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Short communication

Regulation of IFNα-induced expression of the short ACE2 isoform by ULK1

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ABSTRACT

The novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been shown to hijack angiotensin converting enzyme 2 (ACE2) for entry into mammalian cells. A short isoform of ACE2, termed deltaACE2 (dACE2), has recently been identified. In contrast to ACE2, the short dACE2 isoform lacks the ability to bind the spike protein of SARS-CoV-2. Several studies have proposed that expression of ACE2 and/or dACE2 is induced by interferons (IFNs). Here, we report that drug-targeted inhibition or silencing of Unc51-like kinase 1 (ULK1) results in repression of type I IFN-induced expression of the *dACE2* isoform. Notably, dACE2 is expressed in various squamous tumors. In efforts to identify pharmacological agents that target this pathway, we found that fisetin, a natural flavonoid, is an ULK1 inhibitor that decreases type I IFN-induced *dACE2* expression. Taken together, our results establish a requirement for ULK1 in the regulation of type I IFN-induced transcription of *dACE2* and raise the possibility of clinical translational applications of fisetin as a novel ULK1 inhibitor.

1. Introduction

Angiotensin converting enzyme 2 (ACE2) is a key component of the renin-angiotensin system (RAS), primarily expressed in lung, heart, and kidney tissues (Andersen et al., 2020; De et al., 2021). Within the lungs, single-cell RNA sequencing (scRNAseq) has shown that *ACE2* is highly expressed in type II pneumocytes, ciliated cells, and transient secretory cells (Lukassen et al., 2020; Ziegler et al., 2020). Recently, two independent studies provided evidence that interferons (IFNs) induce *ACE2* expression (Ziegler et al., 2020; Zhuang et al., 2020). Follow-up studies have revealed that IFNs and viruses induce the expression of a previously unknown short *ACE2* isoform, termed deltaACE2 (dACE2). This short ACE2 lacks the first 356 amino acids, the carboxypeptidase function, and the ability to bind the spike protein of SARS-CoV-2 compared to the long ACE2 isoform (Blume et al., 2021; Ng et al., 2020; Onabajo et al., 2020). Additionally, screening of the Cancer Genome Atlas identified high expression of the short form of the *ACE2* isoform in squamous tumors of the respiratory, gastrointestinal, and urogenital tracts (Onabajo et al., 2020). The function of this short ACE2 isoform remains unknown, as do the roles of IFNs in the regulation of the two ACE2 isoforms.

IFNs are critical regulators of an immune response (Fenton et al., 2021; Wang and Fish, 2019). Binding of IFN-α/β to the type I IFN receptor, IFNAR, comprised of IFNAR1 and IFNAR2, triggers activation of the canonical Janus activated kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway and non-canonical cellular cascades that lead to expression of IFN-stimulated genes (ISGs). Non-canonical signaling pathways include mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/mechanistic target of rapamycin (PI3K/mTOR) pathways (Mazewski et al., 2020). Earlier studies from our laboratory provided evidence that IFNs activate a novel Unc51-like kinase 1 (ULK1)-dependent signaling cascade, controlling transcription of specific ISGs in a manner definitively unrelated to regulation of autophagy (Saleiro et al., 2018, 2015). Based on these observations, we examined the role of ULK1 in IFNα-mediated transcription of *ACE2* isoforms. We provide evidence that, *in vitro,* IFNα

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induces the expression of the newly identified isoform of *ACE2, dACE2,* in human endothelial, small airway epithelial, and renal epithelial cells. Studies evaluating the effects of small interfering RNA (siRNA)-mediated reduction of *dACE2* suggest a potential role for dACE2 in regulation of tumor cell viability. Moreover, we demonstrate that inhibition of ULK1 reduces IFNα-induced transcription of *dACE2.* Fisetin, a flavonoid compound, is identified as a potent inhibitor of ULK1 kinase activity and IFNα-induced expression of *dACE2*.

2. Material and methods

2.1. Cell lines and reagents

The following human primary cells and cell culture reagents were purchased from Lonza (Alpharetta, GA, USA): Human Umbilical Vein Endothelial Cells (HUVECs) (Catalog#: C2519A) were grown in Endothelial Growth Media-2 (EGM-2) (Catalog#: CC-3162); Human Small Airway Epithelial Cells (SAECs) (Catalog#:CC-2547) were grown in Small Airway Epithelial Growth Medium (SAGM) (Catalog#: CC-3118); Human Renal Epithelial Cells (HREs) (Catalog#: CC-2556) were grown in Human Renal Epithelial Growth Medium (REGM) (Catalog#: CC-3190). The following cell lines were purchased from ATCC: glioblastoma cell line LN229, grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and gentamicin; and the squamous cell carcinoma cell lines SCC9 and SCC25, grown in DMEM:F12 medium supplemented with 10% FBS, 15 mM HEPES and gentamicin. All cell lines were cultured at 37 °C and 5% CO_2. Primary cell lines were used up to passage 9. LN229 cells were routinely subjected to short-tandem repeat (STR) analysis and authenticated using published reference STR profiles. Human Interferon alpha (Infergen) was obtained from Kadmon Pharmaceuticals (Warrendale, PA, USA). The ULK1/2 inhibitor SBI-0206965 and the natural compound Fisetin were purchased from Cayman Chemicals (Ann Arbor, MI, USA); the selective ULK1 inhibitor ULK101 was purchased from Selleckchem (Houston, TX, USA).

