# Enteroviral 3C protease activates the human NLRP1 inflammasome in airway epithelia

Kim S. Robinson<sup>1,2</sup>, Daniel Eng Thiam Teo<sup>3\*</sup>, Kai Sen Tan<sup>4\*</sup>, Gee Ann Toh<sup>3\*</sup>, Hsiao Hui Ong<sup>4\*</sup>, Chrissie Kaishi Lim<sup>5</sup>, Kenneth Lay<sup>2,6</sup>, Bijin Veonice Au<sup>5</sup>, Tian Sheng Lew<sup>7</sup>, Justin Jang Hann Chu<sup>5,7</sup>, Vincent Tak Kwong Chow<sup>7</sup>, De Yun Wang<sup>4</sup>, Franklin L. Zhong<sup>1,2,3,5</sup>†, Bruno Reversade<sup>2,5,6,8,9</sup>†

<sup>1</sup>Skin Research Institute of Singapore (SRIS), 8A Biomedical Grove, #06-06 Immunos, 138648, Singapore. <sup>2</sup>Institute of Medical Biology, Agency of Science, Technological and Research, 8A Biomedical Grove, #06-06 Immunos, 138648, Singapore. <sup>3</sup>Lee Kong Chian School of Medicine, Nanyang Technological University, 11 Mandalay Road, 308232, Singapore. <sup>4</sup>Department of Otolaryngology, Infectious Diseases Translational Research Programme, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 10 Medical Drive, 117597, Singapore. <sup>5</sup>Institute of Molecular and Cell Biology, 61 Biopolis Drive, 138673, Singapore. <sup>6</sup>Genome Institute of Singapore, 60 Biopolis Street, 138672, Singapore. <sup>7</sup>Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 10 Medical Drive, 117597, Singapore. <sup>8</sup>Department of Paediatrics, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 10 Medical Drive, 117597, Singapore. <sup>9</sup>The Medical Genetics Department, Koç University School of Medicine, 34010 Istanbul, Turkey.

\*These authors contributed equally to this work.

+Corresponding author. Email: franklin.zhong@ntu.edu.sg (F.L.Z.); bruno@reversade.com (B.R.)

Immune sensor proteins are critical to the function of the human innate immune system. The full repertoire of cognate triggers for human immune sensors is not fully understood. Here, we report that human NLRP1 is activated by 3C proteases (3Cpros) of enteroviruses, such as human rhinovirus (HRV). 3Cpros directly cleave human NLRP1 at a single site between Glu130 and Gly131. This cleavage triggers N-glycine-mediated degradation of the autoinhibitory NLRP1 N-terminal fragment via the cullin<sup>ZER1/ZYG11B</sup> complex, which liberates the activating C-terminal fragment. Infection of primary human airway epithelial cells by live human HRV triggers NLRP1-dependent inflammasome activation and IL-18 secretion. Our findings establish 3Cpros as a pathogen-derived trigger for the human NLRP1 inflammasome and suggest that NLRP1 may contribute to inflammatory diseases of the airway.

The human innate immune system employs a multitude of sensor proteins to detect microbial infections and kickstart the immune response (1). Nod-like receptor (NLR) proteins are a family of innate immune sensors that can detect pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) (1-3). Upon activation, NLR proteins nucleate the assembly of inflammasome complexes, leading to pyroptotic cell death and secretion of processed inflammatory cytokines, such as IL-1 $\beta$  and IL-18 (4). Among human NLR sensors, NLRP1 remains one of the few whose cognate trigger has not been identified. Human NLRP1 differs from rodent homologs in terms of domain organization, ligand specificity, and tissue distribution (5) and its exact role in human antimicrobial response is still unclear. However, NLRP1 does play an important role in inflammatory disorders. Germline activating mutations in NLRP1 cause a number of Mendelian syndromes characterized by multiple self-healing keratoacanthomas of the skin and hyperkeratosis in the larvngeal and corneal epithelia (6-8). Carriers of certain common NLRP1 single-nucleotide polymorphisms (SNPs) experience increased risks for autoimmune diseases such as asthma and vitiligo (9, 10).

Anthrax lethal factor (LF) is the most well-characterized trigger for murine NLRP1B (11-15). LF directly cleaves NLRP1B close to its N terminus (13–15). This cleavage causes N-degron-mediated degradation of the autoinhibitory N-terminal fragment, freeing the non-covalently bound FIIND<sup>UPA-</sup> CARD (a.a.1213-1474) fragment to activate caspase-1 (16-18) (Fig. 1A). The consensus LF cleavage site is absent in human NLRP1. As a result, LF does not cleave or activate human NLRP1. Human NLRP1 also contains a N-terminal PYRIN domain not found in rodents (Fig. 1A). Despite these differences, both human NLRP1 and rodent homologs can be activated by chemical inhibitors of dipeptidases DPP8 and DPP9, although the underlying mechanisms remain to be fully elucidated (19-21). Rodent Nlrp1 can also be activated by Toxoplasma gondii infection in a process that does not appear to involve protease-mediated cleavage (22, 23). Thus multiple modes of activation exist for NLRP1 from different species. It remains an open question as to whether any naturally occurring pathogen-derived molecules can activate human NLRP1. In this study, we set out to identify the cognate pathogen-derived signal(s) that can activate human NLRP1

, 2020

and assess the mechanisms by which they activate the NLRP1 inflammasome.

# 3Cpros activate human NLRP1 in vitro

In a survey of common human pathogens, we considered the human rhinovirus (HRV), which is the major causative agent for the common cold. HRV is a member of the Picornaviridae family of single-stranded RNA viruses, which cause a wide range of human diseases, including hand-foot-and-mouth disease, peri/myocarditis, and poliomyelitis (24). HRV infection of primary human bronchial epithelial cells can induce caspase-1 activation and IL-1 secretion (24, 25), although the upstream sensing mechanisms are unclear. Notably, all picornaviruses, including HRVs, encode two well-defined proteases termed 2Apro and 3Cpro. These proteases are responsible for cleaving the viral genomic precursor proteins into individual components (26), and have also been shown to cleave host proteins to facilitate immune evasion (27–30).

To test whether HRV-3Cpro can activate human NLRP1, we expressed Myc-tagged HRV-3Cpro in a 293T reporter cell line that stably expressed ASC-GFP and NLRP1-FLAG (termed 293T-ASC-GFP-NLRP1-FLAG). As compared to vector-transfected cells, 3Cpros derived from two strains of HRV (HRV-14, serotype B and HRV16, serotype A) and a closely related enterovirus (coxsackie B3) induced a significant increase in the percentage of cells aggregating ASC-GFP specks (Fig. 1B and fig. S1, A and B), similar to the dipeptidase inhibitor, talabostat. By contrast, none of the other viral proteases tested were able to do so (Fig. 1B and fig. S1, A and B), despite similar or even higher levels of expression. The ability of HRV14-3Cpro to activate human NLRP1 was entirely dependent on its enzymatic activity, since mutating its catalytic cysteine residue (p.C146A) abrogated ASC-GFP speck formation (Fig. 1B and fig. S1B). In an orthogonal assay for NLRP1 activation, HRV14-3CPro and HRV16-3Cpro directly induced the formation of high-molecular weight NLRP1 oligomers by native polyacrylamide gel electrophoresis (PAGE) at the expense of monomeric NLRP1 (fig. S1C). These findings establish enteroviral 3Cpros, such as those encoded by HRV, as robust activators for the reconstituted human NLRP1 inflammasome in vitro.

We and others have recently demonstrated that primary and immortalized human keratinocytes express components of the NLRP1 inflammasome complex endogenously and can undergo rapid pyroptosis in response to inflammasome agonists (7, 19). This provides a robust cellular system to determine if 3Cpros can activate the endogenous NLRP1 inflammasome. Immortalized human keratinocytes stably expressing GFP-tagged ASC and transfected with HRV14-3Cpro formed ASC-GFP specks and underwent membrane permeabilization marked by propidium iodide (PI) staining (fig. S1D). Using a doxycycline-inducible expression system, we showed that only keratinocytes expressing active HRV14-3Cpro demonstrated cardinal features of pyroptosis, including the secretion of cleaved, mature IL-1 $\beta$  and the formation of detergent-insoluble ASC oligomers (Fig. 1D). This occurred despite the lower expression of wild-type 3Cpro compared to its inactive mutant (p.C146A) (Fig. 1D). The effect of 3Cpro requires continuous proteasome activity, as the proteasomal inhibitor MG132 significantly blocked HRV-3Cpro-induced ASC-GFP speck formation (Fig. 1C) in 293T-ASC-GFP-NLRP1 cells, as well as NLRP1 self-oligomerization (fig. S1C) and ASC oligomerization and IL-1 $\beta$  cleavage (Fig. 1D). Therefore, 3Cpro-induced human NLRP1 activation may also involve a functional degradation step that is analogous to LF-induced mouse NLRP1B activation.

HRV14-3Cpro-induced pyroptosis was entirely dependent on NLRP1, as its genetic ablation by CRISPR-Cas9 (NLRP1<sup>-/-</sup> Tet-ON HRV14-3Cpro) abrogated IL-1ß secretion, ASC oligomerization (Fig. 1E and fig. S2A), and lytic cell death (Fig. 1F). In experiments with transiently transfected HRV14-3Cpro and HRV16-3Cpro, NLRP1, ASC, and CASP1 deletion all had similar inhibitory effects (fig. S2, B to D). Therefore, HRV-3Cpro can act as a potent trigger of pyroptotic cell death in immortalized human keratinocytes by activating the endogenous NLRP1 inflammasome. Similar to reconstituted NLRP1 in 293T cells (Fig. 1C and fig. S1C), this effect also required intact proteasome activity (fig. S1D). We also observed that a subset of 3Cpro-expressing keratinocytes underwent apoptotic cell death with Annexin V staining but without PI inclusion (fig. S2E), in agreement with the reported pro-apoptotic roles of 3Cpros in other cell types (31, 32).

