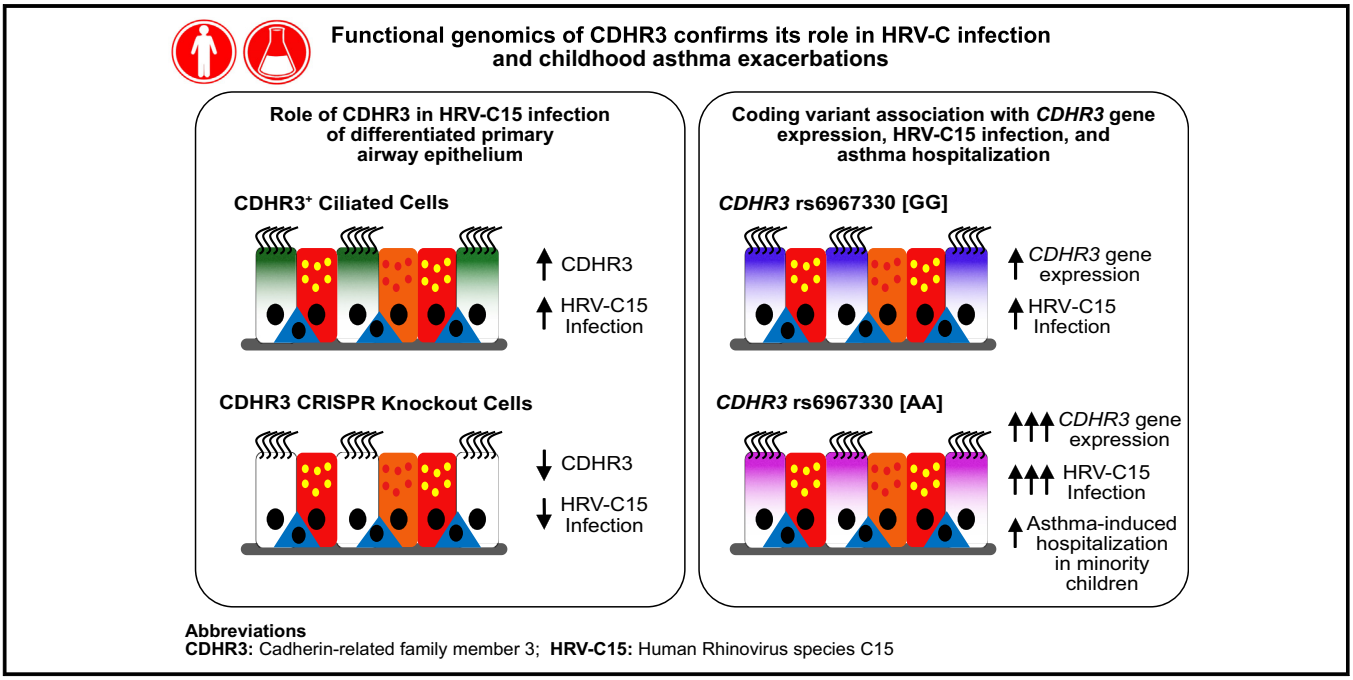
Functional genomics of CDHR3 confirms its role in HRV-C infection and childhood asthma exacerbations

Jamie L. Everman, PhD,[a] Satria Sajuthi, PhD,[a] Benjamin Saef, MS,[a] Cydney Rios, BS,[a] Ari M. Stoner, BS,[a] Mari Numata, MD, PhD,[b] Donglei Hu, PhD,[c] Celeste Eng, MS,[c] Sam Oh, PhD,[c] Jose Rodriguez-Santana, MD,[d] Eszter K. Vladar, PhD,[e,f,g] Dennis R. Voelker, PhD,[b] Esteban G. Burchard, MD, MPH,[c,h] and Max A. Seibold, PhD[a,e,i]

Denver and Aurora, Colo, San Francisco, Calif, and San Juan, Puerto Rico

GRAPHICAL ABSTRACT



Background: Research in transformed immortalized cell lines indicates the cadherin-related family member 3 (CDHR3) protein serves as a receptor for human rhinovirus (HRV)–C. Similar experiments indicate that the *CDHR3* coding variant rs6967330 increases CDHR3 protein surface expression.

Objective: We sought to determine whether CDHR3 is necessary for HRV-C infection of primary airway epithelial cells (AECs) and to identify molecular mechanisms by which CDHR3 variants confer risk for asthma exacerbations.

Methods: CDHR3 function and influence on HRV-C infection were investigated by using single-cell transcriptomics, CRISPR-Cas9 gene knockout, and genotype-specific donor experiments performed in primary AECs. Nasal airway epithelium cis–expression quantitative trait locus (eQTL) analysis of *CDHR3*

From [a]the Center for Genes, Environment, and Health and the Departments of [b]Medicine and [i]Pediatrics, National Jewish Health, Denver; the Departments of [c]Medicine and [h]Bioengineering and Therapeutic Sciences, University of California–San Francisco; [d]Centro de Neumología Pediátrica, San Juan; and [e]the Division of Pulmonary Sciences and Critical Care Medicine and the Departments of [f]Medicine and [g]Cell and Developmental Biology, University of Colorado School of Medicine, Aurora.

Supported by R01 HL135156, R01 MD010443, R01 HL128439, P01 HL132821, P01 HL107202, and U01 HL138626.

Disclosure of potential conflict of interest: J. L. Everman and M. A. Seibold report patent invention "Methods of Identifying and treating subjects having inflammatory subphenotypes on asthma" in process: File No. 2879-178-PROV pending. M. Numata and D. R. Voelker report grants from the National Institutes of Health (NIH) and the Flight Attendant Medical Research Institute during the conduct of this study focused on mechanisms of antiviral and anti-inflammatory actions of phospholipids and are co-inventors of the patent invention "Surfactant lipids, compositions thereof, and uses thereof" owned by National Jewish Health. M. A. Seibold reports grants from the NIH during the conduct of the study and grants from MedImmune, the Department of Defense, Pfizer, and Genentech outside the submitted work; holds patent invention "Transcriptomic response of airway epithelial cells to IL-13" in process: File No. 2879-190-PROV-1 pending and patent invention "Methods of diagnosing and treating subjects at risk of inflammation and/or exacerbation of a respiratory disease or condition" in process File No. 2879-191-PROV-1 pending; and was an invited lecturer at Genentech. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 16, 2018; revised December 30, 2018; accepted for publication January 30, 2019.

Available online March 29, 2019.

Corresponding author: Max A. Seibold, PhD, Center for Genes, Environment, and Health, 1400 Jackson St A636, Denver, CO 80206. E-mail: seiboldm@njhealth.org.

0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2019.01.052

was performed, followed by association testing for asthma hospitalization in minority children.
Results: CDHR3 lung expression is exclusive to ciliated AECs and associated with basal bodies during and after motile ciliogenesis. Knockout of CDHR3 in human AECs did not prevent ciliated cell differentiation but was associated with a decrease in transepithelial resistance and an 80% decrease in HRV-C infection of the mucociliary epithelium. AECs from subjects homozygous for the risk-associated rs6967330 single nucleotide polymorphism (SNP) exhibited greater HRV-C infection compared with cells homozygous for the nonrisk allele. AEC cis-eQTL analysis indicated that rs6967330 and other SNPs are eQTLs for *CDHR3*. Only the eQTL block containing the rs6967330 SNP showed a significant association with childhood asthma hospitalization.
Conclusions: Genetic deletion and genotype-specific studies in primary AECs indicate CDHR3 is critical to HRV-C infection of ciliated cells. The rs6967330 SNP confers risk of severe childhood asthma exacerbations, likely through increasing HRV-C infection levels and protein surface localization. (J Allergy Clin Immunol 2019;144:962-71.)