2.2. Small interfering RNA (siRNA)

ULK1 siRNA (sc-44182) and control siRNA-B (sc-44230) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *dACE2* (Accession #: NM_001388452.1) siRNAs were designed using Horizon's siDESIGN Center (https://horizondiscovery.com/en/ordering-and-calculation-tools/sidesign-center). The following sense sequences were used: siRNA #1 5'-GGAAGCAGGCUGGGACAAAUU-3', siRNA #2 5'-GGACAAAGGAGGGAGGAUCUU-3'. Cells were transfected using Lipofectamine RNAiMAX (ThermoFisher) according to the manufacturer's protocol.

2.3. RNA isolation and real-time quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Germantown, MD, USA); 2 μg of total RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). The following Taqman Probes from ThermoFisher were used: *ACE2* (Hs01085331_m1) that detects a common region present in both *ACE2 and dACE2*, *ULK1* (Hs00177504_m1), and *GAPDH* (Hs03929097_g1). *GAPDH* was used as a normalization control. Real-time quantitative PCR reactions were performed on a Bio-Rad CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) with the following condition: activation at 95 °C; 30 s, 50 cycles of denaturation at 95 °C, 10 s and annealing/extension at 60 °C; 20 s. SsoAdvanced Universal Probes Supermix (Bio-Rad) was used to carry-out quantitative PCR reactions. Relative gene expression was analyzed by the $^{\Delta\Delta}C_t$ method.

The primers for the long *ACE2* isoform and short *dACE2* isoform for SYBR green reaction were previously described (Onabajo et al., 2020). PCR primers for GAPDH: forward 5'- GAAGGTGAAGGTCGGAGTCA-3', reverse 5'-TTGAGGTCAATGAAGGGGTC-3'. The following conditions were used for SYBR green reactions: activation at 95 °C; 3 mins, 50 cycles of denaturation at 95 °C, 15 s and annealing/extension at 60 °C; 1 min. SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) was used to carry-out quantitative PCR reactions. Relative gene expression was analyzed by the $^{\Delta\Delta}C_t$ method.

2.4. ULK1 Kinase Enzyme System ADP-Glo Assay

ULK1 Kinase Enzyme System ADP-Glo was purchased from Promega (Catalog #:V9191, Madison, WI, USA), and the assay was performed according to the manufacturer's protocol. We screened a custom library selected from the complete TargetMol catalog compromising 1280 natural product compounds used at 10 μM. Each compound was tested in duplicate.

2.5. Cell lysis and immunoblotting

Cells were lysed with RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Na deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP40), supplemented with protease (EMD Millipore Protease Cocktail III) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate), then the proteins resolved by SDS-PAGE and immunoblotting. The anti-ACE2 antibody (# ab15348, 1:500) was purchased from Abcam (Waltham, MA); the anti-ULK1 antibody (# 8054, 1:1000) was from Cell Signaling Technology; and the anti-GAPDH antibody (# MAB374, 1:20,000) was from EMD Millipore. Primary antibodies were detected with anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Bio-Rad), or anti-rabbit HRP-conjugated antibody (Cell Signaling) followed by enhanced chemiluminescence using Amersham ECL Prime western blotting detection reagent (GE Healthcare).

2.6. Cell viability assay

Cell viability was quantified using the Cell Proliferation Reagent WST-1 (Roche), following to the manufacturer's protocol. Fourty-eight hours after transfection, siRNA-transfected cells (2000 cells/well) were plated on a 96-well plate using 5 replicates per experimental condition, and were either left untreated, or were treated with 1, 10, 100, 1000, or 10,000 IU/mL IFNα. After 4 days, WST-1 reagent was added to each well and absorbance was measured using the Synergy HT plate reader and Gen5 software (BioTek).

2.7. Statistical analysis

GraphPad PRISM v8.0 (San Diego, CA, USA) was used for statistical analyses. Each statistical test used is described in detail in each figure legend.

3. Results

3.1. Effects of ULK1 inhibition on IFNα-induced expression of ACE2 isoforms

Recent studies have demonstrated that IFNα induces *ACE2* and/or *dACE2* expression (Ziegler et al., 2020, Zhuang et al. 2020, Blume et al., 2021; Ng et al., 2020; Onabajo et al., 20200). The significance of these findings remains unclear. Thus, we examined the effects of IFNα treatment on *ACE2* and/or *dACE2* expression in three different primary cell lines: HUVECs, SAECs and HRE cells using the TaqMan probe that detects a common region present in both *ACE2 and dACE2*. In all three cell lines, *ACE2* and/or *dACE2* mRNA expression increased following treatment with IFNα for 6 h (Fig. 1A-B). We next examined whether inhibition of ULK1 by the small-molecule ULK1 kinase inhibitors SBI-0206965 (SBI) (Egan et al., 2015) and ULK101 (Martin et al., 2018) would inhibit IFN-inducible *ACE2* and/or *dACE2* expression. Treatment with either

