Methods Collection

Cell culture

Primary human bronchial epithelial cells (HBEpC) were procured from PromoCell GmbH (Heidelberg, Germany) and sub-cultured in media supplemented with growth factors as suggested by the supplier. MDCK cells were cultivated in Eagle's Minimum Essential Medium (ATCC) supplemented to 10% with fetal bovine serum and 100 IU/mL penicillin, and 100 µg/mL streptomycin sulfate. MDCK cells were used for the propagation of influenza viruses H1N1 (A/WSN/33), H9N1 (IWF10), and H9N1 (IP10). H1N1 (A/WSN/33) was from Prof. Robert A. Lamb (Northwestern University, Chicago, IL, USA), and H9N1 viruses were from Daniel Perez (Georgia University, GA).

Viral infections

All infections of HBEpCs were performed in 6-well plates at different multiplicity of infection (MOI) as specified for each experiment. Control cells were mock-infected and used as 'mock". Six-well plates were seeded with 1 x 10^5 cells per well and grown to 80% confluence. All infection experiments were done after washing the cells with phosphate buffered saline (PBS), and the virus diluted in Modified Hanks Buffer Saline Solution (MHBSS) was added as described above. After 45 minutes of incubation, excess virus solution was rinsed off using cold PBS and then fresh F12 media was added with or without 1.0 µg/mL of TPCK-trypsin (Sigma-Aldrich, St Louis, MO, USA), depending on the IAV strains used, and infected cells were incubated at 37°C and 5% CO_2.

Overexpression of hBD1

HBEpCs were transiently transfected with the open reading frame (ORF) of *DEFB1* cloned in pCMV6-Entry vector (Origene, MD, USA) and lipofectamine 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA). Controls are cells transfected with an empty vector pCMV6. Following transfection for 48 h, cells were infected with IAV at different MOI for another 24 h.

Transfection of miRNAs and siRNAs

HBEpC were transfected with miRNA inhibitor oligonucleotide or a mimic oligonucleotide (Thermo Fisher Scientific, Carlsbad, CA, USA) using the lipid-based Lipofectamine 2000 reagent diluted in Opti-MEM-I reduced serum medium (Thermo Fisher Scientific) according to the suppliers' protocol. Briefly, HBEpCs were grown in 6-well plates overnight and the transfection mixture was directly applied to the cells at a final concentration of 25 nM oligonucleotides. Cells were transfected with the same concentrations of scrambled oligonucleotides (Thermo Fisher Scientific) and used as control. The transfected cells were then infected at various MOIs. Following the incubation, cells were harvested and used for RNA extraction using RNeasy kit (Qiagen, MD, USA).

The interactions between *DEFB1* and transcription factors during H1N1 infection were further investigated by silencing *DEFB1* expression in HBEpCs with *DEFB1*.siRNA (Dharmacon, Chicago, IL). Exponentially growing cells were transiently transfected with either 25 nM of *DEFB1*.siRNA or the scrambled siRNA control using lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours following transfection, cells were infected with 1 MOI of H1N1

and incubated for an additional 24 h. Following the incubation, cells were harvested and used for RNA extraction using RNeasy kit.

Gene expression and Transcription factor PCR array

Total RNA was isolated from HBEpCs using RNeasy kit (Qiagen) and following the protocol of the supplier. Total RNA (1 µg) was converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). TaqMan primers for *DEFB1*, *NF-κB*, *BCL-XL,* and glyceraldehyde phosphate dehydrogenase (*GAPDH*) were purchased from Applied Biosystems (Thermo Fisher Scientific, Foster City, CA, USA).

PCR array analysis for expression and identification of different transcription factors that are differentially regulated in response to IAV infection was done using TaqMan™ Array, Human Transcription Factors (Non-Hox), Fast 96-well (Catalogue # 4418784, Thermo Fisher Scientific). Gene expression results were normalized to GAPDH, and data were analyzed using the supplier's online software and the ΔΔCt method. RT-PCR was performed using TaqMan assay with matrix gene-specific primers. IAV Matrix gene expression was quantified using a standard curve and reported as influenza copy number.

Plaque Assay for IAV Detection

For viral plaque assay, confluent MDCK cells were propagated in 6-well plates. Confluent monolayers of MDCK cells were prerinsed with PBS and infected with serially diluted cell culture supernatant (800 µL per well) collected from the HBEpC cells exposed to influenza virus. Infected cells were incubated for 45 min at 35 °C. Cells were then washed with PBS and overlayed with 0.6% agarose (Oxoid Ltd., Hampshire, UK) in DMEM/F12 medium, and plates were kept at 35 °C for another 60 h. Following the incubation, cells were treated with 10% formalin and agarose layer was removed. Cells were stained with 1.0% crystal violet and plaque-forming units (PFU) were counted

Confocal microscopy

HBEpCs overexpressing *DEFB1* were grown on chamber slides overnight (Chamber slide™, Lab-TekII, Thermo Fisher Scientific, Rochester, NY, USA) to 80-90% confluence. Cells were then exposed to H1N1 for 24 h. Subsequently, cells were washed with PBS and fixed with 4% methanol-free formaldehyde (Polysciences Inc., Warrington, PA, USA). Immunofluorescent staining was done with anti-IAV antibodies (Millipore, Billerica, MA, USA), anti-hemagglutinin, anti-influenza PB1, and anti-hBD1 antibodies (Abcam, Waltham, MA, USA) for 1 h, followed by appropriate secondary antibodies (Alexa 488 and Alexa-555; Thermo Fisher Scientific). Photomicrographs were produced using a Zeiss LSM510 (Carl Zeiss, Obertochen, AG Germany).

Western immunoblot analysis

HBEpC cells were transfected with *DEFB1* plasmid or control plasmid for 36-48 h, cells were then exposed to H1N1 for an additional 24 h and protein extracts (30 ug) from the cells were prepared with 30µl of radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) and analyzed by10% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) and probed with anti-

STAT3, anti-phosphorylated-STAT3 and mouse monoclonal anti-GAPDH (Abcam). Appropriate IRDye 680 or 800 secondary antibodies (LI-COR Biosciences) were used. Fluorescence detection was performed on the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and the signal intensities of the individual bands were determined. Protein quantitation was carried out using the Image lite software (LI-COR Biosciences).

Argonaute-immunoprecipitation

Co-immunoprecipitation of Argonaute (Ago) proteins bound to miRNA/mRNA complexes is a powerful tool to validate miRNA targets (21). Ago Immunoprecipitation (Ago-IP) of the miRNA was done according to the instructions from a kit available from Active Motif (Active Motif, Carlsbad, CA, USA). Briefly, HBEpCs were grown to 80% confluency in six-well plates and transfected with 25 nM mimics of miR-186-5p and miR-340-5p or negative control (scrambled oligonucleotide of mimic) for 24 h. An equal number of cells was taken for the Ago-IP to minimize variability. Ago-IP uses G-coupled magnetic beads and a pan-Ago antibody that recognizes Ago 1, Ago 2, and Ago 3 to precipitate the miRNA/mRNA complex. An isotype antibody control was also run in parallel. Ago-IP was done as described in the manufacturer's protocol. The precipitated complex was collected, and total RNA was purified from the complex using trizol reagent (Qiagen). The RNA was converted to cDNA with the High-Capacity cDNA Reverse Transfection Kit (Thermo Fisher Scientific, CA) and specific primers for *DEFB1* (Thermo Fisher Scientific) were used for RT-PCR. The data were analyzed by comparing the cells transfected with mimic miRNA or negative control oligonucleotide and the fold enrichment of *DEFB1* mRNA was calculated as described by the manufacturer.