Key words: *Airway epithelium, CRISPR, rhinovirus, expression quantitative trait loci*

Asthma is the most common chronic disease of childhood, with severe exacerbations representing the cause of most patient burden and associated economic costs.[1,2] Asthma exacerbations are the result of complex interactions between environmental exposures and genetic predisposition, which are poorly understood. The environmental factor most commonly associated with asthma exacerbations is viral upper respiratory tract infection, which occurs in 80% of patients with exacerbations.[3] Specifically, human rhinovirus (HRV) species are found in more than 60% of these virus-associated exacerbations.[4] Multiple studies have established that infection with HRV-A is much more strongly associated with asthma exacerbations than infection with HRV-B.[5-7] More recently, a third species, HRV-C, was discovered and several studies have found HRV-C infections to be more strongly associated with severe asthma exacerbations than HRV-A infections.[8,9]

The viral entry receptor for the majority of HRV-A and all HRV-B serotypes is intercellular adhesion molecule 1, and the low-density lipoprotein receptor acts as the receptor for some HRV-A serotypes.[10,11] Structure binding studies revealed that HRV-C does not use either intercellular adhesion molecule 1 or low-density lipoprotein receptor as its entry receptor.[12] Rather, a recent genomic study identified a set of genes that were differentially expressed between HRV-C–susceptible versus nonsusceptible cell/tissue types. Heterologous expression of these genes in HeLa cells (a nonsusceptible cell type) revealed that expression of cadherin-related family member 3 (CDHR3) allowed viral entry and replication in these cells, providing strong evidence that CDHR3 serves as the receptor for HRV-C.[13] Moreover, although CDHR3 expression has been confirmed in the human airway epithelium, specifically in ciliated cells cultured *in vitro*,[14] it is unclear whether other cells in the lung express CDHR3. Supporting possible expression in other lung cell types, a recent murine study described the mouse *CDHR3* ortholog as a marker of an alveolar progenitor cell type.[15]

Abbreviations used
ACT: Acetylated α-tubulin
AEC: Airway epithelial cell
ALI: Air-liquid interface
CDHR3: Cadherin-like family member 3
eQTL: Expression quantitative trait locus
GALA II: Genes-Environments and Admixture in Latino Americans II
HRV: Human rhinovirus
IRB: Institutional review board
KO: Knockout
LD: Linkage disequilibrium
NJH: National Jewish Health
RNA-seq: RNA-sequencing
SNP: Single nucleotide polymorphism
TEER: Transepithelial electrical resistance

A genome-wide scan for severe childhood asthma exacerbations identified *CDHR3* among 4 genome-wide significant loci.[16] The associated single nucleotide polymorphism (SNP) rs6967330 is a CDHR3-coding variant that results in a cysteine to tyrosine amino acid substitution at position 529 in the amino acid sequence. Heterologous expression of these allelic forms of CDHR3 in HeLa and HEK 293T cells indicated that the asthma risk–associated allelic form exhibited greater surface expression than the nonrisk allelic form.[13,16] Taken together, these data support a model in which the rs6967330 variant increases asthma exacerbation risk by increasing surface expression of the CDHR3 protein and thus the risk and possibly level of an HRV-C respiratory tract infection and illness. Several genetic studies have now associated the rs6967330 SNP with risk of asthma-related illnesses, including in Danish and Japanese patient cohorts.[16-19]

Despite these findings, many questions remain, including (1) whether lung cells other than ciliated cells express *CDHR3*; (2) whether, as a cadherin-like protein, CDHR3 plays a role in cell adhesion or other ciliated cell functions; (3) whether perturbation of CDHR3 expression in human airway epithelial cells (AECs) modulates HRV-C infection levels; (4) whether the rs6967330 variant of CDHR3 modifies HRV-C infection in AECs; and (5) whether rs6967330 or other cis-variants function as *CDHR3* expression quantitative trait loci (eQTLs) and modify risk for childhood asthma exacerbations. Here we use CDHR3 CRISPR-Cas9–edited and CDHR3 risk genotype–specific primary AECs for functional experiments, as well as a comprehensive *CDHR3* nasal airway epithelial eQTL analysis and genetic association analysis for *CDHR3* functional variants with childhood asthma exacerbations, to answer these questions.

METHODS

Human subject information

Human lung cells for single-cell RNA-sequencing (RNA-seq) and tracheal airway epithelia isolated from deidentified lung donors whose lungs were not suitable for transplantation were obtained from the International Institute for the Advancement of Medicine (Edison, NJ) and Donor Alliance of Colorado. The National Jewish Health (NJH) Institutional Review Board (IRB) approved research on lung and tracheal cells under IRB protocols HS-3209 and HS-2240. Nasal AECs for culture and the eQTL study came from subjects recruited as part of the Genes-Environments and Admixture in Latino Americans II (GALA II) childhood asthma cohort, which was approved by local institutional review

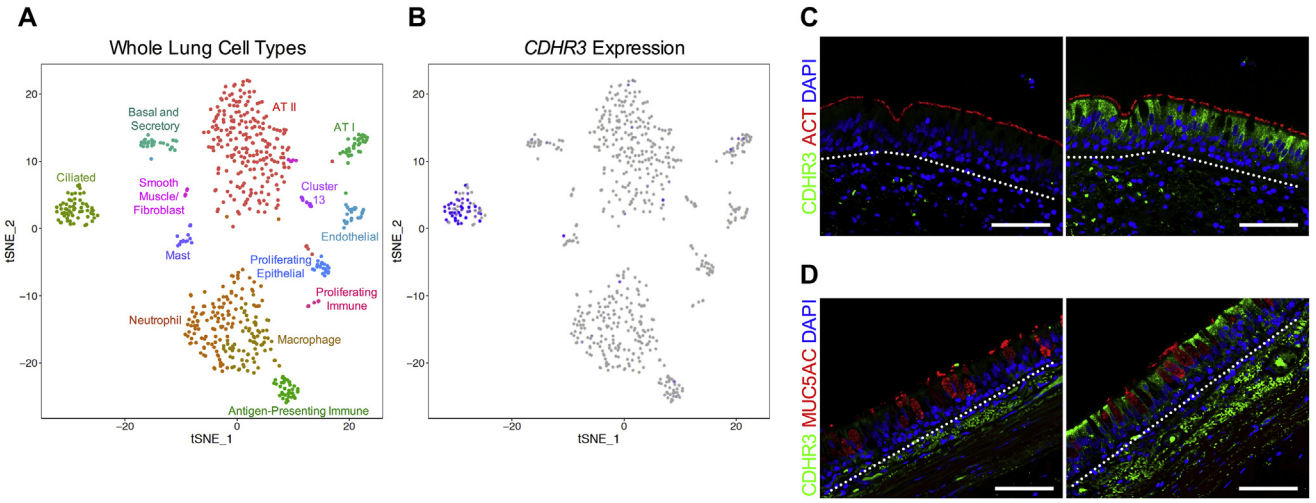


FIG 1. Single-cell RNA-seq of the human lung reveals *CDHR3* is exclusively expressed in ciliated AECs. **A** and **B,** RNA-seq of single cells digested from a human lung identifies 13 distinct cell types by using t-SNE analysis based on cell-specific gene markers (Fig 1, *A*) and demonstrates that *CDHR3* gene expression is highly specific to ciliated cells in the lung (Fig 1, *B*; n = 1142 individual cells analyzed from 1 digested lung and representative of single cell data from 6 total human lungs). **C** and **D,** Immunofluorescence labeling of whole-lung tissue histologic sections shows that CDHR3 localizes to ciliated cells of the airway epithelium (Fig 1, *C*) but not to MUC5AC$^+$ secretory cells in the *in vivo* airway epithelium (Fig 1, *D*). *DAPI*, 4′-6-Diamidino-2-phenylindole dihydrochloride. *Left panels*, Isotype control; *right panels*, CDHR3 antibody. *Bars* = 50 μm. The *white dotted line* designates the basement membrane of the airway epithelium. The image is representative of experiments completed by using histologic sections collected from 3 lung donors.

boards (UCSF, IRB no. 10–00889, reference no. 153543, NJH HS-2627). All subjects and their parents provided written informed assent and written informed consent, respectively.[20,21] Demographic and clinical variables for tissue donors used in this study are listed for all lung, tracheal, and nasal samples (see Table E1 in this article's Online Repository at www.jacionline.org) and for subjects in the genetic eQTL analysis (see Table E2 in this article's Online Repository at www.jacionline.org).