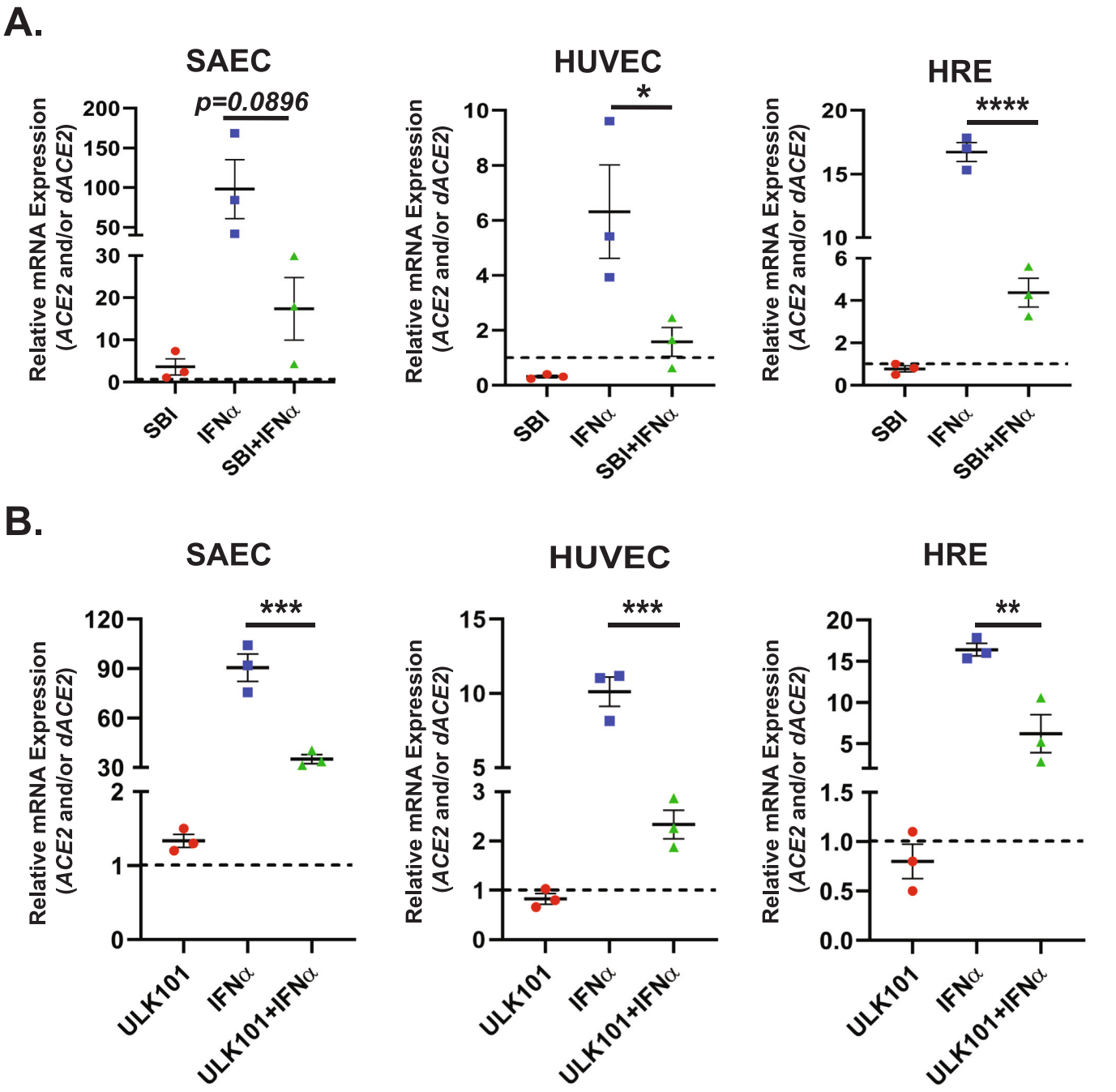

Fig. 1. Drug-targeted inhibition of ULK1 reduces IFN-mediated *ACE2* and/or *dACE2* expression. SAECs, HUVECs, and HRE cells were treated with (A) 10 μM SBI-0206965 (SBI), (B) 10 μM ULK101, and/or 5000 IU/mL IFNα for 6 h. mRNA levels of *ACE2* and/or *dACE2* were assessed by quantitative RT-PCR using the TaqMan probe for *ACE2* (Hs01085331_m1), which detects a common region present in both isoforms, and *GAPDH* for normalization. Data are expressed as relative mRNA expression of *ACE2* and/or *dACE2* over control DMSO-treated cells (dashed line) and represent means ± SEM of three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and *p* values are shown between IFNα-treated and (A) SBI + IFNα-treated cells or (B) ULK101 + IFNα-treated cells. * , $p < 0.05$; * * $p < 0.01$; * ** $p < 0.001$ and * ** * $p < 0.0001$.

inhibitor significantly reduced IFN-inducible *ACE2* and/or *dACE2* expression in all cell lines (Fig. 1A-B). To validate these results, we used siRNA-mediated knockdown of *ULK1* followed by IFNα treatment in all three human primary cell lines. Knockdown of *ULK1* (Fig. 2A-C, left panels and Supplementary Fig. S1) decreased IFNα-induced *ACE2* and/or *dACE2* expression in SAECs, HUVECs and HREs (Fig. 2A-C, right panels), similar to the effects seen by pharmacological ULK1 inhibition (see Fig. 1). These results demonstrate that drug-targeted inhibition or silencing of ULK1 attenuates IFNα-induced *ACE2* and/or *dACE2* expression.