# 3Cpros cleave human NLRP1 between p. Q130 and G131

Picornaviral 3Cpros, including HRV-3Cpro, are cysteine proteases with well-defined catalytic activity and substrate preferences (26, 33). Just as anthrax LF cleaves rodent NLRP1B directly (15, 34), we hypothesized that 3Cpros could activate human NLRP1 via direct cleavage. Overexpressed NLRP1 undergoes auto-cleavage within its FIIND and thus appears as two bands that differ by ~20 kDa when visualized with an Nterminal specific antibody (35, 36) (Fig. 2, A and B). In the presence of HRV14-3Cpro, two additional bands were visualized using the same antibody (Fig. 2B). By contrast, the Cterminal FIIND<sup>UPA</sup>-CARD fragment remained intact, suggesting that the HRV14-3Cpro cleaved NLRP1 at a single site close to the N terminus, and not within the FIIND<sup>UPA</sup>-CARD fragment. To visualize the 3Cpro-specific cleavage more precisely, the same experiment was carried out using the NLRP1<sup>F1212A</sup> mutant, which cannot undergo auto-cleavage within the FIIND and thus remained a single band by SDS PAGE (fig. S3A). With increasing amounts of HRV14-3Cpro, NLRP1F1212A became cleaved into a single proteolytic product, which was approximately 20 kDa smaller than full-length NLRP1 (fig.

S3A). In both experiments, the proteolytic banding patterns could be explained by a single cleavage site approximately 20 kDa from the NLRP1 N terminus. The same cleavage could be observed when NLRP1-expressing cell-free lysate was incubated with recombinant HRV14-3Cpro at 33°C (the preferred temperature for HRV infection) (fig. S3B). Based on these observations, the 3Cpro cleavage site was mapped to the linker region immediately after the PYRIN domain (PYD) (Fig. 2A), a region that is not conserved in rodents. As 3Cpros require a glutamine residue at the  $P_1'$  substrate site (33), each of the 11 glutamine residues present in this linker region was changed to alanine by site-directed mutagenesis. Of all the mutants tested, only the p.Q130A missense mutation abrogated NLRP1 cleavage by HRV14-3Cpro (Fig. 2C) and other 3CPros (fig. S3C). We next generated reporter 293T-ASC-GFP cells that stably expressed either wild-type NLRP1 or the uncleavable Q130A mutant (NLRP1<sup>Q130A</sup>) (Fig. 2D). In contrast to wild-type NLRP1, NLRP1<sup>Q130A</sup>-expressing cells no longer nucleated ASC-GFP specks in response to HRV14-3Cpro expression. However, their response to talabostat remained the same as wild-type NLRP1-expressing cells (Fig. 2E). Similar results were obtained using transfected NLRP1<sup>Q130A</sup> and HRV16-3Cpro (fig. S3D).

To study the endogenous inflammasome response, we rescued human *NLRP1<sup>-/-</sup>* keratinocytes with either wild-type NLRP1 or the cleavage site mutant NLRP1<sup>Q130A</sup>. Only wild-type NLRP1, but not NLRP1<sup>Q130A</sup>, restored HRV14-3Cpro-triggered IL-1β secretion, ASC oligomerization (Fig. 2F and fig. S4, A and B) and lytic cell death (Fig. 2G and fig. S2C). By contrast, both wild-type NLRP1 and NLRP1<sup>Q130A</sup> enabled NLRP1<sup>-/-</sup> cells to respond to talabostat (Fig. 2, F and G, and fig. S4A). Thus, 3Cpro-triggered NLRP1 activation is a result of a discrete cleavage site between a.a. Q130 and a.a. G131, adjacent to the human-specific PYRIN domain. This site is not conserved in rodents, and as expected, HRV14-3Cpro did not induce pyroptotic cell death in murine RAW264.7 cells (fig. S4, C to E). This is consistent with the fact that HRV, like most pathogenic human enteroviruses, do not naturally infect rodents (37).

# Degradation of the 3Cpro cleavage fragment (a.a. 131-1212) is required for NLRP1 activation

We next sought to further dissect how the cleavage of human NLRP1 by 3Cpros triggers proteasome-dependent inflammasome activation. Cleavage of human NLRP1 by HRV-3Cpro removes the entire PYRIN domain (PYD) where most diseasecausing gain-of-function germline mutations occur (Fig. 1A) (6, 7, 38, 39). It is conceivable that 3Cpro cleavage relieves an intrinsic autoinhibitory effect of the non-canonical NLRP1 PYD domain. Alternatively, the cleavage could trigger the destabilization of the entire N-terminal fragment (between the 3Cpro cleavage site and the FIIND auto-proteolysis site, a.a. 131-1212). To distinguish between these two possibilities, we examined a truncation mutant of NLRP1 (a.a. 131-1474), which mimics the major product generated by HRV14-3Cpro cleavage except for the initiating methionine. Unexpectedly, this mutant did not cause increased ASC-GFP speck formation relative to wild-type NLRP1 in 293T-ASC-GFP cells, despite being fully sensitive to talabostat-mediated activation (Fig. 3A and fig. S5A). Thus, the removal of NLRP1 PYD appears to be insufficient by itself to account for HRV-3Cpro triggered NLRP1 activation.

Recently the mechanism by which anthrax LF activates NLRP1B has been reported (16, 17, 40). LF cleavage creates an N-terminal degron that is recognized by the Type II N-degron receptors, such as UBR2, which degrade the inhibitory NLRP1B N-terminal fragment via the proteasome (Fig. 3B). We therefore hypothesized that an analogous pathway may account for the ability of HRV-3Cpro to activate human NLRP1. However, HRV-3Cpro cleavage of human NLRP1 generates an N-terminal glycine, which is not a canonical type II N-terminal degron recognized by related UBR proteins (41, 42). A glycine-specific N-degron pathway has recently been described (43), which consists of receptors ZER1 and ZYG11B and their partner cullins, CUL2 and CUL5 (termed cullin<sup>ZER1/ZYG11B</sup>). The cullin<sup>ZER1/ZYG11B</sup> machinery ubiquitinates substrate proteins with N-terminal glycine residues and causes their degradation via the proteasome. In contrast to the UBR system, the N-glycine degron pathway is not sensitive to type II free amino acids, but can be inhibited by the NEDD8/cullin inhibitor MLN4924 (43) (Fig. 3B). In 293T-ASC-GFP-NLRP1 reporter cells, MLN4924 blocked HRV14-3Cpro-induced ASC-GFP speck formation (Fig. 3C), comparable to proteasomal inhibitors MG132 and bortezomib. MLN4924 similarly blocked HRV14-3Cpro NLRP1 self-oligomerization (Fig. 3D) in 293T-NLRP1-FLAG cells. Notably, MLN4924 stabilized the post-cleavage NLRP1 fragment (corresponding to a.a. 131-1212, taking into account FIIND autoproteolysis) equivalently to MG132 and bortezomib (Fig. 3D). Type II N-degron inhibitor phenylalanine had no effect on either ASC-GFP speck formation or NLRP1 self-oligomerization (Fig. 3, C and D). Similarly, in keratinocytes, both MLN4924 and bortezomib completely blocked mature IL-1ß secretion and ASC oligomerization upon HRV14-3Cpro induction (Fig. 3E and fig. S5C) and stabilized the post-cleavage NLRP1 fragment (fig. S5C). MLN4924 had much smaller effect on talabostat-induced IL-1 $\beta$  secretion in keratinocytes (fig. S5B). Thus, the cullin<sup>ZER1/ZYG11B</sup> system does not appear to fully account for talabostat-mediated NLRP1 activation.

Given the known pleiotropic effects of MLN4924, we used CRISPR-Cas9 to delete ZER1 and ZYG11B (termed ZZ-dKO) in 293T-ASC-GFP reporter cells (data S1). The percentage of ASC-GFP specks in clonal ZZ-dKO 293T-ASC-GFP cells was significantly reduced following co-expression of wild-type NLRP1 and HRV14-3Cpro. By contrast, *UBR2*<sup>-/-</sup> cells were not significantly different from Cas9 control 293T-ASC-GFP cells (Fig. 3F and fig. S5E). As an additional control, we examined the patient-derived gain-of-function NLRP1 mutation, p. M77T, which by itself causes the destabilization of the N-terminal fragment (fig. S5A). Indeed, the percentage of ASC-GFP speck–forming cells induced by NLRP1<sup>M77T</sup> did not differ among *ZZ*-dKO, *UBR2*<sup>-/-</sup>, and control cells (Fig. 3F and fig. S5E). Therefore, cullin<sup>ZER1/ZYG11B</sup> is specifically required for HRV14-3Cpro triggered human NLRP1 activation. The 3Cprocleaved NLRP1 fragment (a.a. 131-1212) was significantly stabilized in *ZZ*-dKO cells relative to control cells (Fig. 3G), but not in *UBR2*<sup>-/-</sup> cells (fig. S5D). Thus, the 3Cpro-cleaved NLRP1 N-terminal fragment is a substrate for the cullin-<sup>ZER1/ZYG11B</sup>-mediated N-terminal glycine degron pathway.

# Live HRV infection activates the NLRP1 inflammasome in primary airway epithelial cells

We next tested the effect of live HRV infection in disease-relevant human epithelial cell types. In HeLa-Ohio cells overexpressing NLRP1-HA, robust HRV16 viral replication was achieved 16 hours post inoculation (MOI = 1), evidenced by the accumulation of the viral capsid protein VP2 (fig. S6A, lanes 4, 5, 10, and 11). This was accompanied by the appearance of the approximately 120-kDa NLRP1 cleavage product in infected cells (fig. S6A). The small molecule pan-HRV-3Cpro inhibitor, rupintrivir completely abrogated NLRP1 cleavage (fig. S6A). Using HeLa-Ohio cells expressing ASC-GFP, we tested whether HRV16 infection could induce NLRP1 inflammasome complex assembly. Although HeLa-Ohio cells demonstrated less NLRP1 inflammasome activation than keratinocytes or HEK293T cells, HRV16 and talabostat caused significant ASC-GFP oligomerization (fig. S6B). These effects were only observed in HeLa-Ohio-ASC-GFP cells expressing wild-type NLRP1, but not in cells expressing the cleavage-resistant NLRP1<sup>Q130A</sup> mutant (fig. S6B). Thus, live HRV16 infection causes 3Cpro-dependent NLRP1 cleavage and activates the reconstituted human NLRP1 inflammasome.