Single-cell RNA-seq and analysis

Single-cell suspensions of elastase-digested lung tissue were obtained, as previously described.[22] Cells were dispensed and imaged by using the ICELL8 Single-Cell System (Takara Bio, Shiga, Japan), and samples were sequenced with the Illumina HiSeq 2500 System (Illumina, San Diego, Calif).

Culture of primary tracheal and nasal AECs

Primary human tracheal and nasal AECs were expanded and differentiated at the air-liquid interface (ALI) *in vitro*,[23-26] and intact and dissociated cultures were harvested for Western blotting, flow cytometry, immunofluorescence, and gene expression analyses.[26]

Lentiviral CRISPR-Cas9 gene editing of airway basal cells

The design of the CRISPR-targeting guide sequences, addition of adaptors and cloning into the lentiCRISPR plasmid backbone, propagation and titration of lentivirus, and AEC transduction and selection were performed, as previously described.[23,25]

Human rhinovirus infection

HRV-C15 virus was propagated by using HeLa-E8 cells, as previously described.[12,13] Cultures were infected with virus at a multiplicity of infection of 0.2 for 4 hours at 34°C, washed, and incubated for a total of 48 hours at 34°C before harvest.

Whole-transcriptome analysis, genotyping, and eQTL analysis

Whole-transcriptome libraries of 695 nasal brushes from the GALA II childhood asthma cohort were constructed by using the Beckman Coulter FX automation system (Beckman Coulter, Fullerton, Calif), and samples were sequenced with the Illumina HiSeq 2500 system. DNA was genotyped with Affymetrix Axiom LAT 1 (World Array 4) and LAT plus HLA genome-wide arrays (Affymetrix, Santa Clara, Calif). eQTL analysis followed the Genotype-Tissue Expression project protocol (version 7) for filtering and analysis.[27]

Immunofluorescence labeling

Intact ALI AEC culture inserts were fixed for 15 minutes at room temperature in 3.2% paraformaldehyde or with ice-cold methanol at −20°C. Human lung tissue samples were fixed in 10% formalin before paraffin embedding and antibody labeling.

RESULTS

CDHR3 is exclusively expressed by ciliated AECs

To identify *CDHR3*-expressing cells within the human lung, we performed single-cell RNA-seq of whole-lung cell suspensions. Using t-Distributed Stochastic Neighbor Embedding (t-SNE) clustering of gene expression in cells, we identified 13 distinct cell populations (Fig 1, *A*). Differentially expressed genes between clusters were intersected with known cell type markers to determine the likely cell type identity of each cluster (see Table E3 in this article's Online Repository at www.jacionline.org). Cell types identified include AEC types (ciliated and secretory), alveolar type 1 and type 2 cells, lung immune cells, and endothelial cells. *CDHR3* was highly and exclusively expressed in ciliated cells (Fig 1, *B*), which were clearly identified as such based on high expression of genes contained in ciliated cell

gene ontology categories (see Fig E1 in this article's Online Repository at www.jacionline.org). Immunofluorescence labeling of lung airway sections confirmed this result at the protein level because CDHR3 was only observed in ciliated AECs identified by using acetylated α-tubulin (ACT) and not secretory cells that are positive for MUC5AC (Fig 1, *C* and *D*).

CDHR3 protein is apically distributed and most highly expressed in immature ciliated cells

To study CDHR3 function, we differentiated basal AECs into a mucociliary epithelium by using the ALI cell-culture model. Using time-course analysis of CDHR3 gene and protein expression across mucociliary culture differentiation (Fig 2, *A*), we found *CDHR3* gene expression was evident by ALI day 5 and protein expression by day 6 before the appearance of motile cilia at day 11. Gene and protein expression increased until day 18 and were maintained at this level through ALI day 30.

Colabeling of CDHR3 and the cilia marker ACT in mature cultures (day 21) revealed the expected coexpression of these proteins (Fig 2, *B*). Despite CDHR3 being a cadherin-related protein, it exhibited a punctate cytoplasmic rather than membrane-localized staining pattern. Confirming this, we colabeled CDHR3 with E-cadherin, revealing no colocalization of these proteins. YZ projection of confocal z-stacks revealed that CDHR3 puncta were localized in the cytoplasm at the apical side of the cell (Fig 2, *C*). Given that CDHR3 is expressed early in ciliated cell development, we also performed immunofluorescence labeling of day 14 cultures, which contain a mix of ciliating and mature ciliated cells. At ALI day 14, we identified many $CDHR3^{+}/ACT^{-}$ cells (32.9% of total cell count; Fig 2, *D*). These cells were rare at day 21 (3.5% of total cell count; Fig 2, *B*), which led us to hypothesize that these were ciliating cells expressing CDHR3 before development of cilia. Moreover, there was an inverse relationship between the mean fluorescence intensity of CDHR3 and ACT within double-positive cells at day 14 ($r = -0.38$, $P = .0002$; Fig 2, *D*).

To support the greater expression of CDHR3 in ciliating rather than mature ciliated cells, we colabeled day 14 cultures for CDHR3 and ezrin, a marker of the apical membrane in mature ciliated cells. The brightest CDHR3-labeled cells were among the cells weakest for ezrin labeling, as reflected by the strong inverse correlation between CDHR3 and ezrin mean fluorescence intensity values ($r = -0.47$, $P < .0001$; Fig 2, *E*). The YZ projection images also indicate that CDHR3 does not colocalize with ezrin to the apical membrane but is localized to the apical submembrane compartment of ciliated cells (Fig 2, *E*).

To further validate that greater CDHR3 expression is found in ciliating cells in the differentiating AECs, we colabeled cultures for centrin, a component of the basal body that forms the intracellular base anchoring the cilium to the apical cell surface. During ciliogenesis, basal bodies are generated in the cytoplasm, traffic to and dock with the membrane, and then elongate the cilium. Centrin has a well-defined pattern of basal body localization within ciliated cells during the course of ciliogenesis, which allows identification of ciliating versus mature ciliated cells.[28] Image analysis revealed that cells with the strongest CDHR3 pattern coexpressed with a cytoplasmic centrin puncta staining pattern indicative of ciliating cells with replicating or trafficking basal bodies. Alternatively, cells exhibiting centrin puncta evenly distributed at the apical surface indicating docked basal bodies of mature ciliated cells exhibited dimmer expression of CDHR3 (Fig 2, *F*).