3.2. Effects of fisetin on IFNα-induced expression of ACE2 isoforms

We next undertook a screen for compounds able to inhibit ULK1. We performed a high throughput screen of 1280 natural compounds, to identify lead compounds that were potent inhibitors of ULK1 kinase activity using an Enzyme System ADP-Glo assay. This screen identified the plant flavanol, fisetin, as one of four compounds that demonstrated greater than 50% ULK1 kinase inhibition (Fig. 3A). We validated this screen in fisetin dose response studies and confirmed the inhibitory effects of fisetin on IFNα-inducible *ACE2* and/or *dACE2* expression in all 3

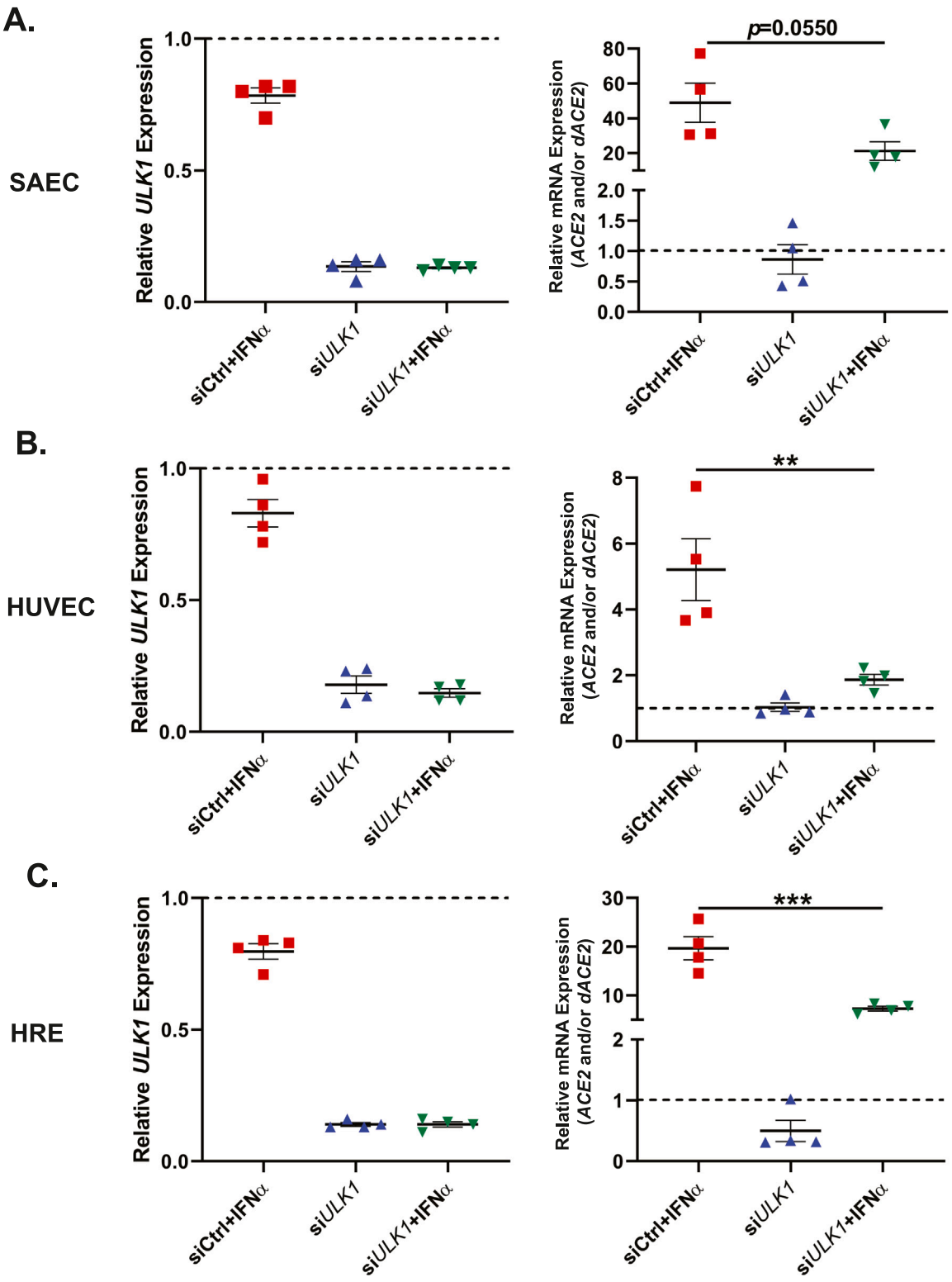

Fig. 2. Knockdown of *ULK1* attenuates IFN-induced *ACE2* and/or *dACE2* expression. (A) SAECs, (B) HUVECs, and (C) HRE cells transfected with either control siRNA (siCtrl) or *ULK1* siRNA (si*ULK1*) were either left untreated or were treated with 5000 IU/mL IFNα for 6 h, and mRNA expression for *ULK1* and *ACE2* isoforms was assessed by quantitative RT-PCR using TaqMan probes and *GAPDH* for normalization. Data are expressed as relative mRNA expression over untreated control siRNA-transfected cells (dashed line) and represent means ± SEM of four independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test and *p* values for *ACE2* and/or *dACE2* expression are shown between IFNα-treated siCtrl-transfected cells and IFNα-treated si*ULK1*-transfected cells. * *, $p < 0.01$; * ** $p < 0.001$.