HRV is one of the most common human viral pathogens responsible for respiratory tract infections. Human airway epithelial cells (AECs) are known to endogenously express multiple dsRNA sensors such as TLR3, MDA5, and RIG-I, which all participate in antiviral defense. However, the repertoire of endogenous inflammasome components expressed in these cells has not been fully characterized. By examining published RNA-seq datasets, we found that primary AECs (44–48) express a very restricted repertoire of NLR sensors (fig. S6C). Known inflammasome sensors such as *MEFV*, *NLRP3*, *NLRC5*, and *AIM2* were expressed at low levels or were undetectable (fig. S6E). We confirmed that *NLRP3* was not expressed in primary human bronchial epithelial cells (TPM < 1) (Fig. 4A and data S2). *NLRP3* was also undetectable in primary human nasal epithelium by RNA in situ staining (fig. S6D). The lack of NLRP3 inflammasome in primary normal human bronchial epithelial cells (NHBEs) was recently confirmed by an independent study (49). Although we cannot rule out that *NLRP3* mRNA may be transcriptionally induced under specific conditions, these results demonstrate that NLRP1, but not NLRP3, is the predominant inflammasome sensor constitutively expressed in human AECs. In addition to *NLRP1*, all AECs endogenously expressed *PYCARD* (ASC), *CASP1*, and the IL-1-family cytokines, *IL1B* and *IL18* (Fig. 4A, data S2, and fig. S6C), suggesting that human AECs are capable of NLRP1-mediated inflammasome activation.

We next profiled the endogenous cytokine and chemokine response of NHBEs to live HRV16 infection (Fig. 4B). IL-18, whose secretion strictly depends on inflammasome activation, was the most highly induced cytokine in both HRV16infected and talabostat-treated NHBEs (Fig. 4, C and D). IL-1ß was also significantly secreted from HRV16-infected and talabostat-treated NHBEs, but its levels were lower and more variable than those of IL-18 (Fig. 4, E and F). This variability is likely due to transcriptional regulation of IL1B mRNA. Similar results were obtained with primary nasal epithelial cells (fig. S6E). In agreement with the lack of endogenous NLRP3 in NHBEs, neither IL-1 $\beta$  nor IL-18 secretion was affected by the NLRP3 inhibitor MCC950 after HRV16 infection or talabostat treatment. By contrast, the 3Cpro inhibitor rupintrivir completely abrogated HRV16-induced IL-18 and IL-18 secretion (Fig. 4, D and E). HRV16-infected NHBEs demonstrated cardinal features of inflammasome activation, including: (i) proteolytic processing of IL-18 and IL-1 $\beta$  into their p17 mature forms (Fig. 4F); (ii) endogenous ASC oligomerization (Fig. 4F); (iii) caspase-1 activation (fig. S7A); (iv) release of intact LDH activity (fig. S7B); and (v) characteristic membrane "ballooning" (fig. S7C). We then performed apical HRV16 infection of 3D air-lifted human bronchial epithelium cultures and confirmed that this serotype induced the secretion of IL-18 (Fig. 5, A and B). Histologically, both HRV16 infection and talabostat treatment induced mucus cell metaplasia (MCM) by alcian blue-periodic acid-Schiff (AB-PAS) staining and gasdermin D (GSDMD) cleavage (Fig. 5C). Thus, HRV16 infection activates the NLRP1, but not NLRP3 inflammasome in primary NHBEs.

We next sought to further validate NLRP1 as the endogenous inflammasome sensor for HRV. CRISPR-Cas9-mediated deletion of *NLRP1*, *ASC*, or pro-caspase-1 in primary NHBEs completely eliminated IL-18 cleavage caused by HRV16 infection or talabostat treatment (Fig. 6A and fig. S7, D and E). Furthermore, HRV16-infected *NLRP1* knockout NHBEs were unable to cleave IL-18 into its mature p17 form (Fig. 6B) or assemble ASC into high-molecular weight oligomers (Fig. 6B). This was not caused by a general defect in viral biogenesis, as the maturation of capsid protein VP2, which itself is dependent on 3Cpro activity (*50*), was unaffected in  $NLRP1^{-/-}$  or  $ASC^{-/-}$  cells (Fig. 6B). These results provide further support that NLRP1 is the primary inflammasome sensor for HRV infection in human NHBEs. HRV-triggered inflammasome activation likely occurs in parallel with other immune sensing pathways, as  $NLRP1^{-/-}$ ,  $ASC^{-/-}$ , and  $CASP1^{-/-}$  NHBEs were still capable of undergoing cell death and producing other cytokines, such as IL-8 (fig. S7, F and G) upon HRV16 infection.

To confirm that HRV-induced NLRP1 activation requires the cleavage between residues Q130 and G131, we infected *NLRP1<sup>-/-</sup>* NHBEs rescued with either FLAG-tagged wild-type NLRP1 or NLRP1<sup>Q130A</sup>. As observed previously with keratinocvtes (Fig. 2, D to G), both wild-type NLRP1 and NLRP1<sup>Q130A</sup> rescued talabostat-dependent IL-18 secretion in NLRP1-/-NHBEs (Fig. 6C and fig. S8B). However, only wild-type NLRP1, and not the uncleavable mutant Q130A, rescued HRV16-induced IL-18 secretion (Fig. 6C), despite similar levels of VP2 maturation. In addition, HRV16 induced strong degradation of the NLRP1 N-terminal fragment only in wildtype NLRP1 rescued NHBEs, but not NLRP1<sup>Q130A</sup>-expressing cells (Fig. 6D). Furthermore, MLN4924 and bortezomib completely blocked HRV16-dependent IL-1ß and IL-18 secretion in NHBEs (Fig. 6E and fig. S8A). MLN4924 pretreatment also stabilized the NLRP1 N-terminal cleavage fragments in HRV16-infected NHBEs expressing wild-type NLRP1 (Fig. 6G) without affecting the accumulation (Fig. 6F) of the activating C-terminal fragment of NLRP1 (Fig. 6G). Thus, both cullin-ZERI/ZYG11B and the proteasome are necessary for HRV-triggered NLRP1 inflammasome activation in NHBEs.

In summary, we report that enteroviral 3C proteases, such as HRV-3Cpro activate the human inflammasome sensor NLRP1 via direct cleavage at a single site between amino acids Q130 and G131. This finding not only provides a unified mechanism for proteolysis-mediated activation of NLRP1 inflammasome in humans and rodents (16-18), but reveals an unexpected role for the recently described N-terminal glycine degron pathway in human innate immunity. Mechanistically, 3Cpro-cleavage leaves a glycine residue (p. G131) on the NLRP1 fragment, which is recognized by the cullin<sup>ZER1/ZYG11B</sup> N-glycine degron machinery and is subsequently degraded by the proteasome (Fig. 3B). This constitutes a key step in unleashing the NLRP1 FIIND<sup>UPA</sup>-CARD fragment to fully assemble the inflammasome complex consisting of ASC and caspase-1. It is noteworthy that a single cleavage site mutation (Q130A) can completely block 3Cpros from activating the NLRP1 inflammasome without affecting talabostat-triggered NLRP1 activation. This suggests that NLRP1 can undergo multiple modes of activation.

The identification of enteroviral 3Cpros as a pathogen-derived trigger for human NLRP1 also sheds light on the evolutionary trajectory of NLRP1. The 3Cpro cleavage site arose in the common ancestor for simians and is absent in prosimians such as tarsiers and lemurs (fig. S9). It is conceivable that the more recently evolved, 3Cpro-responsive *NLRP1* allele has provided a selective survival advantage during the evolution of simian primates including humans, presumably to sense and mount an appropriate immune response against certain simian-tropic enteroviral pathogens. Notwithstanding this notion, our work does not preclude the possibility that human NLRP1 can detect other pathogen- or danger-derived signals besides 3Cpros.

Our findings establish human NLRP1 as part of the repertoire of viral sensors in the airway epithelium and challenges the widely held notion that viral proteases largely serve to disable host immune sensing. As the inflammasome pathway does not require de novo protein synthesis, it is particularly well suited as a fail-safe, second-line defense. The inflammasome can commit the infected cells to pyroptosis and release IL-18 even after host transcription and translation have been shut off by viral virulence factors (51). In keeping with this notion, NLRP1 knockout did not lead to an appreciable increase in infectious viruses 48 hours after inoculation (fig. S8C). Taken together, we propose that the NLRP1 inflammasome plays an important role in antiviral immunity and inflammation in the human airway epithelium. Respiratory viral infections, including HRVs, are well known risk factors for exacerbations for asthma and chronic obstructive pulmonary disease (COPD) (52). Therefore, the NLRP1 inflammasome pathway might serve as a potential therapeutic target to treat these diseases and their complications.

# Materials and methods *Cell culture and chemicals*

293Ts (ATCC #CRL-3216), HeLa-Ohio (ECACC General Cell Collection #84121901), and normal bronchial epithelial cells (NHBE, Lonza #CC-2541) were cultured according to the suppliers' protocols with modifications (53) Immortalized human keratinocytes (N/TERT-1, or N/TERT herein) were a kind gift from H. Reinwald (MTA) (54). Primary nasal epithelial cells were obtained from nasal epithelial biopsies obtained from adult patients scheduled for rhinoplasty and obtained with informed consent at NUH Fresh nasal epithelial tissues were used to derive nasal cells as previously described (55). All cell lines underwent routine Mycoplasma testing with Lonza MycoAlert (Lonza #LT07-118). The following drugs and chemicals were used as part of this study: MCC950 (MCE, #HY-12815), Bortezomib and MLN4924 were a kind gift from Sir D. Lane (p53, A\*STAR, Singapore). Rupintrivir (Sigma, #PZ0315), talabostat (MCE, #HY-13233), MG132 (MCE, #HY13259), A83-01 (MCE, #HY-10432A), Y-27632 (MCE, #HY-10583), isoprenaline (MCE, #HY-B0468).