CDHR3 gene knockout does not affect ciliated cell differentiation but results in reduced transepithelial membrane resistance

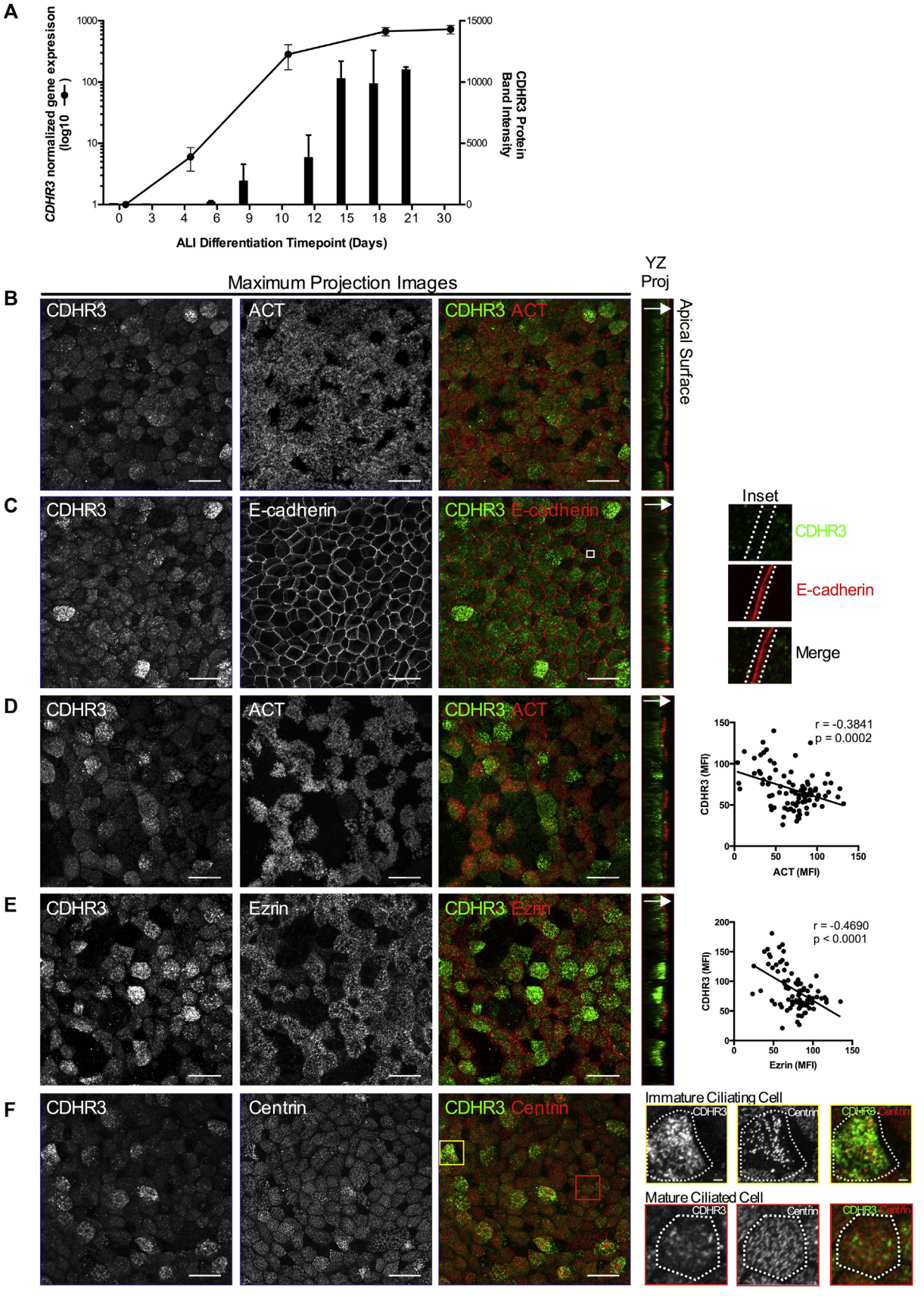
To investigate CDHR3 function in the airway epithelium, we generated tracheal basal AECs knocked out for the *CDHR3* gene using our lentiviral mediated CRISPR-Cas9 gene-editing system (n = 3 donors).[23,25] The CDHR3-edited cells (CDHR3 knockout [KO]) appeared normal and proliferated in culture similarly to the control edited basal cells (control).

To assess whether the edited cells exhibited a loss of CDHR3 protein, we differentiated the CDHR3 KO and control basal cells at ALI for 21 or more days and performed quantitative Western blot analysis. We found the ALI cultures generated from CDHR3 KO cells exhibited 94.57% lower CDHR3 protein expression (SD ± 4.18%; $P < .0001$; Fig 3, *A* and *B*) compared with cultures generated from control cells. Immunofluorescent labeling of cytospin preparations from differentiated ALI cultures also showed a marked decrease in CDHR3 protein within ACT^{+} ciliated cells (Fig 3, *C*), indicating a substantial knockdown of CDHR3.

Given the robust early ciliogenesis expression of CDHR3, we first wanted to determine whether loss of CDHR3 expression affects the differentiation of ciliated cells. To address this, we performed flow cytometric analysis for CDHR3 and ACT in cells isolated from mature ALI cultures differentiated from CDHR3 KO and control basal cells (Fig 3, *D* and *E*). We found 12.9% ± 1.43% of the control cells were $ACT^{+}/CDHR3^{+}$ versus only 2.34% ± 0.40% of the CDHR3 KO cells, and we found an 81.67% ± 2.81% decrease with CDHR3 KO ($P = .0140$; Fig 3, *E*). In the CDHR3 KO cells we observed a corresponding 85.22% ± 1.33% increase in $ACT^{+}/CDHR3^{-}$ cells, which represented 16.43% ± 5.95% of all cells versus only 2.65% ± 1.17% of all cells in the control population ($P = .0032$; Fig 3, *E*). Importantly, we found the total percentage of ciliated cells (ACT^{+}) was unchanged by CDHR3 KO (KO = 18.8% ± 10.8%, control = 15.6% ± 4.4%, $P = .7531$; Fig 3, *E*).

In addition, we performed RNA-seq analysis of ALI-differentiated control and KO gene-edited cells from these 3 donors to determine whether expression of gene markers of ciliated cells and transcription factor drivers of ciliogenesis were affected by CDHR3 KO (see Table E4 in this article's Online Repository at www.jacionline.org). Results supported our flow cytometric data showing that expression of *FOXJ1*, *RFX2*, *RFX3*, *MYB*, *E2F4*, and *TP73* was not differentially expressed in the KO cells, despite significant downregulation of *CDHR3* expression ($P_{adj} = 4.6 \times 10^{-18}$, see Table E4).

Further quantification of immunofluorescence labeling of $FOXJ1^{+}$ nuclei in ALI cultures demonstrated that CDHR3 KO does not affect expression of FOXJ1 or the ciliated cell fate of the airway epithelial cultures during differentiation (see Fig E2 in this article's Online Repository at www.jacionline.org). Moreover, club secretory and mucus secretory marker genes were unaffected by CDHR3 KO, suggesting loss of CDHR3 function has no effect on secretory cell formation. However, we found that among the 27 differentially expressed genes in CDHR3 KO cells (5% false discovery rate and $\log_2$ fold change of >0.5 or <−0.5) that the basal cell marker genes *KRT5*, *KRT14*, and *NGFR* were


A
CDHR3 normalized gene expresison (log10)
CDHR3 Protein Band Intensity
ALI Differentiation Timepoint (Days)
B
Maximum Projection Images
YZ Proj
CDHR3
ACT
CDHR3 ACT
Apical Surface
C
CDHR3
E-cadherin
CDHR3 E-cadherin
Inset
CDHR3
E-cadherin
Merge
D
CDHR3
ACT
CDHR3 ACT
r = -0.3841
p = 0.0002
CDHR3 (MFI)
ACT (MFI)
E
CDHR3
Ezrin
CDHR3 Ezrin
r = -0.4690
p < 0.0001
CDHR3 (MFI)
Ezrin (MFI)
F
CDHR3
Centrin
CDHR3 Centrin
Immature Ciliating Cell
Mature Ciliated Cell

