUD-M that have been shown to be involved in G-quadruplex binding (6) are also essential for the function of the domain in the SARS-CoV replication and transcription (7). PLP2 (corresponding to Nsp3 residues 720–1039) is the C-terminal neighbor to the SUD, PLP2 and 3C-like protease (3CLpro) process the viral replicase polyproteins into 16 replicase proteins. Many CoVs encode two Papain-like proteases (PLPs) [PLP1 (cleaving Nsp1/Nsp2 and Nsp2/Nsp3), PLP2 (cleaving Nsp3/Nsp4)] within Nsp3. SARS-CoV PLP2 and most of other CoV PLP2 display deubiquitinating and deISGylating activities, thus acting as IFN antagonists and contributing to evasion of innate immune response.

RCHY1 is an E3 ubiquitin ligase mediating proteasomal degradation of its target proteins; its targets include the tumor proteins p53, p63, and p73 (8–10). RCHY1 regulates cell-cycle progression and is inducible by p53 (8, 11); it also forms a homodimer and has self-ubiquitination activity (12). RCHY1 is a short-lived protein. Inhibition of RCHY1 ubiquitination via interaction with measles virus phosphoprotein can enhance the stability of RCHY1 (13). In addition, phosphorylation by cyclin-dependent kinase 9 (CDK9) or CAMKII can also regulate the stability of RCHY1. Phosphorylation by CAMKII at Thr154/Ser155 increases instability of RCHY1 and impairs the E3 ubiquitin ligase activity of RCHY1 toward p53 (14).

CAMK2D belongs to the calcium/calmodulin-dependent serine/threonine protein kinase II (CAMKII) family involved in many signaling pathways. CAMKII is a homoenzyme composed of CAMK2A/2B/2C/2D isoforms (15). These have very similar structures, including an N-terminal kinase domain, a central regulatory domain with a calmodulin-binding region, and a C-terminal association domain (16–19). Important substrates of CAMKII include RCHY1 and signal transducer and activator of transcription 1 (STAT1). CAMKII impairs the E3 ligase activity of RCHY1 via phosphorylation (14).

p53 regulates a plethora of target genes that mediate immunosuppression by inducing multiple processes such as cell-cycle arrest, DNA repair, apoptosis, and senescence (18, 19). The proteasomal degradation of p53 is regulated by several E3 ubiquitin ligases such as RCHY1 and MDM2 (8, 20, 21). RCHY1 binds to the central region of p53, ubiquitates p53, and promotes p53 degradation independently of MDM2 (8). Like RCHY1, MDM2 is also a zinc finger and RING-finger domain-containing E3 ubiquitin ligase (22). High levels of MDM2 induce polyubiquitination and degradation of p53, whereas low levels of MDM2 lead to monoubiquitination and nuclear export of p53 (23). CDK9 phosphorylates RCHY1 at Ser211/Thr217 and MDM2 at Ser395, causing degradation of RCHY1 and attenuation of MDM2 toward p53, respectively (24, 25). p53 can also be directly phosphorylated by CDK9 at Ser292, leading to p53 accumulation (26). In addition to its tumor suppressive activity, p53 also participates in the defense of viral infections (27). Notably, p53 was identified by its interaction with SV40 large T antigen (28). Overexpression of p53 reduces HIV-1 replication via preventing phosphorylation of RNA polymerase II and consequently establishing a delayed RNA polymerase II complex at the LTR of HIV-1 (29).

A recent study reported that ectopic expression of HCoV-NL63 PLP2 promotes p53 degradation by interaction with the cellular ubiquitin ligase MDM2 and that p53 exhibits antiviral activity on the replication of Sendai virus (30). Furthermore, p53 functions as an antiviral factor against influenza A virus (31). To antagonize the negative effect of p53, some viruses have evolved strategies to induce p53 degradation. Epstein–Barr virus (EBV) applies a double strategy to counteract p53. First, EBV nuclear antigen 3C deubiquitinates and stabilizes MDM2, thereby preventing phosphorylation of RNA polymerase II and consequently establishing a delayed RNA polymerase II complex (32).

We have recently established a SARS-CoV–host interactome by screening interactions of individual viral proteins against human cDNA libraries, leading to the identification of important pathways and targets for the prevention of CoV replication (34). In the present study, we investigate the SUD–CAMK2D as well as SUD–RCHY1 interactions, identify p53 as a negative regulator of CoV replication, and characterize the RCHY1-dependent mechanism for SARS-CoV to abolish p53 antiviral activity.