# Plasmid transfection and stable cell line generation using lentiviruses

293T-ASC-GFP, N/TERT-ASC-GFP, N/TERT *NLRPI*-KO cells were described previously (*19*). All transient expression plasmids were cloned into the pCS2+ vector using standard restriction cloning using ClaI and XhoI flanking the open reading frames. P Site-directed mutagenesis was carried out with QuickChangeXL II (Agilent #200522). Constitutive lentiviral expression was performed using pCDH vectors (SystemBio). Doxycycline-inducible Tet-ON lentiviral constructs were based on the pTRIPZ backbone (Thermo Fisher).

## Antibodies, staining reagents, and cytokine analysis

The following antibodies were used in this study: c-Myc (Santa Cruz Biotechnology #sc-40), HA tag (Santa Cruz Biotechnology, #sc-805), GAPDH (Santa Cruz Biotechnology, #sc-47724), ASC (Adipogen, #AL-177), CASP1 (Santa Cruz Biotechnology, #sc-622), IL1B (R&D systems, #AF-201), FLAG (SigmaAldrich, #F3165), GFP (Abcam, #ab290), NLRP1 (R&D systems, #AF6788), IL18 (Abcam ab207324), cleaved GSDMD-NT (Asp275) (Cell Signaling Technology, #36425) and HRV-VP2 (QED Bioscience, #18758). HRV16-3Cpro was detected by rabbit serum, a kind gift from A. Palmenberg and J. Gern (University of Wisconsin). All HRP conjugated secondary antibodies were purchased from Jackson Immunoresearch (goat anti-mouse IgG: 115-035-166; goat anti-rabbit IgG: 111-035-144, donkey anti-goat IgG: 705-005-147). The protocol for blue native PAGE and DSS cross-linking were performed as previously described (7). For AB-PAS staining and immunohistochemistry, 5-µm formalin-fixed paraffin-embedded 3D bronchial epithelial sections were deparaffinized in xylenes and rehydrated through a series of alcohol before being hydrated in water. Alcian blue and periodic acid-Schiff (AB-PAS) staining was then carried out using a staining kit (Abacm, ab245876) according to manufacturer's instructions. For immunohistochemistry staining to visualize cleaved GSDMD-N terminal fragment, a previously published protocol was used (56). For trypan blue staining, cells were incubated with trypan blue stain (Thermo Scientific #15250061) and the percentage of positively stained cells versus total cell count calculated. A total of three cell counts were performed per condition. For RNAscope, primary human nasal epithelium sections (normal) were obtained through The National University of Singapore (NUS/NUH, Singapore) with informed consent. Visualization of NLRP1 and NLRP3 RNA was performed according to manufacturer's instructions and recommendations (RNAscope 2.5 HD-Red, ACDbio).

Cytokine and chemokine measurements were carried out with human IL-1 $\beta$  ELISA kit (BD, #557953), human IL-18 ELISA kit (MBL Bioscience, #7620), and Immune Monitoring 65-Plex Human ProcartaPlex Panel (Thermo Fisher EPX650-10065-901) according to the manufacturer's protocol. All

primary antibodies were used at 250 ng/ml.

## Immunoblotting

For whole cell lysates, cells were resuspended in TBS 1% NP-40 with protease inhibitors (Thermo Scientific #78430). Protein concentration was determined using the Bradford assay (Thermo Scientific #23200) and 20  $\mu$ g of protein loaded unless stated otherwise. For analysis of IL-1 $\beta$  and IL-18 cleavage in the media by immunoblotting, samples were 10×-concentrated using filtered centrifugation (Merck, Amicon Ultra, #UFC5003BIK). Protein samples were run using SDS-PAGE system (Bio-Rad), immunoblotted, and then visualized using x-ray film detector or ChemiDoc Imaging system (Bio-Rad).

# Microscopy

Images of ASC-GFP specs were acquired in three random fields in DAPI (358 nm/461 nm) and GFP (469 nm/525 nm) channels using EVOS microscope (FL Auto M5000, Thermo Fisher #AMF5000) with a EVOS 20× Objective (achromat, LWD, phase-contrast, 0.40NA/6.92WD, Thermo Fisher Scientific #AMEP4934) and built-in camera (Sony IMX265 monochrome CMOS) according to manufacturer's protocol (EVOS M5000 Manual, Thermo Fisher Scientific Publication Number MAN0017563). Counting of the number of ASC-GFP specks was performed using ImageJ Fiji "watershed" and "find maxima" built-in functions. Briefly, all DAPI images were subjected to thresholding to distinguish background from foreground to give a binary image. The watershed algorithm was used to separate touching objects, before segmented objects could be analyzed and counted to give a total nuclei count. The number of GFP-ASC specks was calculated by "find maxima" function and noise tolerance set accordingly to count the total number of specks per image. The number of specks was then calculated as a percentage of total nuclei in three representative fields of view. Brightfield images of stained sections were taken at 40× magnification using a widefield microscope (Zeiss AxioImager Z.2, Zeiss) with a 40× oil immersion objective (EC Plan Neofluar Antiflex, Zeiss). Images were acquired with the built-in camera (Axio Cam 506 mono 1.2 Mbps, Zeiss).

# HRV16 virus propagation

HRV used in the study was HRV-A16 (strain 11757; ATCC VR-283, Manassas, VA, USA), and was propagated in HeLa cell line (HeLa Ohio, ECACC 84121901, Porton Down, Salisbury, Wiltshire, UK). HeLa cells were grown in Eagle's Minimum Essential Medium (EMEM) ATCC 30-2003, supplemented with 10% fetal bovine serum (FBS) (BioWest, Kansas City, MO, USA), 2% HEPES and 1% Antibiotic-Antimycotic (Anti-Anti) (Gibco) and incubated at 37°C humidified incubator with 5% CO<sub>2</sub>. To propagate HRV16, HeLa cells were first seeded to achieve 80%-90% confluence in 24-well plates overnight. Cells were rinsed with 1X Dulbecco's phosphatebuffered saline (DPBS) and infected with HRV16 before addition of EMEM with 2% FBS, 2% HEPES, and 1% Anti-Anti. Infected HeLa cells were incubated at 33°C for 2-3 days. Viruses were harvested from the supernatants of infected HeLa cells when about 80% cytopathic effects (CPE) was observed. HRV stocks were centrifuged at 1500g for 10 min at 4°C to remove cellular debris, and aliquoted into cryovials for storage at -80°C.

## Inoculation of human rhinovirus

HRV was diluted using the respective cell culture medium and inoculated at multiplicity of infection (MOI) of 5.0 (NHBE) and 1.0 (HeLa), respectively. Infected cells were incubated at 33°C for 1 hour. Conditioned non-infected cell culture medium from viral propagation was added as an uninfected control. The HRV-infected and control cells were then incubated at 33°C for up to 48 hours post-infection (hpi). Cell culture supernatant and cell lysate were collected to perform relevant assays between 24 and 72 hpi.

#### Viral quantification using rhinovirus plaque assay

HeLa cells (at 85% to 95% confluence) in 24-well plates were incubated with 100  $\mu l$  of serial dilutions from  $10^{\text{--1}}$  to  $10^{\text{--6}}$  of virus-containing conditioned media at 33°C for 1 hour. The plates were rocked every 15 min to ensure equal distribution of virus. The inoculum was removed and replaced with 1 ml of Avicel (FMC Biopolymer) overlay to each well and incubated at 33°C for 65-72 hours. The overlay components were optimized to obtain HRV plaques suitable for counting. Avicel powder was added into double-strength MEM to formulate 1.2% Avicel solution, and with a final concentration comprising 3% FBS, 2% HEPES, 1.5% NaHCO<sub>3</sub>, 3% MgCl<sub>2</sub> and 1% Anti-Anti. Avicel overlay was removed after the incubation period, and cells were fixed with 20% formalin in PBS for 1 hour. Formalin was removed, and cells were washed with PBS. The fixed cells were stained with 1% crystal violet for 15 min and washed. The plaque-forming units (PFU) were calculated as follows: Number of plaques × dilution factor = number of PFU per 100 µl, which is then expressed as PFU/ml.

# HRV16 infection of 3D reconstructed human bronchial epithelium

3D culture of bronchial epithelium was purchased from Mattek (AIR-1484 AIR-100 EpiAirway, 3D Respiratory Epithelial Human MicroTissues) and cultured using the Extended Culture protocol as advised by the supplier.

# CRISPR-Cas9 knockout

CRISPR-Cas9 editing was performed in 293T cells was performed according to the method reported by the Doyon group (57), except that guide RNAs (sgRNAs) were cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene 62988). Single clones of ouabain resistant cells were selected for ZYG11B/ZER1 double knockout. Clones 1-6 were transfected with ZYG11B sg5 and 6. Clones 7-12 were transfected with ZYG11B sg7 and 8. All clones received 3 guides for ZER1, sg5, 6, 8. 293T UBR2 KOs were cultured as polyclonal pools following ouabain selection. N/TERT and NHBE KOs were performed using LentiCRISPR-V2 (Addgene 52961) and stable lentiviral transduction. The sgRNAs used are listed in Table 1. Knockout efficiency was tested with immunoblot or Sanger sequencing of the targeted genomic DNA locus 7-10 days after puromycin selection.

# **RNA-seq of NHBEs**

Library preparation, quality control and high-throughput sequencing were provided by Macrogen, Singapore. Total RNA isolated from NHBEs was processed using Library Kit TruSeq Stranded mRNA LT Sample Prep (Illumina) according the manufacturer's protocol specified in Library Protocol TruSeq Stranded mRNA Sample Preparation Guide, Part # 15031047. Sequencing was carried out with NovaSeq 6000 with NovaSeq 6000 S4 Reagent Kit and Sequencing Protocol NovaSeq 6000 System User Guide Document # 100000019358 v02 (Illumina). Trimmed reads are mapped to reference genome with HISAT2. After the read mapping, Stringtie was used for transcript assembly. Expression profile was calculated for each sample and transcript/gene as read count and FPKM (fragment per kilobase of transcript per million mapped reads).