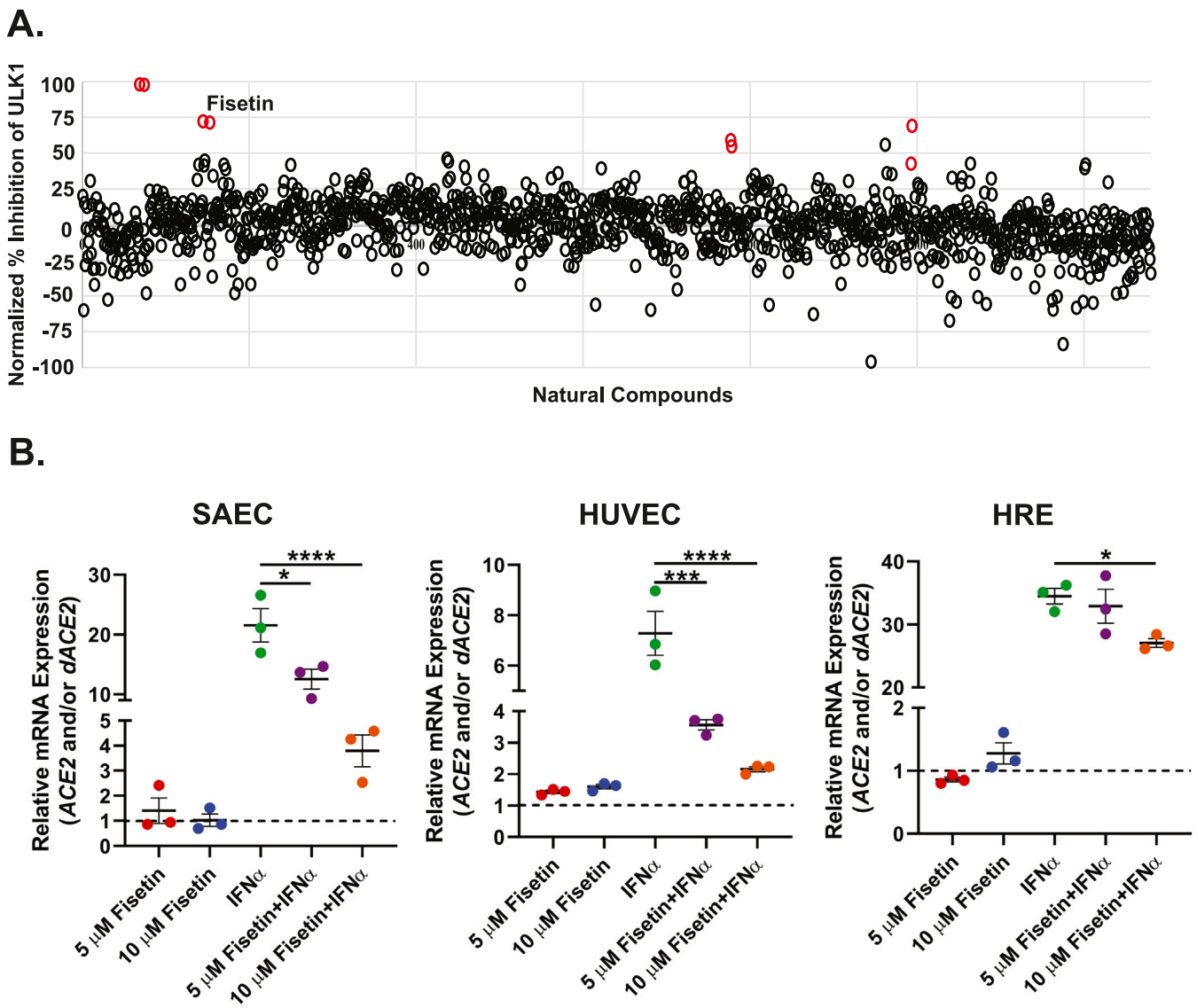

Fig. 3. The natural compound fisetin inhibits ULK1 kinase activity and reduces IFN-induced *ACE2* and/or *dACE2* expression. (A) 1280 natural compounds were screened using the ULK1 Kinase Enzyme System ADP-Glo assay. Depicted are individual circles representing two technical replicates for each compound. Four different natural compounds inhibited ≥ 50% of ULK1 kinase activity (red circles). (B) SAECs, HUVECs, and HRE cells were treated with 5 or 10 μM Fisetin and/or 5000 IU/mL IFNα for 6 h. mRNA expression of *ACE2* isoforms was assessed by quantitative RT-PCR, using the TaqMan probe for *ACE2* (Hs01085331_m1), which detects a common region present in both isoforms, and *GAPDH* for normalization. Data are expressed as relative mRNA expression over control DMSO-treated cells (dashed line) and represent means ± SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test and *p* values are shown between IFNα-treated and Fisetin + IFNα-treated cells. * $p < 0.05$; * ** $p < 0.001$; * ** * $p < 0.0001$.

primary cell lines using the TaqMan probe that detects a common region present in both isoforms (Fig. 3B).