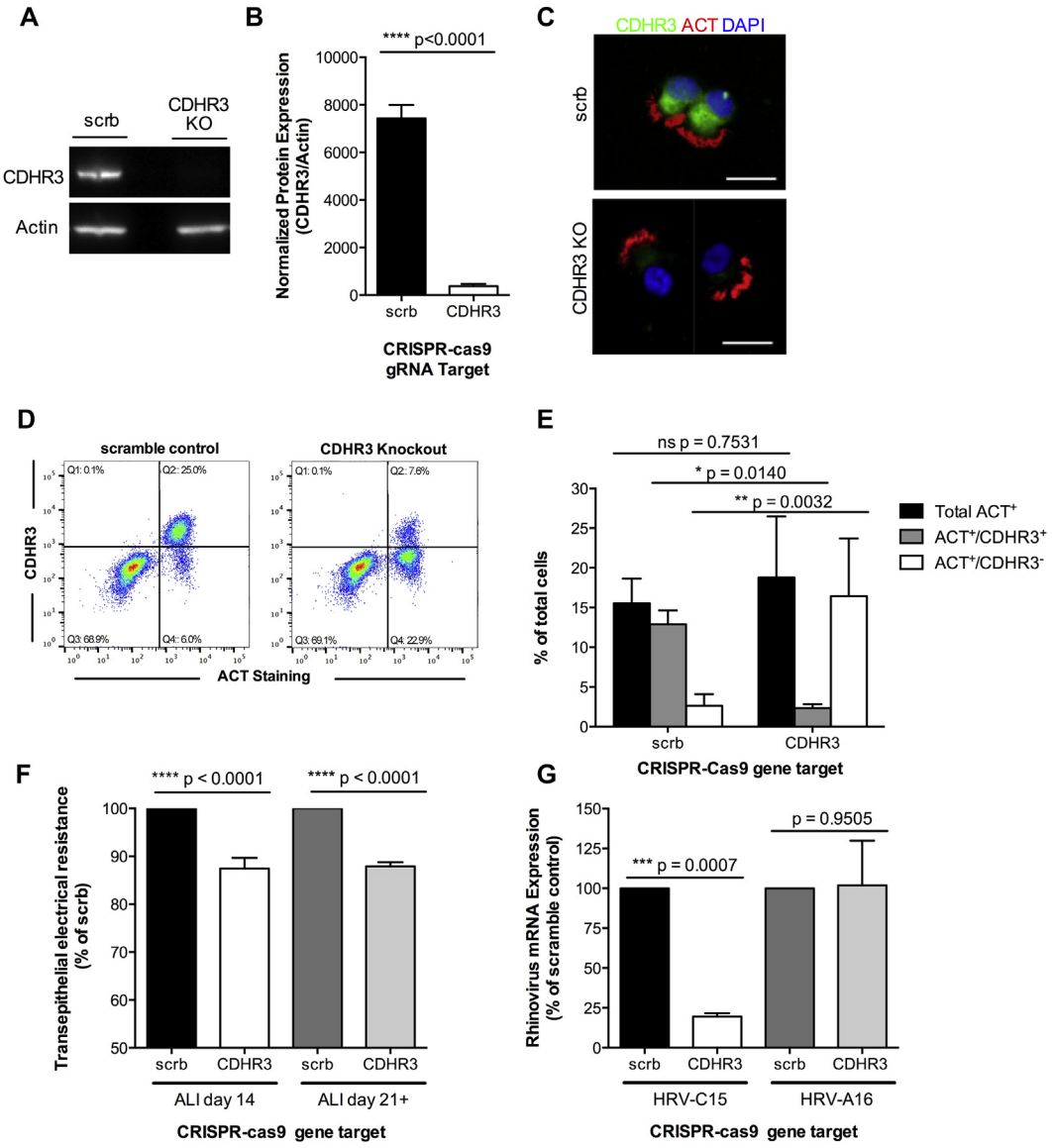


FIG 3. *CDHR3* gene KO decreases epithelial TEER and HRV-C infection. **A-E,** CDHR3 KO or control *(scrb)* tracheal basal AECs were differentiated at ALI for 21 or more days. Dissociated cultures were harvested for CDHR3 protein expression by using Western blotting (Fig 3, *A* and *B*), immunofluorescence labeling for CDHR3 (green) and ACT (red; *bars* = 20 μm; Fig 3, *C*), and flow cytometric analysis (Fig 3, *D* and *E*). *DAPI*, 4′-6-Diamidino-2-phenylindole dihydrochloride. **F,** TEER measurements were taken at day 14 and day 21 or later of ALI differentiation. **G,** Cultures were infected with HRV-C15 or HRV-A16 virus at a multiplicity of infection *(MOI)* of 0.2 for a total of 48 hours at 34°C before harvest and quantification of viral load by means of quantitative PCR. Data are representative of CDHR3 KO or control *(scrb)* cultures grown and analyzed in parallel (n = 3 independent donors).

FIG 2. CDHR3 exhibits an apical and cytoplasmic expression pattern that is greatest in immature ciliating epithelial cells. Tracheal basal AECs were differentiated *in vitro* at ALI for immunofluorescence labeling. **A,** Quantitative PCR and Western blot analyses were completed on cultures harvested over 30 days of differentiation for the *CDHR3* gene (n = 3 donors) and protein expression (n = 2 donors). **B** and **C,** ALI day 21 cultures were colabeled for CDHR3 (green) and either ACT (red; Fig 2, *B*) or E-cadherin (red; Fig 2, *C*). *Magnified inset panels* indicate no colocalization of CDHR3 and E-cadherin at the cell-cell junction (Fig 2, *C*, *far right panel*). **D** and **E,** ALI day 14 cultures were colabeled for CDHR3 (green) and either ACT (red; Fig 2, *D*) or ezrin (red; Fig 2, *E*). Mean fluorescence intensities *(MFIs)* of each protein were quantified in individual cells in culture for correlation analysis of intensities of CDHR3 and ACT or ezrin (n = 90 cells total; Fig 2, *D* and *E*, *far right panels*). **F,** ALI day 21 cultures were colabeled for CDHR3 (green) and centrin (red). Single-cell panels illustrate the CDHR3 pattern in immature *(top)* and mature *(bottom)* cells, as indicated by centrin pattern. *Single-cell inset bars* = 2 μm. YZ projection images show localization of proteins within the apical region of the epithelium (*arrow* indicates direction toward apical surface). *Maximum projection bars* = 20 μm. Images are representative of labeling performed in ALI cultures from 3 donors.

all upregulated, supporting that loss of CDHR3 either increases basal frequency or basal cell marker gene expression (see Table E4).

Although we did not detect junctional localization, as a cadherin-related protein, we speculated that CDHR3 KO might affect apical junctional integrity. We evaluated transepithelial electrical resistance (TEER) in AECs and observed that it was reduced in CDHR3 KO cultures. We found that immature CDHR3 KO cultures (day 14) exhibited a 12.56% decrease in TEER compared with control cultures ($P < .0001$; Fig 3, *F*). Likewise, we found that mature differentiated ALI cultures from CDHR3 KO cells exhibited a 12.11% decrease in transepithelial resistance compared with control cultures ($P < .0001$; Fig 3, *F*). These data indicate that CRISPR-Cas9 KO of CDHR3 in primary AECs does not affect the development or differentiation of ciliated cells in the airway epithelium. However, the effect of CDHR3 KO on epithelial TEER indicates a potential effect on barrier function, ion transport, or other epithelial organization, despite a lack of exclusive CDHR3 junctional localization.