# Analysis of published RNA-seq datasets

RNA-seq data for human airway epithelial cells was extracted from the following published datasets: Nasal #1-GSE107898; Nasal#2- GSE55458; Bronchial#1-GSE107971; Bronchial#2-GSE107897; Alveolar#1: GSE61220. In datasets where multiple treatment conditions were reported, only the basal/mock conditions were selected. RPM values Nasal#2- GSE55458; Bronchial#1-GSE107971 were logged for heatmap generation. Color mapping was performed using the "Double gradient" method in Graphpad Prism 8.

# Caspase-1 GLO and LDH release

Caspase-1 GLO (Promega, #G9951) and LDH release assay (Promega CytoTox96, #G1780) were both carried out according to protocols provided by the manufacturers.

## Statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad). The methods for analysis were included in the figure legends. Error bars show mean values with SEM.

#### **REFERENCES AND NOTES**

- J. D. G. Jones, R. E. Vance, J. L. Dangl, Intracellular innate immune surveillance devices in plants and animals. *Science* **354**, aaf6395 (2016). <u>doi:10.1126/science.aaf6395 Medline</u>
- M. H. Shaw, T. Reimer, Y.-G. Kim, G. Nuñez, NOD-like receptors (NLRs): Bona fide intracellular microbial sensors. *Curr. Opin. Immunol.* 20, 377–382 (2008). doi:10.1016/j.coi.2008.06.001 Medline
- T.-D. Kanneganti, M. Lamkanfi, G. Núñez, Intracellular NOD-like receptors in host defense and disease. *Immunity* 27, 549–559 (2007). doi:10.1016/j.immuni.2007.10.002 Medline
- 4. E. Latz, The inflammasomes: Mechanisms of activation and function. *Curr. Opin. Immunol.* **22**, 28–33 (2010). doi:10.1016/j.coi.2009.12.004 Medline
- J. Sand, E. Haertel, T. Biedermann, E. Contassot, E. Reichmann, L. E. French, S. Werner, H.-D. Beer, Expression of inflammasome proteins and inflammasome activation occurs in human, but not in murine keratinocytes. *Cell Death Dis.* 9, 24 (2018). doi:10.1038/s41419-017-0009-4 Medline
- S. Grandemange, E. Sanchez, P. Louis-Plence, F. Tran Mau-Them, D. Bessis, C. Coubes, E. Frouin, M. Seyger, M. Girard, J. Puechberty, V. Costes, M. Rodière, A. Carbasse, E. Jeziorski, P. Portales, G. Sarrabay, M. Mondain, C. Jorgensen, F. Apparailly, E. Hoppenreijs, I. Touitou, D. Geneviève, A new autoinflammatory and autoimmune syndrome associated with NLRP1 mutations: NAIAD (*NLRP1*-associated autoinflammation with arthritis and dyskeratosis). *Ann. Rheum. Dis.* **76**, 1191–1198 (2017). doi:10.1136/annrheumdis-2016-210021 Medline
- F. L. Zhong, O. Mamaï, L. Sborgi, L. Boussofara, R. Hopkins, K. Robinson, I. Szeverényi, T. Takeichi, R. Balaji, A. Lau, H. Tye, K. Roy, C. Bonnard, P. J. Ahl, L. A. Jones, P. J. Baker, L. Lacina, A. Otsuka, P. R. Fournie, F. Malecaze, E. B. Lane, M. Akiyama, K. Kabashima, J. E. Connolly, S. L. Masters, V. J. Soler, S. S. Omar, J. A. McGrath, R. Nedelcu, M. Gribaa, M. Denguezli, A. Saad, S. Hiller, B. Reversade, Germline NLRP1 mutations cause skin inflammatory and cancer susceptibility syndromes via inflammasome activation. *Cell* 167, 187–202.e17 (2016). doi:10.1016/j.cell.2016.09.001 Medline
- O. Mamaï, L. Boussofara, M. Denguezli, N. Escande-Beillard, W. Kraeim, B. Merriman, I. Ben Charfeddine, G. Stevanin, S. Bouraoui, A. Amara, A. Mili, R. Nouira, D. H'mida, B. Sriha, M. Gribaa, A. Saad, B. Reversade, Multiple self-healing palmoplantar carcinoma: A familial predisposition to skin cancer with primary palmoplantar and conjunctival lesions. *J. Invest. Dermatol.* **135**, 304–308 (2015). doi:10.1038/jid.2014.311 Medline
- J. Sui, H. Li, Y. Fang, Y. Liu, M. Li, B. Zhong, F. Yang, Q. Zou, Y. Wu, NLRP1 gene polymorphism influences gene transcription and is a risk factor for rheumatoid arthritis in han chinese. *Arthritis Rheum.* 64, 647–654 (2012). doi:10.1002/art.33370 Medline
- C. B. Levandowski, C. M. Mailloux, T. M. Ferrara, K. Gowan, S. Ben, Y. Jin, K. K. McFann, P. J. Holland, P. R. Fain, C. A. Dinarello, R. A. Spritz, NLRP1 haplotypes associated with vitiligo and autoimmunity increase interleukin-1β processing via the NLRP1 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 2952–2956 (2013). doi:10.1073/pnas.1222808110 Medline
- E. D. Boyden, W. F. Dietrich, *Nalp1b* controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat. Genet.* 38, 240–244 (2006). doi:10.1038/ng1724 <u>Medline</u>
- Z. L. Newman, M. P. Printz, S. Liu, D. Crown, L. Breen, S. Miller-Randolph, P. Flodman, S. H. Leppla, M. Moayeri, Susceptibility to anthrax lethal toxin-induced rat death is controlled by a single chromosome 10 locus that includes *rNlrp1*. *PLOS Pathog.* 6, e1000906 (2010). doi:10.1371/journal.ppat.1000906 Medline
- K. A. Hellmich, J. L. Levinsohn, R. Fattah, Z. L. Newman, N. Maier, I. Sastalla, S. Liu, S. H. Leppla, M. Moayeri, Anthrax lethal factor cleaves mouse NIrp1b in both toxinsensitive and toxin-resistant macrophages. *PLOS ONE* 7, e49741 (2012). doi:10.1371/journal.pone.0049741 Medline
- J. L. Levinsohn, Z. L. Newman, K. A. Hellmich, R. Fattah, M. A. Getz, S. Liu, I. Sastalla, S. H. Leppla, M. Moayeri, Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLOS Pathog.* 8, e1002638 (2012). doi:10.1371/journal.ppat.1002638 Medline
- J. Chavarría-Smith, R. E. Vance, Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLOS Pathog.* 9, e1003452 (2013). doi:10.1371/journal.ppat.1003452 Medline
- 16. A. J. Chui, M. C. Okondo, S. D. Rao, K. Gai, A. R. Griswold, D. C. Johnson, D. P. Ball,

C. Y. Taabazuing, E. L. Orth, B. A. Vittimberga, D. A. Bachovchin, N-terminal degradation activates the NLRP1B inflammasome. *Science* **364**, 82–85 (2019). doi:10.1126/science.aau1208 Medline