3.3. *ULK1 activity mediates IFNα-induced expression of the short dACE2 isoform*

While our original studies were ongoing, several laboratories described the novel IFN and virus-inducible shorter isoform of ACE2, dACE2 (Blume et al., 2021; Ng et al., 2020; Onabajo et al., 2020). To clearly determine which isoform is affected by ULK1 inhibition, we used distinct primers to discriminate the long *ACE2* and the short *dACE2* isoforms by qRT-PCR analysis (Onabajo et al., 2020). IFNα treatment increased substantially the mRNA levels of *dACE2* compared to those of *ACE2* in SAECs (Fig. 4A-C). Additionally, drug-targeted inhibition of ULK1 using SBI-0206965 or fisetin and siRNA-mediated knockdown of *ULK1* significantly reduced IFNα-induced *dACE2* mRNA expression in SAECs (Fig. 4A-C). In contrast to dACE2, ACE2 protein expression was barely detectable by immunoblotting analysis in SAECs (Fig. 4D). However, gene-targeted inhibition of *ULK1* suppressed the IFNα-mediated increase of dACE2 protein in SAECs (Fig. 4D).

In earlier studies, we provided evidence for IFNα-induced growth inhibition mediated by signaling associated with ULK1 in malignant cells (Saleiro et al., 2015). The role of dACE2 in tumor cell growth is unknown. Accordingly, we designed siRNAs against the sequence encoding the first ten unique amino acids of dACE2, which are absent in ACE2 (Onabajo et al., 2020). To validate the specificity of the siRNAs designed, we assessed the protein levels for both ACE2 and dACE2 isoforms using siRNA-transfected human glioblastoma LN229 cells (Fig. 4E). ACE2 protein expression was barely detectable by immunoblotting in LN229 cells (Fig. 4E). Nonetheless, *dACE2* siRNA #2 was found to reduce dACE2 expression compared to control siRNA-transfected cells (Fig. 4E). *dACE2* siRNA #1 more potently reduced dACE2 expression (Fig. 4E), however it also substantially induced cell death of LN229 cells. Thus, we next evaluated the