Deficiency in CDHR3 protein downregulates HRV-C15 infection of AECs

Our CDHR3 KO AECs allowed us to directly test whether HRV-C infection is dependent on CDHR3 protein expression in well-differentiated ALI-cultured human AECs. We examined both viral binding at 4 hours after apical infection and infection at 48 hours by using quantitative PCR. We found no difference in HRV-C15 viral binding between control and KO cells ($P = .1836$, data not shown). However, at 48 hours after infection, the CDHR3 KO epithelium exhibited 80.42% lower HRV-C15 RNA levels compared with control infected cultures (SD ± 2.30%, $P < .0001$; Fig 3, *G*). To test whether this effect on HRV-C15 infection was specific to the rhinovirus C species, we performed the same experiment on CDHR3 KO cells with the HRV-A16 virus. We found no difference in HRV-A16 RNA levels between control and CDHR3 KO-infected cultures ($P = .9505$; Fig 3, *G*).

The *CDHR3* rs6967330 genotype is associated with increased HRV-C infection of AECs

Our CDHR3 KO experiments are supportive of CDHR3 levels influencing HRV-C infection. The CDHR3 coding risk variant rs6967330 has been shown to increase CDHR3 protein surface localization when allelic forms of the protein are expressed heterologously in HeLa and 293T cells.[13,16] Therefore we hypothesized that donor AECs natively expressing the CDHR3 variant (A) allele would exhibit higher HRV-C infection levels than donor AECs expressing the nonrisk-associated CDHR3 (G) allele.

To test this hypothesis, we compared HRV-C15 infection levels between nasal AEC ALI cultures generated from 6 pediatric donors homozygous for the G allele (poorer surface localization) versus 6 donors homozygous for the A allele (better surface localization). After HRV-C15 infection, we found a 4.3-fold increase in viral RNA isolated from the AA donor cells compared with the GG donor cells at 4 hours after infection ($P = .0021$, Fig 4). Likewise, at 48 hours, we observed a 7.5-fold higher infection level in the AA versus GG subjects ($P = .0390$, Fig 4). For the first time, these results show in primary human AECs differentiated from donors with different alleles for the CDHR3 SNP rs6967330 that carriage of the homozygous (A) genotype increases the risk of HRV-C15 infection at both the early and late stages of rhinovirus infection of the nasal airway epithelium.

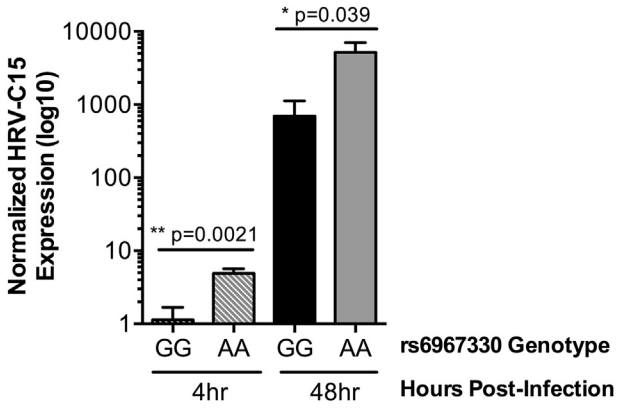


FIG 4. HRV-C15 infection level is higher in AECs expressing the risk-associated CDHR3 rs6967330 genotype. Nasal AECs homozygous for the rs6967330 G allele (n = 6) or homozygous for the A allele (n = 6) were differentiated at the ALI. Cultures were infected with HRV-C15 at a multiplicity of infection *(MOI)* of 0.2 for 4 hours or a total of 48 hours at 34°C before quantitation of HRV-C15 by means of quantitative PCR.

The rs6967330 variant is an eQTL associated with asthma hospitalization in minority children

We also considered whether the rs6967330 SNP can function as an eQTL in the airway epithelium to increase *CDHR3* expression levels and thus susceptibility to HRV-C infection. To investigate this, we extracted *CDHR3* eQTL data from the ongoing, genome-wide nasal airway epithelial eQTL study of the GALA II cohort (unpublished data). In this study we are using whole-transcriptome sequencing expression data on nasal brushings from 431 asthmatic children and 242 healthy control children paired with genome-wide genotype data to perform a cis-eQTL analysis. We tested all genic SNPs and those 1 Mb upstream and downstream of the gene for association with *CDHR3* expression. We identified 11 independent eQTL loci for the *CDHR3* gene (see Table E5 in this article's Online Repository at www.jacionline.org). A locus zoom plot revealed the gene localization and linkage disequilibrium (LD) structure for the top 4 loci, all with association *P* values of less than 1×10^{-10} (Fig 5, *A*). The *CDHR3* eQTL SNP with the fourth strongest effect was part of an LD block that also included the rs6967330 coding risk SNP. We found the rs6967330 A allele, which we found to be associated with increased HRV-C infection in primary AECs, was also associated with greater *CDHR3* expression levels ($P = 2.16 \times 10^{-9}$; Fig 5, *B*). In fact, we found there was a linear relationship between the number of variant alleles (higher expressed allele) for these top 4 eQTL SNPs a subject carried and *CDHR3* gene expression levels ($P = 2.86 \times 10^{-81}$; Fig 5, *C*). We tested the top 4 eQTL SNPs, as well as a variable for the total number of risk alleles carried, for association with asthma hospitalization risk in 670 Mexicans, 1153 Puerto Ricans, and 420 other Latino children with asthma by using a meta-analysis. We found only the rs73195680 SNP, which is located in the LD block with the rs6967330 loci, and rs6967330 itself were associated with asthma hospitalization risk ($P = 5.0 \times 10^{-3}$ and 7.0×10^{-3}, respectively; see Table E6 in this article's Online Repository at www.jacionline.org). These results reveal strong