- A. Sandstrom, P. S. Mitchell, L. Goers, E. W. Mu, C. F. Lesser, R. E. Vance, Functional degradation: A mechanism of NLRP1 inflammasome activation by diverse pathogen enzymes. *Science* **364**, eaau1330 (2019). <u>doi:10.1126/science.aau1330</u> <u>Medline</u>
- H. Xu, J. Shi, H. Gao, Y. Liu, Z. Yang, F. Shao, N. Dong, The N-end rule ubiquitin ligase UBR2 mediates NLRP1B inflammasome activation by anthrax lethal toxin. *EMBO J.* 38, e101996 (2019). <u>doi:10.15252/embj.2019101996 Medline</u>
- F. L. Zhong, K. Robinson, D. E. T. Teo, K.-Y. Tan, C. Lim, C. R. Harapas, C.-H. Yu, W. H. Xie, R. M. Sobota, V. B. Au, R. Hopkins, A. D'Osualdo, J. C. Reed, J. E. Connolly, S. L. Masters, B. Reversade, Human DPP9 represses NLRP1 inflammasome and protects against autoinflammatory diseases via both peptidase activity and FIIND domain binding. *J. Biol. Chem.* **293**, 18864–18878 (2018). doi:10.1074/jbc.RA118.004350 Medline
- M. C. Okondo, S. D. Rao, C. Y. Taabazuing, A. J. Chui, S. E. Poplawski, D. C. Johnson, D. A. Bachovchin, Inhibition of Dpp8/9 Activates the NIrp1b Inflammasome. *Cell Chem. Biol.* 25, 262–267.e5 (2018). doi:10.1016/j.chembiol.2017.12.013 Medline
- N. M. de Vasconcelos, G. Vliegen, A. Gonçalves, E. De Hert, R. Martín-Pérez, N. Van Opdenbosch, A. Jallapally, R. Geiss-Friedlander, A.-M. Lambeir, K. Augustyns, P. Van Der Veken, I. De Meester, M. Lamkanfi, DPP8/DPP9 inhibition elicits canonical NIrp1b inflammasome hallmarks in murine macrophages. *Life Sci. Alliance* 2, e201900313 (2019). doi:10.26508/lsa.201900313 Medline
- K. M. Cirelli, G. Gorfu, M. A. Hassan, M. Printz, D. Crown, S. H. Leppla, M. E. Grigg, J. P. J. Saeij, M. Moayeri, Inflammasome sensor NLRP1 controls rat macrophage susceptibility to *Toxoplasma gondii*. *PLOS Pathog*. **10**, e1003927 (2014). doi:10.1371/journal.ppat.1003927 Medline
- S. E. Ewald, J. Chavarria-Smith, J. C. Boothroyd, NLRP1 is an inflammasome sensor for *Toxoplasma gondii*. Infect. Immun. 82, 460–468 (2014). doi:10.1128/IAI.01170-13 Medline
- 24. R. Zell, *Picornaviridae*—the ever-growing virus family. *Arch. Virol.* **163**, 299–317 (2018). doi:10.1007/s00705-017-3614-8 Medline
- S. C. Piper, J. Ferguson, L. Kay, L. C. Parker, I. Sabroe, M. A. Sleeman, E. Briend, D. K. Finch, The role of interleukin-1 and interleukin-18 in pro-inflammatory and antiviral responses to rhinovirus in primary bronchial epithelial cells. *PLOS ONE* 8, e63365 (2013). doi:10.1371/journal.pone.0063365 Medline
- A. C. Palmenberg, Proteolytic processing of picornaviral polyprotein. Annu. Rev. Microbiol. 44, 603–623 (1990). doi:10.1146/annurev.mi.44.100190.003131 Medline
- E. J. Walker, P. Younessi, A. J. Fulcher, R. McCuaig, B. J. Thomas, P. G. Bardin, D. A. Jans, R. Ghildyal, Rhinovirus 3C protease facilitates specific nucleoporin cleavage and mislocalisation of nuclear proteins in infected host cells. *PLOS ONE* 8, e71316 (2013). doi:10.1371/journal.pone.0071316 Medline
- S. N. Croft, E. J. Walker, R. Ghildyal, Human Rhinovirus 3C protease cleaves RIPK1, concurrent with caspase 8 activation. *Sci. Rep.* 8, 1569 (2018). doi:10.1038/s41598-018-19839-4 Medline
- S. de Breyne, J. M. Bonderoff, K. M. Chumakov, R. E. Lloyd, C. U. T. Hellen, Cleavage of eukaryotic initiation factor eIF5B by enterovirus 3C proteases. *Virology* 378, 118–122 (2008). doi:10.1016/j.virol.2008.05.019 Medline
- A. Mukherjee, S. A. Morosky, E. Delorme-Axford, N. Dybdahl-Sissoko, M. S. Oberste, T. Wang, C. B. Coyne, The coxsackievirus B 3C<sup>pro</sup> protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. *PLOS Pathog.* 7, e1001311 (2011). <u>doi:10.1371/journal.ppat.1001311 Medline</u>
- E. J. Buenz, C. L. Howe, Picornaviruses and cell death. Trends Microbiol. 14, 28–36 (2006). doi:10.1016/j.tim.2005.11.003 Medline
- S. N. Croft, E. J. Walker, R. Ghildyal, Picornaviruses and apoptosis: Subversion of cell death. mBio 8, e01009-17 (2017). doi:10.1128/mBio.01009-17 Medline
- 33. D. A. Matthews, W. W. Smith, R. A. Ferre, B. Condon, G. Budahazi, W. Slsson, J. E. Villafranca, C. A. Janson, H. E. McElroy, C. L. Gribskov, S. Worland, Structure of human rhinovirus 3C protease reveals a trypsin-like polypeptide fold, RNA-binding site, and means for cleaving precursor polyprotein. *Cell* **77**, 761–771 (1994). doi:10.1016/0092-8674(94)90059-0 Medline
- J. Chavarría-Smith, P. S. Mitchell, A. M. Ho, M. D. Daugherty, R. E. Vance, Functional and evolutionary analyses identify proteolysis as a general mechanism

for NLRP1 inflammasome activation. *PLOS Pathog.* **12**, e1006052 (2016). doi:10.1371/journal.ppat.1006052 Medline.

- J. N. Finger, J. D. Lich, L. C. Dare, M. N. Cook, K. K. Brown, C. Duraiswami, J. Bertin, P. J. Gough, Autolytic proteolysis within the function to find domain (FIIND) is required for NLRP1 inflammasome activity. *J. Biol. Chem.* 287, 25030–25037 (2012). doi:10.1074/jbc.M112.378323 Medline
- 36. A. D'Osualdo, C. X. Weichenberger, R. N. Wagner, A. Godzik, J. Wooley, J. C. Reed, CARD8 and NLRP1 undergo autoproteolytic processing through a ZU5-like domain. PLOS ONE 6, e27396 (2011). doi:10.1371/journal.pone.0027396 Medline
- R. B. Register, C. R. Uncapher, A. M. Naylor, D. W. Lineberger, R. J. Colonno, Human-murine chimeras of ICAM-1 identify amino acid residues critical for rhinovirus and antibody binding. *J. Virol.* 65, 6589–6596 (1991). doi:10.1128/JVI.65.12.6589-6596.1991 Medline
- 38. S. B. Drutman, F. Haerynck, F. L. Zhong, D. Hum, N. J. Hernandez, S. Belkaya, F. Rapaport, S. J. de Jong, D. Creytens, S. J. Tavernier, K. Bonte, S. De Schepper, J. van der Werff Ten Bosch, L. Lorenzo-Diaz, A. Wullaert, X. Bossuyt, G. Orth, V. R. Bonagura, V. Béziat, L. Abel, E. Jouanguy, B. Reversade, J.-L. Casanova, Homozygous *NLRP1* gain-of-function mutation in siblings with a syndromic form of recurrent respiratory papillomatosis. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 19055–19063 (2019). doi:10.1073/pnas.1906184116 Medline
- T. Herlin, S. E. Jørgensen, C. Høst, P. S. Mitchell, M. H. Christensen, M. Laustsen, D. A. Larsen, F. I. Schmidt, M. Christiansen, T. H. Mogensen, Autoinflammatory disease with corneal and mucosal dyskeratosis caused by a novel NLRP1 variant. *Rheumatology* 59, 2334–2339 (2020). <u>doi:10.1093/rheumatology/kez612</u> <u>Medline</u>
- H. Xu, J. Shi, H. Gao, Y. Liu, Z. Yang, F. Shao, N. Dong, The N-end rule ubiquitin ligase UBR2 mediates NLRP1B inflammasome activation by anthrax lethal toxin. *EMBO J.* 38, e101996 (2019). <u>doi:10.15252/embj.2019101996 Medline</u>
- K. E. Wickliffe, S. H. Leppla, M. Moayeri, Killing of macrophages by anthrax lethal toxin: Involvement of the N-end rule pathway. *Cell. Microbiol.* **10**, 1352–1362 (2008). doi:10.1111/j.1462-5822.2008.01131.x Medline
- A. Varshavsky, N-degron and C-degron pathways of protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 358–366 (2019). <u>doi:10.1073/pnas.1816596116</u> <u>Medline</u>
- R. T. Timms, Z. Zhang, D. Y. Rhee, J. W. Harper, I. Koren, S. J. Elledge, A glycinespecific N-degron pathway mediates the quality control of protein Nmyristoylation. Science 365, eaaw4912 (2019). doi:10.1126/science.aaw4912 Medline
- M. L. Landry, E. F. Foxman, Antiviral response in the nasopharynx identifies patients with respiratory virus infection. *J. Infect. Dis.* **217**, 897–905 (2018). doi:10.1093/infdis/jix648 Medline
- 45. V. T. Mihaylova, Y. Kong, O. Fedorova, L. Sharma, C. S. Dela Cruz, A. M. Pyle, A. Iwasaki, E. F. Foxman, Regional differences in airway epithelial cells reveal tradeoff between defense against oxidative stress and defense against rhinovirus. *Cell Rep.* 24, 3000–3007.e3 (2018). doi:10.1016/j.celrep.2018.08.033 Medline
- 46. J. G. Clark, K.-H. Kim, R. S. Basom, S. A. Gharib, Plasticity of airway epithelial cell transcriptome in response to flagellin. *PLOS ONE* **10**, e0115486 (2015). doi:10.1371/journal.pone.0115486 Medline
- U. Hedström, O. Hallgren, L. Öberg, A. DeMicco, O. Vaarala, G. Westergren-Thorsson, X. Zhou, Bronchial extracellular matrix from COPD patients induces altered gene expression in repopulated primary human bronchial epithelial cells. *Sci. Rep.* 8, 3502 (2018). doi:10.1038/s41598-018-21727-w Medline
- 48. B. Tian, X. Li, M. Kalita, S. G. Widen, J. Yang, S. K. Bhavnani, B. Dang, A. Kudlicki, M. Sinha, F. Kong, T. G. Wood, B. A. Luxon, A. R. Brasier, Analysis of the TGFβinduced program in primary airway epithelial cells shows essential role of NFxB/RelA signaling network in type II epithelial mesenchymal transition. *BMC Genomics* 16, 529 (2015). doi:10.1186/s12864-015-1707-x Medline
- S. Lee, A. Ishitsuka, M. Noguchi, M. Hirohama, Y. Fujiyasu, P. P. Petric, M. Schwemmle, P. Staeheli, K. Nagata, A. Kawaguchi, Influenza restriction factor MxA functions as inflammasome sensor in the respiratory epithelium. *Sci. Immunol.* 4, eaau4643 (2019). doi:10.1126/sciimmunol.aau4643 Medline
- M. G. Cordingley, P. L. Callahan, V. V. Sardana, V. M. Garsky, R. J. Colonno, Substrate requirements of human rhinovirus 3C protease for peptide cleavage in vitro. *J. Biol. Chem.* 265, 9062–9065 (1990). <u>Medline</u>
- 51. L. Carrasco, A. E. Smith, Sodium ions and the shut-off of host cell protein synthesis