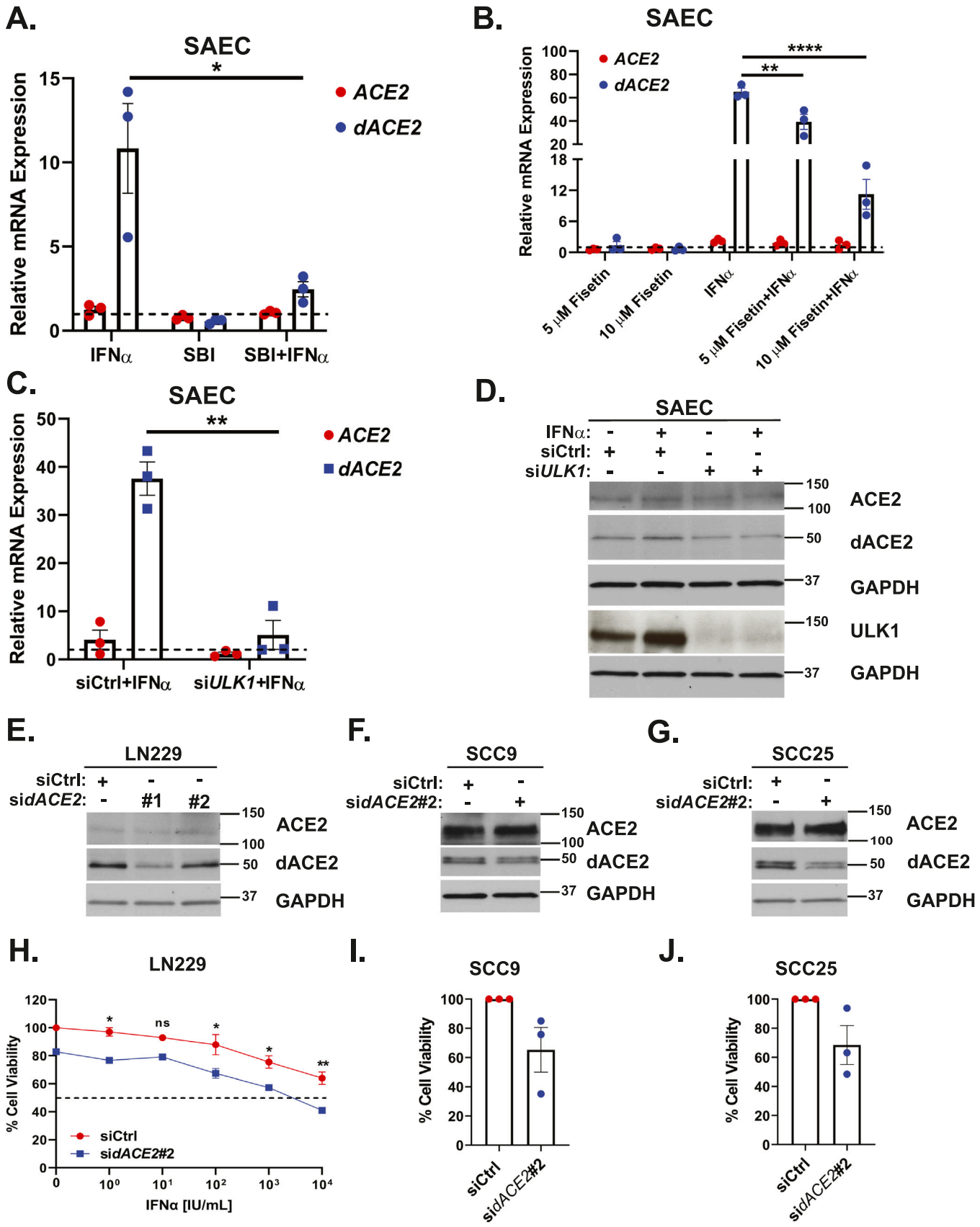

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Fig. 4. *dACE2* as an IFN-stimutated gene in primary small airway epithelial cells and its role in cancer cells. (A) SAECs were either treated with vehicle control (DMSO), 10 μM SBI-0206965 (SBI) and/or with 5000 IU/mL IFNα for 6 h. mRNA expression for the long *ACE2* and short *dACE2* isoforms was assessed by quatitative RT-PCR using specific primers for each isoform (described in Onabajo et al., 2020) and *GAPDH* for normalization. (B) SAECs were treated with or without 5000 IU/mL IFNα in the presence of fisetin (5 or 10 μM) or DMSO (vehicle control) for 6 h. mRNA expression of *ACE2* and *dACE2* was assessed by quantitative RT-PCR using specific primers for each isoform (Onabajo et al., 2020) and *GAPDH* for normalization. (C) SAECs transfected with control siRNA (siCtrl) or *ULK1* siRNA (si*ULK1*) were either left untreated or were treated with 5000 IU/mL IFNα for 6 h, and mRNA expression of *ACE2* and *dACE2* was assessed by quantitative RT-PCR using specific primers for each isoform (Onabajo et al., 2020) and *GAPDH* for normalization. (A-C) Data are expressed as relative mRNA expression over (A-B) DMSO-treated cells or (C) untreated siCtrl-transfected cells (dashed lines) and represent means ± SEM of three independent experiments. Statistical analyses were performed using (A-B) one-way ANOVA followed by Tukey's multiple comparisons test and significant p values are shown between IFNα-treated and (A) SBI + IFNα-treated cells or (B) Fisetin + IFNα-treated cells and (C) using two-sample two-tailed t test between IFNα-treated siCtrl-transfected cells and IFNα-treated si*ULK1*-transfected cells. * , $p < 0.05$; * *, $p < 0.01$ and * ** *, $p < 0.0001$. (D) Equal amounts of total cell lysates from SAECs transfected with control siRNA (siCtrl) or *ULK1* siRNA (si*ULK1*) either left untreated or treated with 1000 IU/mL IFNα for 24 h were immunoblotted with anti-ACE2 and anti-GAPDH antibodies. The same cell lysates were ran in parallel and immunoblotted for ULK1 and GAPDH. (E-G) Immunoblotting analyses of dACE2 and ACE2 in lysates from LN229 glioblastoma cells (E), SCC9 (F) and SCC25 (G) cells 48 h after transfection with siCtrl or specific *dACE2* siRNAs (si*dACE2*#1 or si*dACE2*#2). (H) 48 h after transfection with siCtrl or si*dACE2*#2, LN229 cells were plated in a 96-well plate and treated with increasing concentrations of IFNα for 4 days. Cellular viability was assessed using WST-1 assay. Data are expressed as percentage of untreated siCtrl-transfected cells and represent means ± SEM of three independent experiments, each done in 5 replicates. Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparisons test between IFNα-treated siCtrl-transfected cells and IFNα-treated si*dACE2*-transfected cells. * , $p < 0.05$; * *, $p < 0.01$; ns, $p > 0.05$. (I-J) SCC9 (I) and SCC25 (J) cells were transfected with control siRNA (siCtrl) or si*dACE2*#2. After 48 h, transfected cells were plated in a 96-well plate and cellular viability was assessed 4 days later using WST-1 assay. Data are expressed as percentage of siCtrl-transfected cells and represent means ± SEM of three independent experiments, each done in 5 replicates.

specificity of *dACE2* siRNA #2 in the squamous cell carcinoma cell lines SCC9 and SCC25, known to express high levels of ACE2 and dACE2 (Onabajo et al., 2020). Similar to LN229 cells, *dACE2* siRNA #2 specifically reduced dACE2 protein levels and not the long ACE2 isoform (Fig. 4F-G). In cell viability assays, we observed that siRNA-mediated knockdown of *dACE2* reduces the proliferation of all three cell lines compared to control siRNA-transfected cells (Fig. 4H-J). Albeit modest, siRNA-mediated reduction of dACE2 was found to decrease the viability of LN229, SCC9 and SCC25 cells on average by 17.16% ± 0.26, 34.69% ± 15.34% and 32.06% ± 12.82, respectively (Fig. 4H-J). Moreover, knockdown of *dACE2* in combination with IFNα treatment resulted in additive anti-proliferative effects in LN229 cells (Fig. 4H).

4. Discussion

ACE2 expression is critical for SARS-CoV-2 entry into the cells (Hoffmann et al., 2020; Lukassen et al., 2020; Yan et al., 2020). In early studies, human and non-human primate type II pneumocytes were shown to express detectable levels of *ACE2* mRNA compared to other respiratory cell types, suggesting that type II pneumocytes could represent a vulnerable point of entry for SARS-CoV-2 infection (Ziegler et al., 2020). Additionally, ACE2 expression was detected in cells from human immunodeficiency virus (HIV)+ donors, which also presented increased levels of other ISGs consistent with the presence of chronic IFN signaling (Utay and Douek, 2016), suggesting that *ACE2* expression could be induced by IFNs (Ziegler et al., 2020). Using *in silico*, *in vitro*, and *in vivo* analyses, Ziegler et al. showed that *ACE2* expression is induced by IFN treatment and viral infections. In follow-up studies, several groups have identified a novel isoform of ACE2 (dACE2, MIRb-ACE2, or short ACE2) inducible by type I, II, and III IFNs and by several types of viruses including SARS-CoV-2 (Blume et al., 2021; Ng et al., 2020; Onabajo et al., 2020). Thus, fully discerning which *ACE2* isoform is induced by IFNs is essential to determine its role on IFN-mediated biological responses.