**Results**

**SUD Interacts with RCHY1 and CAMK2D in Vivo.** Studying virus–host interactions, we have recently performed Y2H screens of a complete SARS-CoV ORF collection (ORFeome) with human cDNA libraries (34). Several E3 ubiquitin protein ligases including RCHY1 were identified as interacting partners of SUD. Independently, plasmids expressing an ectopic GFP–SUD fusion were transfected into HEK293 cells. Together with its host-binding partners, the GFP–SUD fusion protein was subsequently enriched with a GFP-trap consisting of beads coated with anti-GFP antibodies. After quantitative mass-spectrometry analysis of the proteins bound to the beads (35, 36), RCHY1 showed up again as an interacting partner of SUD (Fig. 1 A–C). In addition, CAMK2D was also identified to bind to SUD in the same assay. RCHY1–SUD interaction was further confirmed by fluorescence-3-hybrid assay (Fig. S1). Most interestingly, RCHY1 is a substrate of CAMKII (14).

To confirm that SUD also binds to those novel partners in vivo, split-YFP protein–protein interaction assays were carried out in human living cells (HEK-293). SUD and its interacting candidates were fused to the C-terminal (YFPc) and N-terminal (YFPN) YFP fragments, respectively. If SUD binds to a tested candidate in this assay, the N-terminal and C-terminal YFP fragments assemble, leading to fluorescence emission (37). As a result, SUD was demonstrated to bind to both RCHY1 and CAMK2D in the cytosol of HEK293 living cells (Fig. 1 D and E).

CAMKII isoforms can phosphorylate RCHY1 at T154/S155 in vitro (14), but a physical interaction between RCHY1 and CAMK2D has never been examined in vivo. For this reason, a split-YFP assay was performed. However, no clear YFP signal representing association of RCHY1 and CAMK2D was detected. The in vivo expression level of RCHY1 was subsequently found to be extremely low in the presence of CAMK2D (Fig. 1F), indicating a repressive expression level in the presence of SUD. Alternatively, the RCHY1–CAMK2D interaction was tested by a highly sensitive communo-precipitation (coIP) assay. As shown in Fig. 1 F and G, the SUD–RCHY1 and SUD–CAMK2D interactions were further confirmed (Fig. 1F, compare lanes 2 and 4 with lane 1; Fig. 1G, compare lanes 6 and 5). Furthermore, the RCHY1–CAMK2D interaction was detected (Fig. 1F, compare lane 3 and 4 with lane 1). Negative controls (lane 1 in Fig. 1F and lane 5 in Fig. 1G) showed no unspecific binding of CAMK2D–HA or SUD–RFP to the GFP-trap. In conclusion, there is direct physical interaction between each pair of proteins among CAMK2D, RCHY1, and SUD.

**RCHY1 Residues 95–144 Bind to SUD.** The RCHY1 protein as an interacting partner of SUD consists of 261 aa residues. The N-terminal fragment comprising residues 1–94 contains a CHY zinc-finger domain (residues 20–94) that is responsible for binding to calmodulin (14). The C-terminal fragment comprising residues 145–261 contains a RING-finger domain (residues 145–186) with intrinsic E3 ubiquitin ligase activity and a zinc ribbon (residues 190–250). The middle region of RCHY1 comprising residues 120–138 is required for binding to tumor protein p53 and to CDK9 (8, 24). Phosphorylation by CAMKII at T154/S155 or by CDK9 at Ser211/Thr217 enhances self-ubiquitination of RCHY1 and shortens its half-life (14, 24). A schematic domain presentation of RCHY1 is shown in Fig. 2A.

To determine which region is crucial for interaction with SUD, we cloned two truncated RCHY1 fragments into split-YFP destination vectors and tested their interactions with SUD. One fragment contained residues 1–144 and was thus lacking the RING-finger domain and the zinc ribbon; the other contained residues 95–261 and was devoid of the calmodulin-binding region (Fig. 2A). Interestingly, both truncated RCHY1 fragments interacted with SUD (Fig. 2B). The region in common between these two fragments, residues 95–144, was consequently supposed to be required for binding to SUD. Indeed, it was subsequently demonstrated that the RCHY1 fragment 95–144 interacted with SUD in the cytosol of HEK293 cells (Fig. 2B).
Of note, the RCHY1 binding sites for p53 and CDK9 are within this region (8, 24).

**SUD-NM Interacts with RCHY1.** SUD is located between the X-domain and the PL$^{p30}$ domain of SARS-CoV Nsp3 (Fig. S24); it consists of three subdomains: SUD-N, SUD-M, and SUD-C. SUD-N and, in particular, SUD-M have been demonstrated to bind oligo(G) nucleotides capable of forming G-quadruplexes (6). In addition, SUD-C exhibits RNA-binding activity (38). The interaction between RCHY1 and diverse SUD subdomain fragments was tested by split-YFP microscopy (Fig. S2B). SUD-NM did bind to RCHY1, whereas neither SUD-N nor SUD-M alone did. SUD-MC did not bind to RCHY1 either. The results indicate that both SUD-N and SUD-M are indispensable for the interaction with RCHY1.