by picornaviruses. Nature 264, 807–809 (1976). doi:10.1038/264807a0 Medline

- S. E. Jacobs, D. M. Lamson, K. St. George, T. J. Walsh, Human rhinoviruses. *Clin. Microbiol. Rev.* 26, 135–162 (2013). doi:10.1128/CMR.00077-12 Medline
- C. Zhang, H. J. Lee, A. Shrivastava, R. Wang, T. J. McQuiston, S. S. Challberg, B. A. Pollok, T. Wang, Long-term *in vitro* expansion of epithelial stem cells enabled by pharmacological inhibition of PAK1-ROCK-Myosin II and TGF-β signaling. *Cell Rep.* 25, 598–610.e5 (2018). doi:10.1016/j.celrep.2018.09.072 Medline
- 54. M. A. Dickson, W. C. Hahn, Y. Ino, V. Ronfard, J. Y. Wu, R. A. Weinberg, D. N. Louis, F. P. Li, J. G. Rheinwald, Human keratinocytes that express hTERT and also bypass a p16<sup>INK4a</sup>-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell. Biol.* **20**, 1436–1447 (2000). doi:10.1128/MCB.20.4.1436-1447.2000 Medline
- 55. Y. Yan, K. S. Tan, C. Li, T. Tran, S. S. Chao, R. J. Sugrue, L. Shi, V. T. Chow, D.-Y. Wang, Human nasal epithelial cells derived from multiple subjects exhibit differential responses to H3N2 influenza virus infection in vitro. *J. Allergy Clin. Immunol.* **138**, 276–281.e15 (2016). doi:10.1016/j.jaci.2015.11.016 Medline
- 56. K. Wang, Q. Sun, X. Zhong, M. Zeng, H. Zeng, X. Shi, Z. Li, Y. Wang, Q. Zhao, F. Shao, J. Ding, Structural mechanism for GSDMD targeting by autoprocessed caspases in pyroptosis. *Cell* **180**, 941–955.e20 (2020). <u>doi:10.1016/j.cell.2020.02.002</u> <u>Medline</u>
- 57. D. Agudelo, A. Duringer, L. Bozoyan, C. C. Huard, S. Carter, J. Loehr, D. Synodinou, M. Drouin, J. Salsman, G. Dellaire, J. Laganière, Y. Doyon, Marker-free coselection for CRISPR-driven genome editing in human cells. *Nat. Methods* 14, 615–620 (2017). doi:10.1038/nmeth.4265 Medline

#### ACKNOWLEDGMENTS

We thank all members of the Reversade laboratory for support and helpful scientific discussion. We also acknowledge the generous support and advice from L. Dahai (NTU), W. Bin (NTU), R. Sobota (IMCB), and S. Masters (WEHI). Funding: This work is supported by Agency for Science, Technology and Research (GODAFIT Strategic Positioning Fund, Bruno Reversade), National Medical Research Council, Singapore (NMRC/OFYIRG/0046/2017, F.L.Z.), Concern Foundation's Conquer Cancer Now Award (F.L.Z.), and National Research Foundation, Singapore (F.L.Z.). T.K.S. is a recipient of fellowship support from the EAACI Research Fellowship, 2019. B.R. is a fellow of the Branco Weiss Foundation and a recipient of the A\*STAR Investigatorship, the senior NRF investigatorship, and EMBO Young Investigatorship. F.L.Z. is a Nanyang Assistant Professor and National Research Foundation Fellow. Author contributions: F.L.Z. initiated the project. K.S.R. designed and performed most of the experiments and analyzed the data with help from D.E.T.T., T.G.A., K.L., and K.S.T. K.S.R., T.K.S., O.H.H., and L.T.S. performed the viral infection experiments. B.A. performed Luminex assays. C.K.L., J.C.J.H., V.T.K.C., and W.D.Y. supervised virology experiments. F.L.Z. and B.R. jointly conceived of the study, supervised experiments, analyzed the data, and wrote the manuscript. Competing interests: B.R., F.L.Z., and K.S.R. are joint inventors of provisional patent application "Blocking human airway inflammation by pharmacological inhibition of components of the NLRP1 inflammasome and N-glycine degron pathway" (10202002526P, Singapore). Data and materials availability: Immortalized N/TERT keratinocytes are obtained under an MTA from Brigham and Women's Hospital. All other data are available in the manuscript and supplementary materials.

#### SUPPLEMENTARY MATERIALS

science.sciencemag.org/cgi/content/full/science.eaay2002/DC1 Figs. S1 to S9 MDAR Reproducibility Checklist Data S1 and S2

20 June 2019; resubmitted 11 February 2020 Accepted 8 October 2020 Published online 22 October 2020 10.1126/science.aay2002

sgRNA used (Gene #sgRNA number)	Sequence (5' to 3')
NLRP1#1	GATAGCCCGAGTGACATCGG
NLRP1#2	AGCCCGAGTGACATCGGTGG
CASP1#1	ACAGACAAGGGTGCTGAACA
CASP1#2	ATTGACTCCGTTATTCCGAA
CASP1#3	TGACTCCGTTATTCCGAAAG
ASC#2	GCTAACGTGCTGCGCGACAT
ASC#3	CATGTCGCGCAGCACGTTAG
UBR2#1	TGCATAACTTGAACTTTGAG
UBR2#2	TCCATGCACAAAACACAAGT
UBR2#3	ACTGTGGTGATACTGAAGCC
UBR2#4	TGATACTGAAGCCTGGAAAG
UBR2#5	TTGCATGCTGTTTAATGATG
ZER1#5	CAGGGACCCAATCAATCATG
ZER1#6	GGCAGGACGAGTCTATCCAG
ZER1#7	AGGCTGAAGAAGCTCTCGTG
ZER1#8	TGTAGGCTGGAATGTGCGTG
ZYG11B#5	AGCGCTCGTAAGGATCCTCG
ZYG11B#6	GCAGTGGCTTTGCAACCATG
ZYG11B#7	CTTGGTTAAGTTAAATACAC
ZYG11B#8	CAAGTTCCACTAACTTGTGG

 Table 1. CRISPR sgRNA sequences used to generate knockout 293Ts, immortalized keratinocytes, and NHBEs.

 and NHBEs.

 Sequence (5' to 3')



Fig. 1. Enteroviral 3Cpros activate the human NLRP1 inflammasome. (A) Domain structures of human NLRP1 and rodent NLRP1B. Murine NLRP1B is activated by anthrax lethal factor (LF) toxin cleavage, followed by the proteasomal degradation of the autoinhibitory N-terminal fragment. (B) Percentage of 293T-ASC-GFP-NLRP1-FLAG cells with ASC-GFP specks after overexpression of Myc-tagged viral proteases. Cells were fixed 24 hours after transfection of the indicated proteases or empty vector control. Talabostat (2 µM, 24 hours) treatment was used as a positive control. The number of cells with ASC-GFP specks were visually scored with widefield epifluorescence microscopy at 20× magnification. More than 100 cells were scored for ASC-GFP speck formation per condition. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 (two-way ANOVA); n.s., not significant. Error bars represent standard error of the mean from three biological replicates. (C) The effect of MG132 on NLRP1-dependent ASC-GFP speck formation. 293T-ASC-GFP or 293T-ASC-GFP-NLRP1-FLAG cells were transfected with HRV14-3Cpro and treated with 2.5  $\mu$ M MG132 6 hours post transfection. \*\*\*P < 0.001 (two-way ANOVA). Error bars represent standard error of the mean from three biological replicates. (D) HRV14-3Cpro induces mature IL-1<sup>β</sup> secretion and ASC oligomerization in immortalized human keratinocytes. Tet-ON HRV-3Cpro or 3Cpro<sup>C146A</sup> immortalized keratinocytes (N/TERT) were harvested 24 hours after 1 µg/ml doxycycline (DOX). Conditioned media was concentrated 10 times before SDS-PAGE. Endogenous ASC oligomers were extracted by 1% SDS after covalent cross-linking of the 1% NP40-insoluble pellets with 1 mM DSS in PBS. (E) IL-1β ELISA of conditioned media from NLRP1<sup>-/-</sup> and Cas9 control Tet-ON HRV14-3Cpro N/TERT keratinocytes 24 hours after DOX induction. \*\*\*P < 0.001; \*\*\*\*P < 0.0001 (two-way ANOVA). n = 3 independent cell seedings and inductions. Error bars represent the standard error of the mean. (F) Trypan blue exclusion assay of NLRP1<sup>-/-</sup> and Cas9 control Tet-ON HRV14-3Cpro N/TERT keratinocytes 24 hours after DOX induction. \*\*P < 0.01; \*\*\*P < 0.001 (two-way ANOVA), n = 3 independent cell seedings and inductions as in (E). Error bars represent the standard error of the mean.



Fig. 2. 3CPros activate NLRP1 by direct cleavage at a single site between p. Glu130 and Gly131. (A) HRV14-3Cpro cleaves NLRP1 close to its N terminus. Schematic shows full length NLRP1 and the antibodies used to detect the NLRP1 auto-proteolytic fragments. The epitope of the N-terminal NLRP1 antibody is between NLRP1 a.a. 130 and a.a. 230. (B) 293T cells were transfected with C-terminal HA-tagged NLRP1 and Myc-tagged HRV14-3Pro. Full-length NLRP1 and its cleavage products were visualized with the N-terminal fragment-specific NLRP1 antibody and an antibody against the C-terminal HA tag. Red arrows indicate the proposed proteolytic relationship between the observed NLRP1 fragments. Note that the presence of catalytically active 3Cpro decreased the expression of all transfected plasmids [see also (C)]. (C) To map the 3Cpro cleavage site, 293T cells were co-transfected with the indicated NLRP1 alanine mutants and HRV14-3Cpro. 3Cpro<sup>C146A</sup> was used as a negative control. Total cell lysates were harvested 48 hours post transfection and analyzed by SDS-PAGE and immunoblotting. (D) Q130A abrogates HRV14-3Cpro cleavage. 293T-ASC-GFP-NLRP1<sup>WT</sup>-FLAG and 293T-ASC-GFP-NLRP1<sup>Q130A</sup>-FLAG cells were transfected with Myc-HRV14-3Cpro or treated with talabostat for 48 hours. Total cell lysates were analyzed by SDS-PAGE. \*, nonspecific NLRP1 degradation product. Red arrow, 3Cpro-dependent cleavage product (a.a. 131-1212). (E) Q130A abrogates 3CPro-dependent, but not talabostat-dependent NLRP1 activation in 293T cells. Cells were transfected or treated with talabostat as in Fig. 1D and fixed 24 hours post transfection. The number of cells with ASC-GFP specks were visually scored with wide-field epifluorescence microscopy at 20× magnification. \*\*P < 0.01; \*\*\*\*P < 0.0001 (two-way ANOVA). n = 3independent transfections/drug treatment. n.s., not significant. Error bars represent the standard error of the mean. (F) Representative immunoblots demonstrate that wild-type NLRP1, but not NLRP1<sup>Q130A</sup> restores 3Cpro-dependent IL-1 $\beta$  secretion and ASC oligomerization. (G) Trypan blue exclusion assay demonstrates that wild-type NLRP1, but not NLRP1<sup>Q130A</sup> restores 3Cpro-triggered lytic cell death in  $NLRP1^{-/-}$  human keratinocytes. \*\*\*\*P < 0.0001 (twoway ANOVA). n = 3 independent transfections/drug treatment. n.s., not significant. Error bars represent the standard error of the mean.