Once secreted, IFNs bind to their respective cell surface transmembrane receptors, which interact with JAK family members activating the classical STAT signaling cascades (Mazewski et al., 2020; Platanias, 2005). Additionally, engagement of IFN receptors activates other signaling pathways, including MAPK cascades (Mazewski et al., 2020; Platanias, 2005). Also, we have previously shown that the autophagy initiator ULK1 is necessary for the ideal type I IFN-dependent phosphorylation of p38 MAP kinase and subsequent transcription of ISGs in an autophagy-independent manner (Saleiro et al., 2015). It is the concerted activation of these multiple signaling cascades that controls optimal expression of ISGs, ultimately driving IFN-dependent biological responses by the products of these genes (Mazewski et al., 2020; Platanias, 2005; Saleiro et al., 2015). In the current study, we identify ULK1 as an essential component of the IFN signaling pathway that stimulates *dACE2* expression. The *dACE2* promoter region exhibits binding sites for transcription factors that are known to be activated downstream of the IFN-p38 MAPK pathway, suggesting that ULK1 controls transcription of *dACE2* via regulation of p38 MAPK signaling pathways (Ng et al., 2020; Saleiro et al., 2015). Future studies are necessary to fully characterize the pathways that contribute to IFN-ULK1-mediated *dACE2* expression.

Recent efforts have aimed at identifying compounds that constrain IFN-mediated *ACE2* expression. For instance, a recent study found that fludarabine, which inhibits STAT1, among other proteins, was able to dampen type I IFN-induced expression of *ACE2* (Xiu et al., 2021). Another study reported that IFNs mainly activate transcription of *dACE2* and, to a lower degree, *ACE2* in human airway epithelial cells and this was mitigated by the JAK inhibitors ruxolitinib and baricitinib (Lee et al., 2021). Here, we identified ULK1 as a key and essential component of the IFN-ULK1-dACE2 axis, suggesting ULK1 as a potential drug target to block this cascade. Hence, we examined 1280 natural compounds for their ability to inhibit ULK1 kinase activity and identified fisetin as one of the most potent ULK1 inhibitors tested in our screen. Fisetin is a natural flavonoid found in fruits and vegetables and it has been studied for its anti-oxidant (Hanneken et al., 2006), anti-inflammatory (Higa et al., 2003), and anti-neoplastic (Imran et al., 2021; Jia et al., 2019) properties and is available as a dietary health supplement (Pal et al., 2016). Our data show that fisetin inhibits IFN-induced expression of *dACE2*, reminiscent of specific small-molecule ULK1 inhibitors, raising the possibility of future clinical-translational applications using this compound.

Undoubtedly, the biological role of the recently discovered dACE2 isoform needs further investigation. dACE2 lacks ACE2's carboxypeptidase function and the ability to interact with the spike protein of SARS-CoV-2 (Blume et al., 2021; Ng et al., 2020; Onabajo et al., 2020). Busnadiego et al. (2020) have shown that all three types of IFN block SARS-CoV-2 replication, indicating that any IFN-inducible increase in dACE2 expression does not facilitate SARS-CoV-2 entry into the cells. Moreover, accumulating evidence suggests that dACE2 is a bona fide ISG (Blume et al., 2021; Ng et al., 2020, Onabajo et al., 2020, Scagnolari C et al.,2021). In addition, dACE2 may also play a role during tumor progression as indicated by dACE2 overexpression in squamous and other solid tumors (Onabajo et al., 2020). In line with this, our data showed that siRNA-mediated reduction of *dACE2* decreases cellular viability of LN229 glioblastoma cells and of the squamous cell carcinoma cell lines SCC9 and SCC25, albeit modestly. This inhibition in LN229 cells adds to the suppressive effects of Type I IFN treatment. Future studies are warranted to examine the role of dACE2 in the growth

of malignant cell lines of diverse neoplastic origin, as well as primary malignant cells.

5. Conclusion

IFNs induce expression of both immunostimulatory and immunosuppressive genes, as well as genes that promote pro- and anti-tumor effects (Arslan et al., 2017; Fenton et al., 2021; Fischietti et al., 2021; Saleiro and Platanias, 2019). The identification of an IFN-ULK1-dACE2 axis adds an additional layer to the complexity of IFN signaling in human disease progression. The recent discovery of dACE2 and the crucial role of ULK1 in *dACE2* expression as an ISG invites further exploration of this isoform in human malignancies and in response to pathogens.

CRediT authorship contribution statement

R.E.P, D.S, F.E, and L.C.P designed research; R.E.P, D.S, L.I, and F.E performed research, R.E.P, D.S, L.I, F.E, G.E.S, E.N.F. and L.C.P analyzed data/interpreted experimental results. R.E.P, D.S, F.E, E.N.F, and L.C.P wrote/edited the manuscript. All authors read and approved the final manuscript.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon request.

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Declaration of competing interest

The authors declare that they have co conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2022.04.008.

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