MERS-CoV is another highly pathogenic coronavirus. The MERS Nsp3 contains a homolog to SUD-MC, but not to SUD-N (Fig. S3). The interaction between RCHY1 and MERS-MC (homologous to SUD-MC) was also analyzed by split-YFP fluorescence microscopy. Consistent with the lacking interaction between SUD-MC and RCHY1, MERS-MC did not bind to RCHY1 (Fig. S2C).

**SUD Augments Ubiquitination of p53.** As shown previously, the E3 ubiquitin ligase RCHY1 physically interacts with SUD. Because p53 is subject to ubiquitination by RCHY1, both in vivo and in vitro (8), we examined whether binding to SUD influences the E3 ubiquitin ligase activity of RCHY1. As mentioned previously, the region of RCHY1 involved in binding to p53 is within the RCHY1 fragment (residues 95–144) that binds to SUD. Therefore, we first assumed SUD to compete with the RCHY1-mediated ubiquitination of p53. However, the in vivo ubiquitination assay revealed that SUD dramatically promoted the ubiquitination of p53 (Fig. 3A). The negative control (Fig. 3A, lane 1) shows that GFP was not ubiquitinated by RCHY1. Thus, the multiple bands appearing in the anti-HA blot of Fig. 3A, lane 2, only represent p53 ubiquitination; compared with lane 2, lane 3 shows that SUD strongly augmented ubiquitination of p53. As shown in Fig. S24, the PL$^{p30}$ domain occurs next to the C terminus of the SUD within SARS-CoV Nsp3. The PL$^{p30}$ releases the nonstructural proteins Nsp1, Nsp2, and Nsp3 from the viral polyproteins. In addition to being a protease, PL$^{p30}$ possesses deubiquitinating (DUB) and desISGylating activities (39, 40). Initially, we thought that SUD promoting p53 ubiquitination was in conflict with the DUB activity of PL$^{p30}$. The SARS-CoV PL$^{p30}$ and the SUD–PL$^{p30}$ fusion were therefore cloned to test whether p53 is a substrate of the PL$^{p30}$. The in vivo ubiquitination assay demonstrated that the PL$^{p30}$ was not able to deubiquitinate p53 (Fig. S3B). The two anti-HA
SUD Stabilizes RCHY1. Phosphorylation of RCHY1 by CAMKII abrogates the E3 ubiquitin ligase activity of RCHY1 toward p53; it also leads to enhanced self-ubiquitination and instability of RCHY1 (14). However, SUD does not block the phosphorylation of RCHY1 despite the fact that SUD augments ubiquitination of p53. To find out the mechanism of how SUD promotes RCHY1-mediated ubiquitination of p53, the protein level of RCHY1 was examined in the absence and presence of SUD. RCHY1 has a short half-life of ∼3.5 h (14). Therefore, the expression level of the RCHY1–RFP fusion was very weak, despite the presence of the strong CMV promoter (Fig. 4B, Upper). However, SUD clearly enhanced the protein level of RCHY1–RFP fusion (Fig. 4B, Lower). Western blot has also demonstrated that SUD leads to accumulation of Myc–YFP–RCHY1 (Fig. 4C).

SUD–PLpro Forms a Complex with RCHY1 and p53. p53 could be pulled down by RCHY1 in a co-IP assay (8). RCHY1 is located in both the nucleus and the cytosol, whereas p53 can also shuttle between these two compartments (14, 41). It has not been reported where RCHY1 and p53 interact with each other. Therefore, we performed a split-YFP assay to answer this question. However, the YFP signal was not detectable due to the low expression level of RCHY1. The RCHY1 fragment 1–144 containing both the SUD and p53 binding sites displayed a much higher expression level. The split-YFP assay revealed that p53 interacts with RCHY1 fragment 1–144 mainly in the nucleus and only to a small extent in the cytosol (Fig. 4D). Thus, in the cytosol, RCHY1 can interact with both SUD and p53. To investigate whether p53 and SUD compete for binding to RCHY1, an excessive amount of plasmids ectopically expressing the p53–RFP fusion was cotransfected with YFP–RCHY1 and YFP–SUD fusion proteins (Fig. 4E). As a result, the split-YFP signal representing the interaction between RCHY1 and SUD was not diminished, indicating that there was no competition between p53 and SUD for binding to RCHY1.

