



NRF2 controls a diverse network of antiviral effectors with p62 acting as a central restriction factor effective across virus families

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ABSTRACT

The transcription factor erythroid 2 (NFE2)-related factor 2 (NRF2) is a key regulator of cellular homeostasis. Recent discoveries have identified agonists of NRF2 as inducers of broad cellular resistance to viral infection including SARS-CoV-2. Nevertheless, it is still unclear to what extent NRF2 itself is an inducer of anti-viral immunity and its downstream antiviral effectors have not been mapped. Here, we first demonstrate through specific genetic activation and silencing that NRF2 restricts SARS-CoV-2 replication. We then used a focused CRISPR-activation screen to map antiviral NRF2-inducible effector genes that restrict replication of SARS-CoV-2, Influenza A virus (IAV), Herpes Simplex virus 1 (HSV1) and Vaccinia virus (VACV). This approach allowed us to identify a range of antiviral effectors each of which restrict members of one or more virus families. Importantly, we identified the NRF2-inducible selective autophagy receptor p62/SQSTM1 as a broadly effective restriction factor across all the tested viruses. Importantly, p62 inhibited SARS-CoV-2 replication in cells treated with the lysosomal inhibitor bafilomycin A1, as well as in cells deficient in the autophagy protein ATG5. Similarly, p62 inhibited replication of HSV1 and IAV independently of ATG5 and ATG16L1 respectively. Thus, NRF2 restricts viral replication through a hitherto underappreciated network of antiviral restriction factors effective across multiple virus families. Importantly, we identify p62 as a broadly acting antiviral effector that restricts viral replication independently of canonical autophagy.

1. Significance statement

This manuscript demonstrates that NRF2 is a restriction factor for SARS-CoV-2 in human cells. Further, the manuscript significantly expands the portfolio of known antiviral effector by identifying >20 new NRF2-driven antiviral genes. Importantly, we identify p62/SQSTM1 as a broadly effective restriction factor against mutable virus families.

CRediT authorship contribution statement

Alice Pedersen: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Julia Blay-Cadanet:** Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing –

review & editing. **Jacob Storgaard:** Data curation, Formal analysis, Investigation. **Bruno Hernaez:** Data curation, Formal analysis, Investigation. **Jacob Thyrsted:** Data curation, Investigation. **Cecilie S. Bach-Nielsen:** Data curation, Formal analysis, Investigation. **Krishna Twayana:** Data curation, Investigation. **Sofie E. Jørgensen:** Data curation, Formal analysis. **Clàudia Rio-Bergé:** Data curation. **Cecilie Poulsen:** Data curation. **Anne L. Thielke:** Data curation, Investigation. **Emil A. Thomsen:** Data curation, Formal analysis. **Joanna Kalucka:** Supervision, Writing – review & editing. **David Olganier:** Supervision, Writing – review & editing. **Yonglun Luo:** Formal analysis, Supervision, Writing – review & editing. **Fulvio Reggiori:** Supervision, Writing – review & editing. **Trine H. Mogensen:** Supervision, Writing – review & editing. **Antonio Alcamí:** Supervision, Writing – review & editing. **Anne Louise Hansen:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Christian K. Holm:** Conceptualization, Funding acquisition, Supervision, Writing – original

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The nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2) is a key regulator of cellular homeostasis by regulating redox balance, metabolic homeostasis, and iron dynamics, which are vital for cellular fitness and resilience to stress. In normal conditions, NRF2 is bound by its repressor Kelch-like ECH-Associated protein 1 (KEAP1) [1,2], which effectively locks NRF2 in place for ubiquitination by the cullin 3 (Cul 3) complex, ultimately leading to NRF2 proteasomal degradation [3,4]. During conditions of oxidative stress, KEAP1 releases NRF2 to induce expression of antioxidant and detoxifying genes like *NAD(P)H quinone dehydrogenase 1* (NQO1), *glutamate-cysteine ligase catalytic subunit* (GCLC), and *heme oxygenase-1* (HO-1) through binding to antioxidant response elements (AREs) in gene promoter regions [5–7].

Recently, we and others have identified NRF2 as a regulator of the anti-viral type I interferon (IFN) response [8,9], suggesting that NRF2 induction would support viral replication. Surprisingly, we subsequently learned that several NRF2 agonists including 4-octyl-itaconate (4-OI), di-methyl-fumarate, and sulforaphane, induced cellular antiviral resistance to infection with several viruses including SARS-CoV-2, Herpes Simplex virus (HSV1), Vaccinia virus (VACV), Zika virus, Influenza A virus (IAV), Rotavirus, and Hepatitis B virus [10–17]. Recent reports have demonstrated that in some cases, NRF2-independent mechanisms contribute significantly to the antiviral properties of NRF2-agonists due to off-target effects of the agonists themselves [18,19]. For instance, Waqas and co-workers demonstrated that several NRF2 agonists restricted IAV replication independently of NRF2 [18]. Instead, their electrophilic properties suggested direct binding to nuclear export protein XPO1, which is necessary for viral egress from the nucleus [18].

Because NRF2 agonists display lack of target specificity, it is still therefore unclear to which extend NRF2 itself restricts viral replication, and if so, which downstream effector genes mediate its antiviral effects. In this study we used genetic approaches to directly induce or inhibit endogenous NRF2 expression to demonstrate that NRF2 induces broad cellular resistance to SARS-CoV-2. We then performed a focused CRISPR-activation (CRISPRa) screen to identify a diverse range of NRF2-driven restriction factors effective against SARS-CoV-2, IAV, HSV1, and VACV. Importantly, among the hits, the selective autophagy receptor p62/SQSTM1 restricted the replication of all the tested viruses independently of canonical autophagy. Altogether, these findings demonstrate that NRF2 controls an unappreciated and diverse network of antiviral genes that restrict viral replication across virus families.