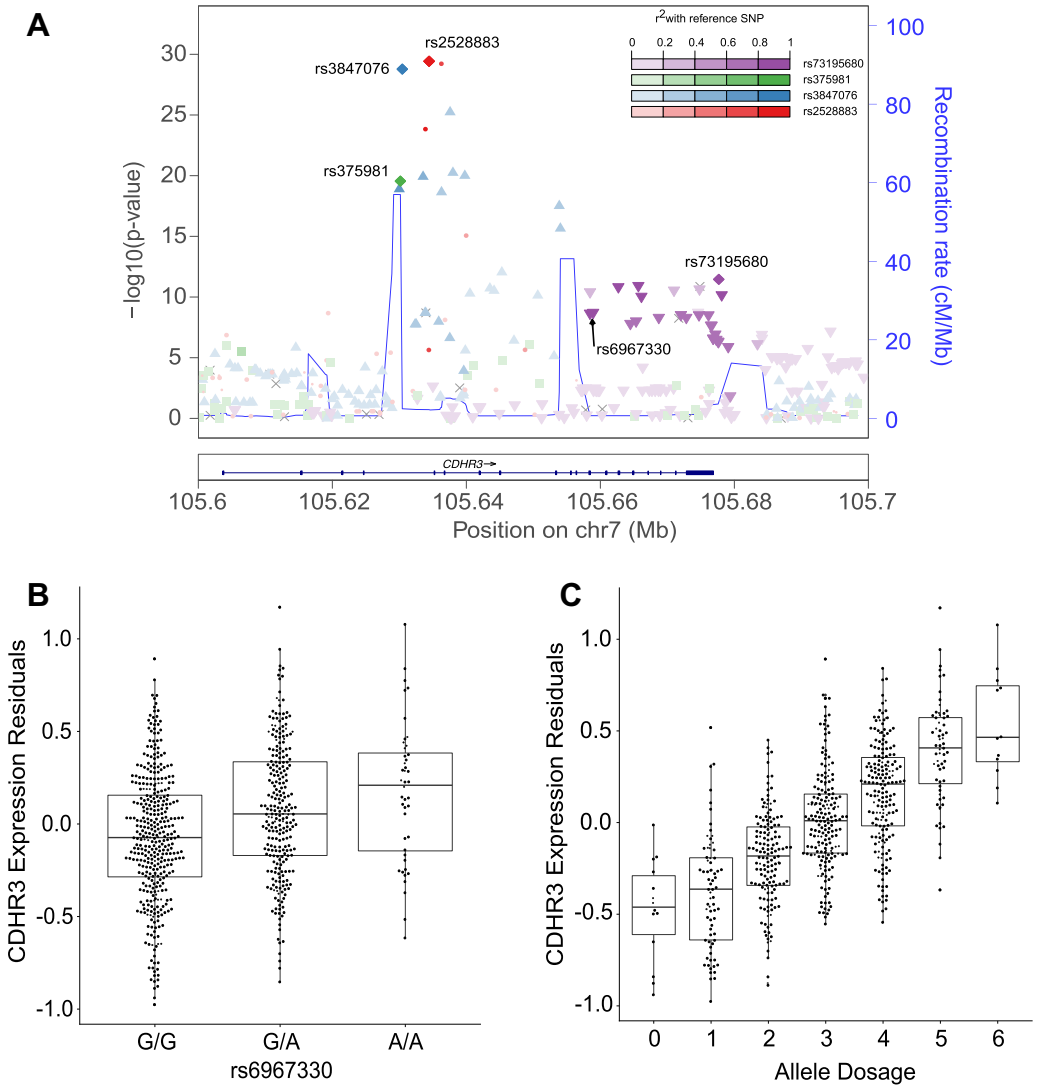


FIG 5. The rs6967330 risk variant and other cis-variants are eQTLs for the *CDHR3* gene in the nasal airway epithelium of children. **A,** The top 4 independent cis-eQTLs for *CDHR3* are depicted in the *CDHR3* regional plot. **B,** The *CDHR3* risk variant rs6967330 acts as an eQTL for *CDHR3* gene expression. **C,** Additive effect of *CDHR3* cis-eQTL variants on *CDHR3* expression. Allele dosage was calculated as the sum of all eQTL-associated (higher expression) effect alleles across the top 4 independent *CDHR3* cis-eQTL variants.

genetic regulation of *CDHR3* gene expression but that only the rs6967330 coding variant LD block confers greater risk of asthma hospitalization in minority children.

DISCUSSION

The translation of novel asthma genetic findings into better understanding of molecular disease mechanisms has been impeded by a lack of suitable research methodologies in human airway tissue and primary cells. We applied a series of novel approaches to human AECs, including single-cell transcriptomics, CRISPR gene editing, genotype-specific primary tissue culture, and eQTL studies to better understand the role of CDHR3 in virus-induced asthma exacerbations.

Our single-cell transcriptomic data identifying most cell types in the human lung confirm a prior report of CDHR3 expression being restricted to ciliated cells within the airway epithelium.[14] A recent report demonstrated that the mouse *CDHR3* ortholog is expressed in a subset of alveolar cells; however, we did not detect *CDHR3* in alveolar cells, and thus this is likely not a conserved pattern of expression.[15] Because CDHR3 has been shown to be necessary for HRV-C infection, our data firmly establish the tropism of HRV-C exclusively to ciliated cells in the human lung.

We leveraged the power of CRISPR-Cas9 technology to generate CDHR3 KO AECs. Although KO did not abolish HRV-C infection, it was reduced by 80%, strongly supporting a significant role for CDHR3 in HRV-C infection. Surprisingly, we did not observe an effect of KO on viral binding (although we did observe an effect of the CDHR3 variant risk on HRV-C binding). This result could be related to the timing or technical limitations of the binding assay, as well as greater sensitivity of the binding assay to incomplete KO. However, the reduced but measurable level of HRV-C infection we observed might also suggest the

existence of coreceptors or weak alternative receptors among the mature mucociliary epithelium and/or other roles for CDHR3 in HRV-C infection, including viral replication. In the future, development of an antibody against the extracellular domain of CDHR3 will allow a more complete investigation of the status of CDHR3 as the exclusive receptor for HRV-C in human AECs. Nonetheless, our CRISPR KO of CDHR3 allowed us to directly determine in primary well-differentiated AECs that robust HRV-C infection is dependent on CDHR3 expression.

Because our data strongly support a role for CDHR3 in HRV-C infection of the human mucociliary airway epithelium, we addressed whether the rs6967330 asthma exacerbation risk variant could modulate HRV-C infection in well-differentiated AECs from genotyped donors. Our finding of a 7.5-fold increase in HRV-C infection among AECs derived from donors carrying the AA risk genotype strongly supports increased HRV-C infection as a mechanism for exacerbation risk associated with this variant. A prior study reported that the CDHR3 protein carrying the rs6967330 risk allele (AA) results in greater protein surface localization than the nonrisk allele,[13,16] providing a possible mechanism for our finding of increased HRV-C infection of AECs differentiated from donors with the AA genotype and consequently exacerbation risk. Unfortunately, the lack of an extracellular CDHR3 antibody has prevented formal confirmation of this increased surface localization of the endogenously expressed risk-associated rs6967330 allele in differentiated AECs.

Given that a large number of complex disease risk variants are regulatory in nature, we decided to explore whether rs6967330 or other cis variants function as eQTLs for the *CDHR3* gene. Our comprehensive cis-eQTL analysis of the nasal airway epithelium from more than 600 children established that *CDHR3* gene expression is modulated by genetic variation, with 11 independent eQTLs identified. For the first time, this analysis allowed us to establish that the rs6967330 SNP is part of a CDHR3 eQTL LD block. Because the risk A allele of the rs6967330 SNP was associated with greater *CDHR3* gene expression, this presented the possibility that eQTL function rather than the previously suggested increase in CDHR3 protein localization at the cell surface could explain the increased HRV-C infection and exacerbation risk associated with the rs6967330 variant. If this hypothesis was correct, we would have expected the rs6967330 LD block, which was the fourth strongest eQTL loci, and the 3 stronger *CDHR3* cis-eQTLs to be associated with asthma exacerbation risk. Instead, our finding that only the rs6967330 eQTL was associated with asthma hospitalization risk strongly supports an alternative mechanism for the variant risk, likely the reported change in surface localization. Moreover, our association between the rs6967330 variant and asthma-related hospitalization presents the first evidence that this variant confers risk in Hispanic populations. This extends a growing body of data implicating the rs6967330 variant in the risk of asthma exacerbations/hospitalization and related traits, including early-life bronchiolitis, early-onset asthma, and chronic rhinosinusitis.[16-19,29]

Monitoring of gene and protein expression throughout AEC differentiation indicated robust CDHR3 expression before the appearance of cilia. Our finding of CDHR3 localization near the basal bodies in ciliating and mature ciliated cells raised the possibility of its involvement in ciliogenesis. CDHR3 KO AECs differentiated at ALI allowed us to conclusively determine that CDHR3 loss does not block ciliated cell formation. We also localized CDHR3 to the apical surface in the vicinity of ezrin, a protein that links the apical cytoskeleton to the apical membrane. We speculate that CDHR3 might also be a component and possibly regulator of this network of actin and microtubules that is in constant contact with cilia, the cell surface, and apical cell junctions. Through these interactions, CDHR3 can affect many vital ciliated cell processes, including barrier function, which is supported by the consistent decrease in transmembrane resistance on CDHR3 KO. If the reduced transmembrane resistance is mediated by a change in barrier function, this could allow greater penetration of allergens, toxins, and microorganisms in the airway, providing a potential mechanism for CDHR3 function influencing asthma exacerbation risk. Further work will be needed to confirm an effect of CDHR3 on barrier function.