Subsequently, we tested whether p53 can form a complex simultaneously with RCHY1 and SUD. To better mimic the real situation of the viral protein interacting with RCHY1 in SARS-CoV-infected cells, the SUD–PLpro fusion protein instead of SUD was applied. p53–GFP was able to pull down SUD–PLpro in the co-IP assay only if RCHY1 was also expressed (Fig. 4F, compare lane 3 with lane 2); this indicates that p53 forms a complex with RCHY1 and SUD–PLpro but there is no direct interaction between p53 and SUD–PLpro. The negative control (Fig. 4F, lane 1) shows no unspecific binding of RCHY1 or SUD–PLpro to the GFP-trap.

CDK9 is another important protein kinase involved in RCHY1–p53 signaling. Similar to CAMK2D, CDK9 phosphorylates RCHY1 and enhances the self-ubiquitination of this protein. However, differently from CAMK2D, CDK9 can directly phosphorylate p53 at Ser392 (24). We therefore tested a possible binding of SUD–PLpro to CDK9 but could not detect any interaction (Fig. 4F, lane 4).

PLpro Binds to RCHY1 and Promotes Its Accumulation. To confirm that SUD–PLpro binds to RCHY1 in vivo, plasmids expressing YFP–SUD–PLpro and YFP–RCHY1 were transfected into HEK293 cells for a split-YFP assay. A vector expressing YFP–PLpro was applied as one of the controls. By chance, an interaction between RCHY1 and SARS–CoV PLpro was discovered although the

![Diagram](https://www.pnas.org/cgi/doi/10.1073/pnas.1603435113)
A coIP assay was subsequently performed to further confirm the binding of PLpro to RCHY1. HA-tagged PLpro was pulled down together with GFP–RCHY1 by the GFP-trap containing beads coated with anti-GFP antibodies (Fig. 5B), implying a physical interaction between SARS-CoV PLpro and RCHY1. Similarly to SUD, PLpro also influences the stability of RCHY1 (Fig. 5C). Interestingly, both SUD and PLpro led to accumulation of RCHY1 at the protein level but not the mRNA level (Fig. 5C, compare lanes 2 and 3 with lane 1). Compared with the SUD–PLpro fusion and PLpro alone, SUD promoted a slightly higher accumulation of RCHY1 when expressed separately (Fig. 5C, compare lane 4 with lanes 2 and 3). Because it is known that viral SARS-CoV PLpro acts as a deubiquitinase on cellular proteins and inhibits innate immune responses, we tested the effect of PLpro on RCHY1 in an in vitro deubiquitination assay (Fig. S4). As RCHY1 was not deubiquitinatated by PLpro, the protein must be stabilized by a different, as-yet-unknown mechanism.

**SUD–PLpro Leads to Degradation of p53.** Ubiquitination of p53 can regulate the protein in many different aspects, such as proteasome-mediated degradation, degradation-independent nuclear export, and functional alteration (41). To find out the consequence of enhanced p53 ubiquitination promoted by SUD and SUD–PLpro, the endogenous p53 protein level was tested (Fig. 5D). Ectopic HA-tagged RCHY1 was transfected into cells as a positive control. Overexpression of RCHY1 resulted in p53 degradation (Fig. 5D, compare lane 2 with lane 1). Like the phenotype obtained from the positive control, SUD–PLpro also led to dramatic enhancement of the degradation of p53 (Fig. 5D, compare lane 3 with lane 1). Subsequently, PLpro alone was also found to cause p53 degradation (Fig. 5D, compare lane 5 with lane 4). Moreover, the SUD–PLpro fusion resulted in further degradation of p53 compared with PLpro alone (Fig. 5D, compare lane 6 with lane 5), which indicates that SUD augments p53 degradation.

**P53 Inhibits Replication of a SARS-CoV.** The tumor suppressor protein p53 has been demonstrated to possess an inhibitory effect on some viruses such as HIV-1 and human papillomavirus (42, 43). Ectopic expression of HCoV-NL63 PLP2 was shown to promote p53 degradation by interaction with the cellular ubiquitin ligase MDM2, leading to reduced replication of Sendai virus (30). However, to our knowledge, a direct effect of p53 on coronavirus replication has not been shown before. To investigate how SARS-CoV benefits from p53 degradation promoted by SUD–PLpro, the influence of p53 on viral growth of SARS-CoV was examined. Human ACE2-transgenic p53-expressing HCT116 cells (HCT116/AEC2 p53+) as well as p53 knockout cells (HCT116/AEC2 p53−/−) were infected with SARS-CoV, and viral replication was monitored by real-time PCR. As shown in Fig. 6, SARS-CoV grew to an ~1,000-fold higher titer in p53−/− cells compared with p53+/+ (Fig. 6A). To exclude that differential metabolism or cell growth was responsible for differences in virus replication, cells were infected with a second virus in parallel. Vesicular stomatitis virus (VSV) grew equally well in both p53−/− and p53+/+ HCT116/AEC2 cells, suggesting that facilitation growth was SARS-CoV specific (Fig. 6B).