Fig. 3. 3Cpro-triggered NLRP1 activation requires the N-terminal glycine degron pathway. (A) Overexpressed NLRP1 (a.a. 131-1474) does not cause spontaneous ASC-GFP speck formation. 293T-ASC-GFP cells were transfected with wild-type NLRP1 or mutants and fixed 48 hours post transfection. \*\*\*\*P < 0.0001 (two-way ANOVA). n = 3 independent transfections/drug treatment. n.s., not significant. Error bars represent the standard error of the mean. (B) Summary of the distinct pathways regulating post-cleavage N-terminal fragment degradation in human NLRP1 and murine NLRP1B. The two types of the N-degron pathway require distinct recognition receptors and demonstrate distinct sensitivities to small-molecule inhibitors. (C) MLN4924 and proteasomal inhibitors abrogate 3Cpro-induced ASC-GFP specks. 293T-ASC-GFP-NLRP1-FLAG cells were transfected with HRV14-3Cpro and treated with the indicated drugs for 24 hours: MLN4924 (1  $\mu$ M), MG132 (2.5  $\mu$ M), bortezomib (0.5  $\mu$ M), or phenylalanine (1 mM). Wild-type NLRP1, but not NLRP1<sup>Q130A</sup> restores 3Cpro-dependent IL-1B secretion and ASC oligomerization. \*\*\*\*P < 0.0001 (one-way ANOVA). n = 3 independent transfections/drug treatment. n.s., not significant. Error bars represent the standard error of the mean. (D) MLN4924 and bortezomib inhibit NLRP1 self-oligomerization and stabilize the 3Cpro cleavage fragment. 293T-NLRP1-FLAG cells were transfected with HRV14-3Cpro and treated with the indicated drugs 16 hours post-transfection for another 24 hours. Lysates were analyzed by SDS-PAGE and immunoblot. The red arrowhead indicates the HRV14-3Cpro cleavage product (a.a. 131-1212). (E) IL-1B ELISA of conditioned media. \*\*\*\*P < 0.0001, one-way ANOVA. n = 3 independent doxycycline inductions/drug treatment. n.s., not significant. Error bars represent the standard error of the mean. (F) Cullin<sup>ZER1/ZYG11B</sup>, but not UBR2, is genetically required for 3Cpro-induced NLRP1 activation. The indicated CRISPR KO 293T-ASC-GFP cells were transfected with wild-type NLRP1 + HRV14-3Cpro (or vector) or with NLRP1<sup>M77T</sup>. Cells were fixed 24 hours post-transfection. ASC-GFP specks were scored using wide-field microscopy at 20×.\*\*\*\**P* < 0.0001, one-way ANOVA. *n* = 3 transfections, >100 cells. n.s., not significant. Error bars represent the standard error of the mean. (G) Cullin<sup>ZER1/ZYG11B</sup>, but not UBR2 is responsible for the degradation of the 3Cpro cleavage product. CRISPR KO 293T-ASC-GFP cells stably expressing NLRP1-FLAG were transfected with HRV14-3Cpro. Cell lysates were harvested 48 hours post transfection. The red arrowhead indicates the 3Cpro cleavage product (a.a. 131-1212).



Fig. 4. HRV infection activates inflammasome-driven pyroptosis in NHBEs independently of NLRP3. (A) NLRP1, NLRP3, PYCARD (ASC), and CASP1 mRNA expression (RNA-seq TPM) in NHBEs. n = 11, independent cultures of NHBEs. \*\*\*\*P < 0.0001, two-tailed Student's t test. Error bars represent the standard error of the mean. (B) Overview of cytokine profiling of HRV16infected and talabostat-treated NHBEs. (C) Cells were inoculated with HRV16 at MOI = 5 and cultured at 33°C for 48 hours. Luminex array was performed on conditioned media after removing cell debris. n = 3 independent infections and treatment. Cytokines/chemokines that were induced by at least fivefold, such as IL-18 (P < 0.05, Student's t test, lognormal values), are highlighted in red. (**D**) MCC950 did not affect HRV16 triggered IL-1β secretion in NHBEs. NHBEs were pretreated with MCC950 (5 µM) or rupintrivir (10 nM) before HRV16 inoculation or talabostat (2  $\mu$ M) treatment. \*P < 0.05 (two-way ANOVA). n = 3 independent drug treatments. n.s., not significant. Error bars represent the standard error of the mean. (E) MCC950 did not affect HRV16 triggered IL-18 secretion in NHBEs. NHBEs were pretreated with MCC950 (5 µM) or rupintrivir (10 nM) before HRV16 inoculation or talabostat (2  $\mu$ M) treatment. \*P < 0.05 (twoway ANOVA). n = 3 independent drug treatments. n.s., not significant. Error bars represent the standard error of the mean. (F) Representative immunoblot demonstrates the secretion of cleaved IL-1ß and IL-18 and ASC oligomerization in HRV16-infected primary NHBEs. Cells were inoculated with HRV16 (MOI = 5) or treated with talabostat (2 μM) for 48 hours at 33°C.



Fig. 5. HRV infection leads to inflammasome activation and IL-18 secretion in 3D bronchial epithelial cultures. (A) Overview of apical HRV infection 3D human bronchial epithelial cultures. (B) IL-18 levels in apical or basal media 48 hours post-infection or talabostat treatment. \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 (two-way ANOVA). n = 3 independent infected or treated cultures. n.s., not significant. Error bars represent the standard error of the mean. (C) Representative alcian blue (AB)/periodic acid–Schiff (PAS) staining (top) and immunohistochemical staining for cleaved (N-terminal) GSDMD (bottom) of HRV16-infected (MOI = 3) and talabostat (2  $\mu$ M)-treated 3D human bronchial epithelial cultures. Staining performed on two sections from three independent 3D bronchial epithelial cultures. Scale bar = 50  $\mu$ m.



Fig. 6. NLRP1 cleavage and the N-glycine degron pathway are indispensable for HRVdependent inflammasome activation in NHBEs. (A) Endogenous NLRP1, ASC, and caspase-1 are indispensable for HRV16-induced IL-18 secretion in NHBEs. Cas9-control, NLRP1-/-, ASC-/-, and CASP1<sup>-/-</sup> NHBEs were infected with HRV16 MOI = 5. Conditioned media were harvested 48 hours post infection. \*P < 0.05, \*P < 0.001 (two-way ANOVA). n = 3 independent live viral infections/drug treatment. n.s., not significant. Error bars represent the standard error of the mean. (B) NLRP1 is genetically required for HRV16-induced IL-18 cleavage and ASC oligomerization. Cas9 control,  $NLRP1^{-/-}$ , and  $ASC^{-/-}$  NHBEs were infected with HRV16 as before. Cleaved IL-18 are marked with arrows. (C) Mutating the NLRP1 cleavage site abrogates HRV16triggered IL-18 secretion in NHBEs. NLRP1-/- NHBEs were rescued with lentiviral constructs expressing FLAG-tagged NLRP1<sup>WT</sup> or NLRP1<sup>Q130A</sup>, which carried silent mutations at the PAM site. The rescued cells were infected with HRV16 or treated with talabostat. Conditioned media was analyzed by IL-18 ELISA. \*\*P < 0.01, \*\*\*\*P < 0.0001 (two-way ANOVA), n = 3 replicates from one of two independent infections. Error bars represent the standard error of the mean. (D) HRV infection leads to NLRP1 cleavage and degradation of NLRP1 N-terminal fragments.  $NLRP1^{-/-}$  or NLRP1<sup>WT</sup>- or NLRP1<sup>Q130A</sup>- rescued cells were infected with HRV16 or treated with talabostat. Cells were harvested 48 hours post infection. (E) MLN4924 and bortezomib block HRV16-induced IL-18 secretion. NHBEs were pretreated with MLN4924 (1 µM) or bortezomib (1 µM) before HRV16 infection or talabostat treatment. IL-18 levels were measured with ELISA. \*\*\*P < 0.001 (two-way ANOVA), n = 3 independent infections/treatments. Error bars represent the standard error of the mean. (F) MLN4924 does not affect 3Cpro-dependent VP2 maturation in the course of HRV16 infection. Lysates from HRV16-infected NHBEs in Fig. 5F were analyzed by immunoblotting 48 hours post HRV16 inoculation. (G) MLN4924 stabilizes the 3Cpro cleavage fragment (a.a. 131-1212). NHBEs were pretreated with MLN4924 and infected with HRV as in (D).



#### Enteroviral 3C protease activates the human NLRP1 inflammasome in airway epithelia

Kim S. Robinson, Daniel Eng Thiam Teo, Kai Sen Tan, Gee Ann Toh, Hsiao Hui Ong, Chrissie Kaishi Lim, Kenneth Lay, Bijin Veonice Au, Tian Sheng Lew, Justin Jang Hann Chu, Vincent Tak Kwong Chow, De Yun Wang, Franklin L. Zhong and Bruno Reversade

published online October 22, 2020

ARTICLE TOOLS	http://science.sciencemag.org/content/early/2020/10/21/science.aay2002
SUPPLEMENTARY MATERIALS	http://science.sciencemag.org/content/suppl/2020/10/21/science.aay2002.DC1
REFERENCES	This article cites 57 articles, 20 of which you can access for free http://science.sciencemag.org/content/early/2020/10/21/science.aay2002#BIBL
PERMISSIONS	http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title Science is a registered trademark of AAAS.

Copyright © 2020, American Association for the Advancement of Science