In summary, our work here has provided some of the first data on CDHR3 expression, biology, and molecular genetics in the human airway epithelium. We have revealed the likely mechanism underlying the risk of asthma exacerbations conferred by the CDHR3 rs6967330 variant while extending this risk to minority children with asthma. These data form the basis for both further human genetics and molecular mechanistic studies of CDHR3 involvement in childhood asthma and asthmatic airway epithelium.

We thank Shirley Sobus and Josh Loomis at the National Jewish Health Flow and Microscopy Core for guidance with instrument use and analysis.

Key messages

- **Genetic deletion of CDHR3 in human mucociliary epithelial cultures strongly supports involvement of CDHR3 in HRV-C infection of human AECs.**
- **A CDHR3-coding SNP (rs6967330) increases HRV-C infection of AECs and is associated with asthma hospitalization in minority children.**

REFERENCES

1. Ivanova JI, Bergman R, Birnbaum HG, Colice GL, Silverman RA, McLaurin K. Effect of asthma exacerbations on health care costs among asthmatic patients with moderate and severe persistent asthma. J Allergy Clin Immunol 2012;129:1229-35.
2. Nurmagambetov T, Kuwahara R, Garbe P. The economic burden of asthma in the United States, 2008-2013. Ann Am Thorac Soc 2018;15:348-56.
3. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995;310:1225-9.
4. Johnston SL, Sanderson G, Pattemore PK, Smith S, Bardin PG, Bruce CB, et al. Use of polymerase chain reaction for diagnosis of picornavirus infection in subjects with and without respiratory symptoms. J Clin Microbiol 1993;31:111-7.
5. Khetsuriani N, Lu X, Teague WG, Kazerouni N, Anderson LJ, Erdman DD. Novel human rhinoviruses and exacerbation of asthma in children. Emerg Infect Dis 2008;14:1793-6.
6. Zhao M, Zhu WJ, Qian Y, Sun Y, Zhu RN, Deng J, et al. Association of different human rhinovirus species with asthma in children: a preliminary study. Chin Med J (Engl) 2016;129:1513-8.
7. Chen WJ, Arnold JC, Fairchok MP, Danaher PJ, McDonough EA, Blair PJ, et al. Epidemiologic, clinical, and virologic characteristics of human rhinovirus infection among otherwise healthy children and adults: rhinovirus among adults and children. J Clin Virol 2015;64:74-82.
8. Bizzintino J, Lee WM, Laing IA, Vang F, Pappas T, Zhang G, et al. Association between human rhinovirus C and severity of acute asthma in children. Eur Respir J 2011;37:1037-42.
9. Miller EK, Khuri-Bulos N, Williams JV, Shehabi AA, Faouri S, Al Jundi I, et al. Human rhinovirus C associated with wheezing in hospitalised children in the Middle East. J Clin Virol 2009;46:85-9.

10. Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, et al. The major human rhinovirus receptor is ICAM-1. Cell 1989;56:839-47.
11. Hofer F, Gruenberger M, Kowalski H, Machat H, Huettinger M, Kuechler E, et al. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. Proc Natl Acad Sci U S A 1994;91:1839-42.
12. Bochkov YA, Palmenberg AC, Lee WM, Rathe JA, Amineva SP, Sun X, et al. Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C. Nat Med 2011;17:627-32.
13. Bochkov YA, Watters K, Ashraf S, Griggs TF, Devries MK, Jackson DJ, et al. Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication. Proc Natl Acad Sci U S A 2015; 112:5485-90.
14. Griggs TF, Bochkov YA, Basnet S, Pasic TR, Brockman-Schneider RA, Palmenberg AC, et al. Rhinovirus C targets ciliated airway epithelial cells. Respir Res 2017;18:84.
15. Zacharias WJ, Frank DB, Zepp JA, Morley MP, Alkhaleel FA, Kong J, et al. Regeneration of the lung alveolus by an evolutionarily conserved epithelial progenitor. Nature 2018;555:251-5.
16. Bonnelykke K, Sleiman P, Nielsen K, Kreiner-Moller E, Mercader JM, Belgrave D, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. Nat Genet 2014;46:51-5.
17. Stenberg Hammar K, Niespodziana K, van Hage M, Kere J, Valenta R, Hedlin G, et al. Reduced CDHR3 expression in children wheezing with rhinovirus. Pediatr Allergy Immunol 2018;29:200-6.
18. Kanazawa J, Masuko H, Yatagai Y, Sakamoto T, Yamada H, Kaneko Y, et al. Genetic association of the functional CDHR3 genotype with early-onset adult asthma in Japanese populations. Allergol Int 2017;66:563-7.
19. Husby A, Pasanen A, Waage J, Sevelsted A, Hodemaekers H, Janssen R, et al. CDHR3 gene variation and childhood bronchiolitis. J Allergy Clin Immunol 2017;140:1469-71.e7.
20. Neophytou AM, White MJ, Oh SS, Thakur N, Galanter JM, Nishimura KK, et al. Air pollution and lung function in minority youth with asthma in the GALA II (Genes-Environments and Admixture in Latino Americans) and SAGE II (Study of African Americans, Asthma, Genes, and Environments) studies. Am J Respir Crit Care Med 2016;193:1271-80.
21. Nishimura KK, Galanter JM, Roth LA, Oh SS, Thakur N, Nguyen EA, et al. Early-life air pollution and asthma risk in minority children. The GALA II and SAGE II studies. Am J Respir Crit Care Med 2013;188:309-18.
22. Kosmider B, Mason RJ, Bahmed K. Isolation and characterization of human alveolar type II cells. Methods Mol Biol 2018;1809:83-90.
23. Chu HW, Rios C, Huang C, Wesolowska-Andersen A, Burchard EG, O'Connor BP, et al. CRISPR-Cas9-mediated gene knockout in primary human airway epithelial cells reveals a proinflammatory role for MUC18. Gene Ther 2015;22:822-9.
24. Reynolds SD, Rios C, Wesolowska-Andersen A, Zhuang Y, Pinter M, Happoldt C, et al. Airway progenitor clone formation is enhanced by Y-27632-dependent changes in the transcriptome. Am J Respir Cell Mol Biol 2016;55:323-36.
25. Everman JL, Rios C, Seibold MA. Primary airway epithelial cell gene editing using CRISPR-Cas9. Methods Mol Biol 2018;1706:267-92.
26. Everman JL, Rios C, Seibold MA. Utilization of air-liquid interface cultures as an in vitro model to assess primary airway epithelial cell responses to the type 2 cytokine interleukin-13. Methods Mol Biol 2018;1799:419-32.
27. GTEx Consortium; Laboratory, Data Analysis &Coordinating Center (LDACC)—Analysis Working Group; Statistical Methods groups—Analysis Working Group; Enhancing GTEx (eGTEx) groups; NIH Common Fund; NIH/NCI, et al. Genetic effects on gene expression across human tissues. Nature 2017;550:204-13.
28. Vladar EK, Stearns T. Molecular characterization of centriole assembly in ciliated epithelial cells. J Cell Biol 2007;178:31-42.
29. Chang EH, Willis AL, McCrary HC, Noutsios GT, Le CH, Chiu AG, et al. Association between the CDHR3 rs6967330 risk allele and chronic rhinosinusitis. J Allergy Clin Immunol 2017;139:1990-2.e2.