Loss-of-function assays were also performed to examine the effect of p53 on replication of the SARS-CoV replicon. The SARS-CoV replicon DNA pBAC-SARS-Rep-(enillia)Luc (7) and the SARS-CoV replicon RNA in vitro transcribed from pBAC-SARS-Rep-(M(etrilida))Luc (34) carrying a secreted Metridia luciferase reporter were tested in p53-expressing HCT116 lysates were purified with GFP-trap consisting of beads coated with anti-GFP antibodies. The samples of IP and input were analyzed by 12% (wt/vol) SDS/PAGE and WB with anti-HA, anti-GFP, anti-SARS-CoV Nsp3, anti-Myc, and anti-lamin A antibodies. (B) Plasmids expressing indicated proteins were cotransfected into HEK293 cells growing in 10-cm dishes. At 24 h after transfection, cells were lysed and the interaction was weaker than the interaction between the SUD–PLpro fusion and RCHY1 (Fig. 5A).

Fig. 3. SUD augments p53 ubiquitination and stabilizes RCHY1. (A) Plasmids expressing HA-tagged ubiquitin, c-Myc-tagged YFPR–RCHY1, GFP or p53–GFP fusion, and RFP or SUD–RFP fusion were cotransfected into HEK293 cells growing in 10-cm dishes. At 24 h after transfection, cells were lysed and the samples of IP and input were analyzed by 12% (wt/vol) SDS/PAGE and WB with anti-HA, anti-GFP, anti-SARS-CoV Nsp3, anti-Myc, and anti-lamin A antibodies. (B) Plasmids expressing indicated proteins were cotransfected into HEK293 cells growing in 10-cm dishes. The in vivo ubiquitination assay was carried out as described in A.
p53+/− and HCT116 p53−/− knockout p53−/− cells (44) as well as in a doxycycline (DOX)-dependent p53-inducible cell line SW480 (19) (Fig. 6 C and D). p53 expression was monitored by Western blot using anti-p53 antibodies in the respective cell lines (Fig. S5). Both of the replicons replicated better in HCT116 p53−/− than in HCT116 p53+/−, and better in SW480 without DOX induction than in SW480 with DOX induction. The observed fold differences between the use of replicons and virus infection are attributed to the commonly lower replication efficiencies of coronavirus replicons (>27 kb), because transfection/ electroporation efficiencies do not reach 100% of cells. On the contrary, viable virus is much more efficient due to multicycle infection and exponential growth when using low multiplicities of infection (MOIs, 0.001).

To explore the efficiency of genome replication of SARS-CoV replicon under the pressure of p53 the plasmid pBAC-SARS-Rep-RLuc was transfected into HEK293 cells at increasing concentrations of a p53-expressing plasmid. p53 strongly reduced replication of the SARS-CoV replicon by 65%, whereas it had no effect on the pcDNA3.0 Renilla luciferase (pcDNA3.0 RLuc) control (Fig. S6). The results demonstrate that p53 is a negative regulator of SARS-CoV replication.

MERS-CoV PLpro and HCoV-NL63 PLP1/2 Interact with and Stabilize RCHY1 and Cause Endogenous p53 Degradation. As its name implies, the SUD is unique for the SARS-CoV, whereas PLpro domains are relatively well-conserved in coronaviruses (39). Among the human coronaviruses, only SARS-CoV and MERS-CoV have one PLpro domain, whereas most other HCoVs have two PLpro domains, PLP1 and PLP2 (39, 45, 46). To investigate whether other HCoV PLpros also target RCHY1, similarly to what the SARS-CoV PLpro does, we cloned the MERS-CoV PLpro and the HCoV-NL63 PLP1 to perform a coIP RCHY1-binding assay. As a result, MERS-CoV PLpro and NL63 PLP1 showed clear interaction with RCHY1 (Fig. 7A, compare lanes 2 and 3 with lane 1).
Most of the GFP-NL63 PLP2 was degraded with only a little intact protein remaining (Fig. 7A, lane 4). Nevertheless, a small amount of HA–RCHY1 could be coimmunoprecipitated by GFP-NL63 PLP2 (Fig. 7A, compare lane 4 with lane 1), implying interaction between these proteins. To further confirm the interaction, a split-YFP assay was performed (Fig. S7). Both MERS-CoV PLP2 and NL63 PLP2 showed clear interaction with RCHY1 in the nucleus and the cytosol, whereas NL63 PLP1 interacted with RCHY1 at the endoplasmic reticulum (ER).

Subsequently, we examined whether MERS-CoV PLP2 and NL63 PLP2/1 also stabilize RCHY1. As shown in Fig. 7B, all of the tested PLP2s stimulated RCHY1 accumulation (compare lanes 2, 3, and 4 with lane 1). Indeed, the stabilization of RCHY1 by the NL63 PLP2 was dramatic (Fig. 7B, compare lane 4 with lane 1). Consistent with this result, all of the tested PLP2s led to degradation of endogenous p53 as well (Fig. 7C). Thus, similarly to SARS-CoV PLP2, MERS-CoV PLP2 and NL63 PLP2/1 also targeted RCHY1 for p53 degradation.

To find out whether p53 inhibits viral replication of other HCoVs in addition to SARS-CoV, the influence of p53 on NL63 replication was tested. Due to an extremely low transfection efficiency of p53, NL63 receptor ACE2 were used as a substitute for Caco2. Real-time quantitative PCR (q-PCR) analysis revealed that NL63 replication was reduced by ~30% in cells overexpressing p53, compared with control (Fig. 7D); this implies that p53 is an inhibitory factor not only for SARS-CoV but also for other HCoVs.

Discussion

PLP1/2 of the alphacoronavirus NL63 as well as the PLP2s of SARS-CoV and MERS-CoV from the genus betacoronavirus physically interact with and stabilize RCHY1, and lead to p53 degradation. Because PLP2s and PLPs are relatively well conserved among coronaviruses, it is highly likely that all of the coronaviruses share this strategy to antagonize the antiviral host factor p53. SUd alone strongly binds to and stabilizes RCHY1, leading to stimulation of p53 ubiquitination and degradation. The SUd–PL2 fusion interacts with RCHY1 more intensively and causes stronger endogenous p53 degradation than SARS-CoV PLP2 alone. Hence, SUd functions as an enhancer for SARS-CoV Nsp3 to counteract p53, and this might contribute to the high virulence of SARS-CoV.

The mechanism of how SUd and PLP2/PLP2s stimulate the accumulation of RCHY1 remains unclear. It is known that phosphorylation by CAMKII or CDK9 leads to polyubiquitination and subsequent degradation of RCHY1 (14, 24), but SUd does not impair the phosphorylation activity of CAMK2D toward RCHY1. Because papain-like proteases of SARS-CoV, MERS-CoV, and NL63 possess deubiquitinating activity (39, 46, 47) and can stabilize RCHY1, we originally hypothesized that the latter may be a possible substrate for these enzymes. However, an in vitro deubiquitination assay has shown that SARS-CoV PLP2 does not deubiquitinate RCHY1. Thus, SUd and PLP2 should stabilize RCHY1 through other mechanisms, e.g., by counteracting the binding of RCHY1 to CDK9 or interfering with the homodimerization of RCHY1 for self-ubiquitination. Because both SUd and PLP2 physically interact with RCHY1, their mechanisms of RCHY1 stabilization might also be different from one another.

The protein level of RCHY1 is dramatically increased in the presence of SUd. As a result, RCHY1–mediated ubiquitination of p53 is enhanced and p53 degradation is stimulated. However, enhanced p53 degradation should not be the only consequence of RCHY1 accumulation. Besides p53, the substrate targets of the three ubiquitin ligase RCHY1 include transcription factors p63, p73, and c-Myc; checkpoint kinase Chk2; DNA polymerase polH; histone deacetylase HDAC1; and CDK inhibitor p27Kip1 (9, 10, 48–52). It can be hypothesized that during HCoV infection, the protein levels of various genes might also be down-regulated. SUd also physically

Fig. 5. SARS PL-pro interacts with RCHY1 and leads to accumulation of RCHY1 as well as p53 degradation. (A) YFP-N and YFP-n were fused to the N terminus of SARS PL-pro and to the N terminus of RCHY1, respectively. At 24 h after transfection, HEK293 cells were fixed with PFA and stained with DAPI. Samples were inspected with a fluorescent microscope (Leica DM4000 B, 40x objective; Upper). Pictures for statistical analysis were taken from at least five randomly chosen areas. Intensities of split-YFP and DAPI signals were measured with ImageJ software (*** P < 0.001; Lower). (B) Plasmids expressing HA-tagged SARS PL-pro and GFP or GFP–RCHY1 fusion were transfected into HEK293 cells grown in 10-cm culture dishes. The coloP assay was performed as described before. (C) WB (Upper) Plasmids ectopically expressing SUd, PL-pro, or SUd–PL-pro fusion were transfected into HEK293 cells grown in a six-well plate. Cell lysates were prepared 24 h posttransfection and subjected to Western blotting with anti-Myc and anti-lamin A antibodies. qPCR: (Lower) Plasmids ectopically expressing SUd, PL-pro, or SUd–PL-pro fusion were transfected into HEK293 cells grown in a 24-well plate. At 24 h posttransfection, cells were harvested for total RNA extraction, first cDNA synthesis, and subsequent SYBR Green qPCR. The experiment was carried out in quadruplicates. (D) Indicated plasmids were transfected into HEK293 cells grown in a six-well plate. At 24 h posttransfection, cells were lysed for Western blotting with anti-p53 and anti-lamin A antibodies.
interacts with CAMK2D, although it does not disturb phosphorylation of RCHY1 by CAMK2D. However, RCHY1 is neither the only substrate nor the only interacting partner of CAMK2D. The association of SUD and CAMK2D might alter the affinity of CAMK2D for other proteins, e.g., for its candidate binding partner IFN-γ receptor 2 (IFNGR2), which was discovered by Y2H screen (53). Alternatively, the interaction between SUD and CAMK2D might interfere with phosphorylation of STAT1 by CAMK2D, which is required for maximum IFN-γ-stimulated gene (ISG) expression (54). In addition to stimulation of p53 degradation, SUD probably features even more complex interferences with host cellular signaling pathways.

To date, p53 was found to serve as an antiviral factor against diverse positive-sense single-stranded RNA (ssRNA) viruses such as hepatitis C virus (HCV) and poliovirus; negative-sense ssRNA viruses such as influenza A virus; and the retrovirus HIV-1 (29, 31, 55, 56). p53 antiviral activity on Se

Plasmid Constructs. The fragments c-Myc-YFP (c-Myc amino acids 1–155) and HA-YFP (HA amino acids 156–225) were amplified with designed primers (Table S1) from the template plasmids pSYNE-355 and pSYCE-355 (37), respectively. The obtained PCR fragments were subsequently cloned into the pTREX-dest30-PrA Gateway-compatible vector via AgeI/Xhol cleavage sites or into the pTREX-dest30-ct-PrA Gateway-compatible vector (60) via Spel/Apal sites to replace the protein A tag, yielding pDEST-c-Myc-YFP (c-Myc-YFP fused to N terminus of a test gene) and pDEST-ct-HA-YFP (HA-YFP fused to N terminus of a test gene) split-YFP vectors.

Materials and Methods

Cell Cultures and Transfection. HEK293 and HCT116 p53+/+ and p53−/− cells were cultured at standard conditions (SI Materials and Methods). For introduction of the ACE2 receptor into HCT116 p53+/+ and p53−/− cells, 2 × 109 cells/mL were transduced with human ACE2-carrying lentiviruses at an MOI of 0.005. Lentiviruses were provided in McCoy’s 5A supplemented with polybrene (1 μg/mL). After 48 h, the supernatant was removed, cells were washed with PBS, and McCoys’s 5A containing 1 μg/mL puromycin was added for selection. Selective medium was changed every 3 d. After 10 d, surviving cells were expanded and tested for hACE2 protein expression by immunofluorescence assay and Western blot analysis as described previously (62).

Split-YFP. Fluorescence microscopy was performed according to standard procedures and is described in detail in SI Materials and Methods.
CoIP and Western Blot. GFP fusions and complexed proteins were immuno-precipitated by GFP-Trap A beads (ChromoTek) according to manufacturer’s protocol under native conditions (SI Materials and Methods). Detailed Western blot protocols were described elsewhere (63).

qPCR. Quantification NL63 viral RNA (63) and of cellular mRNAs is described in detail in SI Materials and Methods.

In Vivo Ubiquitination Assay. Plasmids expressing HA-tagged ubiquitin, c-Myc-YFP-tagged RCHY1 (c-Myc-YFP–RCHY1), substrate protein (GFP control or p53-GFP), and effector protein (RFP control, Sud-RFP fusion, RFP-SUD-PL** fusion, or RFP-PL** fusion) were cotransfected into HEK293 cells using the polyethylenimine (PEI) method. At 24 h posttransfection, cells were lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 10 mM N-ethylmaleimide, and protease inhibitor (Complete ULTRA Tablets; Roche). Subsequently, GFP-Trap A beads (ChromoTek) were used to purify GFP and p53-GFP from the cell lysates. The purified GFP and p53-GFP were examined with anti-HA and anti-GFP (ChromoTek) antibodies by Western blot.

In Vitro Deubiquitination Assay. HEK293 cells growing on 10-cm dishes were cotransfected with GFP-RCHY1, HA-ubiquitin, calmodulin1-RFP, and CAMK2D-c-Myc-YFP**. At 23 h posttransfection, cells were treated with PMA to a final concentration of 20 ng/mL and ionomycin to a final concentration of 1 µM to activate CAMK2D. At 90 min after the treatment, cells were harvested and GFP-RCHY1 protein was purified with GFP-trap A beads (ChromoTek). The purified bead-bound GFP-RCHY1 was subsequently incubated with 10 µM SARS-CoV PL** protein at 25 °C for 30 min in a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 2 µM DTT. After incubation, the beads were washed twice with washing buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5 mM EDTA. The bead-bound GFP-RCHY1 was finally examined with anti-HA and anti-GFP (ChromoTek) antibodies by Western blot.

Purification of Recombinant SARS-CoV PL**. The PL** of SARS-CoV (strain TOR2; GenBank accession no. AY274119) is part of Nsp3 and comprises 319 aa residues, corresponding to Glu1541–Tyr1859 of p1a. A gene coding for the PL** was amplified by the PCR using the forward primer 5′-CTAGCTAGCGAGGTTAAGACTATAAAAGTGTTC-3′ and the reverse primer 5′-CCGCTCGAGTAAATACGACGAGCTTTGATGTTGATG-3′. The resulting PCR product was digested with restriction enzymes Nhel and Xhol for ligation into pET-28a (Novagen). Cloning was designed to include an N-terminal hexahistidine (His6) tag and a thrombin cleavage site. The recombinant plasmid was verified by DNA sequencing. Expression of the PL** gene and purification of the protein were carried out by a method described previously (64).

In Vivo Kinase Activity Assay. Plasmids expressing calmodulin-HA, CAMK2D-c-Myc-YFP**, substrate protein (GFP control or GFP-RCHY1), and effector protein (RFP control or Sud-RFP fusion) were cotransfected into HEK293 cells using the PEI method. Medium was changed 20 h posttransfection. Then the cells were treated with PMA to a final concentration of 20 ng/mL and ionomycin to a final concentration of 1 µM. Two hours after the treatment, cells were harvested and GFP-Trap A beads (ChromoTek) were used to purify GFP and the GFP-RCHY1 fusion from the cell lysates. Purified GFP and GFP-RCHY1 fusion proteins were subsequently examined with anti-phospho-Ser/Thr (QIAGEN) and anti-GFP (ChromoTek) antibodies by Western blot for in vivo phosphorylation.

Luciferase Activity Assay. Transfected cells were harvested with Renilla Luciferase Assay System Kit (Promega). The luciferase activity was then measured with a FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH).

Statistical Analysis. qPCR and luciferase measurement data are shown as mean ± SD of tri- or quadruplicated values and analyzed by one-way or two-way ANOVA with Bonferroni’s posttest (or by t test) using Prism 5 (GraphPad Software Inc.). P values of 0.05 or less were considered significant.

Virus Infection Experiments. Cell cultures were infected in quadruplicate with either SARS-CoV (strain Frankfurt) and VSV (strain Indiana) at a multiplicity of infection of 0.001. After virus adsorption, cells were washed with PBS and cell culture medium was replenished. Samples were taken at 0 and 24 h postinfection. Viral RNA was extracted by a Macherey-Nagel viral RNA kit. Real-time PCR was performed using virus-specific probes and primers with the One-Step SuperScript III kit (Life Technologies) according to previous protocols (34).

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Fig. 7. MERS PL** and NL63 PLP1/2 lead to accumulation of RCHY1 and p53 degradation. (A) The indicated plasmids were transfected to HEK293 cells growing in 10-cm culture dishes. CoIP was performed as described before. (B) The indicated plasmids were transfected to HEK293 cells growing in a six-well plate. At 24 h posttransfection, cells were lysed. The lysates were Western blotted with anti-HA and anti-lamin A antibodies. (C) At 24 h after transfection with the indicated plasmids into HEK293 cells, cells were harvested and Western blotted with anti-p53 and anti-lamin A antibodies. (D) HEK293 cells were cotransfected with plasmids over-expressing RFP-ACE2 and plasmids overexpressing GFP control or p53-GFP. At 24 h after transfection, cells were inoculated with NL63 viruses of MOI 0.4 at 37 °C for 1 h. At 24 h after infection with NL63 virus, total RNA was extracted from cell lysates. Viral RNA was analyzed by qPCR with NL63-specific primers and standardized with RFP-ACE2 intensity. Experiments were performed in sextuplicates. ***p < 0.001.