2. Results

2.1. NRF2 restricts SARS-CoV-2 replication

As NRF2-inducers have been shown to also restrict viral replication through NRF2-independent mechanisms, we decided to target NRF2 genetically. First, we tested NRF2 potential in restricting SARS-CoV-2 replication using an NRF2-KO 786-O-derived cell line. We observed a clear increase in SARS-CoV-2 replication in comparison to control cells at both RNA level by qPCR and protein level by immunoblotting (Fig. 1A–B). To investigate this connection further, we generated NRF2 KO cell cultures using CRISPR-Cas9 and single guide RNAs (sgRNAs) targeting the NRF2-encoding gene *NFE2L2* in 786-O cells. Again, we could observe that NRF2 deficiency resulted in increased SARS-CoV-2 replication by qPCR for viral RNA, and by TCID₅₀ titration of released progeny virus particles (Fig. 1C–D).

Having established that lack of NRF2 promotes SARS-CoV-2 replication, we then tested whether its overexpression would have the opposite effect, i.e., boost cellular anti-viral resistance and reduce viral replication. To this end, we used promoter-targeting sgRNAs and mRNA encoding nuclease-deficient Cas9 (dCas9) fused with a transcriptional activator complex to induce endogenous overexpression of NRF2 by

CRISPRa. For comparison, we also used the CRISPRa system to induce endogenous expression of the interferon regulatory factor 1 (IRF1), a well-established potent inducer of anti-viral interferon-stimulated genes (ISGs) [20]. Induction of NRF2 by this method suppressed SARS-CoV-2 replication measured by immunoblotting and TCID₅₀ (Fig. 1E–F). Notably, the suppression of SARS-CoV-2 replication by NRF2 was similar in potency to that obtained by inducing IRF1.

Thus, we next decided to identify anti-viral effector genes operating downstream of NRF2. To identify NRF2-dependent candidate genes with anti-viral activities, we selected 65 NRF2-driven genes of interest based on previously published datasets, i.e., a full transcriptome analysis of cells treated with siRNA against NRF2 [8], transcriptome analysis of cells treated with the potent NRF2 agonist 4-octyl-itaconate [21] previously demonstrated to induce anti-viral immunity [13], and a ChIP-seq analysis to identify genes that bind NRF2 at the chromatin level in A549 cells [8] (Extended data Fig. 1A). Using CRISPRa sgRNAs specific for these 65 genes, we then individually induced their expression in Huh-7 cells before infecting them with SARS-CoV-2 and assessing SARS-CoV-2 replication by immunoblotting and qPCR. We included dCas9 alone as the negative control, and induction of IRF1 as the positive control (Fig. 1H). This approach allowed us to identify a substantial number of NRF2-inducible genes that restricted SARS-CoV-2 replication as measured by expression of SARS-CoV-2 nucleocapsid (NC) using immunoblotting. Notably, induction of IRF1 reduced NC expression by roughly two-fold. A subset of genes, including *Sequestosome-1* (SQSTM1), which encodes p62/SQSTM1 lymphocyte antigen 6 family member K (LY6K), *glutamate-cysteine ligase modifier subunit* (GCLM), and *ETS variant transcription factor 4* (ETV4) displayed an even more potent effect, with 8–10 fold reductions in NC expression in comparison with the negative control (Extended data Figs. 2A and 1H). At the RNA level, the median reduction was approximately 20% with a selection of genes showing a reduction of approximately 50%. Notably, genes affecting viral replication at the protein level, e.g. p62, did not necessarily affect the amount of viral RNA, indicating pre- or post-translational effects (Extended data Figs. 2B and 1J).

We then tested the most potent anti-viral genes identified in the CRISPRa screen for their ability to affect the release of progeny SARS-CoV-2. Here, LY6K, p62, and ETV4, but not GCLM, significantly reduced the extracellular release of new viral particles in infected cell cultures (Fig. 1K). LY6K is highly expressed in several malignancies and has not previously been linked to anti-viral resistance itself [22]. By contrast, the related LY6E, which belongs to same gene family, was recently demonstrated to restrict SARS-CoV-2 infection in mice through an undisclosed mechanism [23,24]. ETV4 is a transcription factor and is involved in controlling cell cycle progression and fate specification in epithelial cells and in a different types of malignancies including pancreatic cancer, but has not been associated with anti-viral immunity [25]. P62 is mostly known for its function as a selective autophagy receptor that binds ubiquitinated cytoplasmic structures, including proteins, protein complexes, organelles and invading pathogens, and mediate their degradation via autophagy [26,27]. However, p62 is also important in autophagy-independent pathways such as oxidative stress responses and activation of mammalian target of rapamycin (MTOR) [28–30]. Importantly, p62 was recently shown to bind ectopically overexpressed SARS-CoV-2 M in HEK293 cells [31].

2.2. Several NRF2-inducible genes restrict IAV replication

We then used the same CRISPRa sgRNA library and approach to identify NRF2-driven genes that restrict IAV replication. Immunoblotting of the IAV non-structural protein 1 (NS1) revealed that a selection of genes including p62, *Egl-9 family hypoxia inducible factor 3* (EGLN3), *musculin* (MSC), *glutamate-cysteine ligase catalytic subunit* (GCLC), *UDP-glucose 6-dehydrogenase* (UGDH), and *LysM domain containing 2* (LYSMD2) potentially reduced viral replication when overexpressed (Fig. 2A–B). In contrast to the modest anti-viral effect of IRF1 against

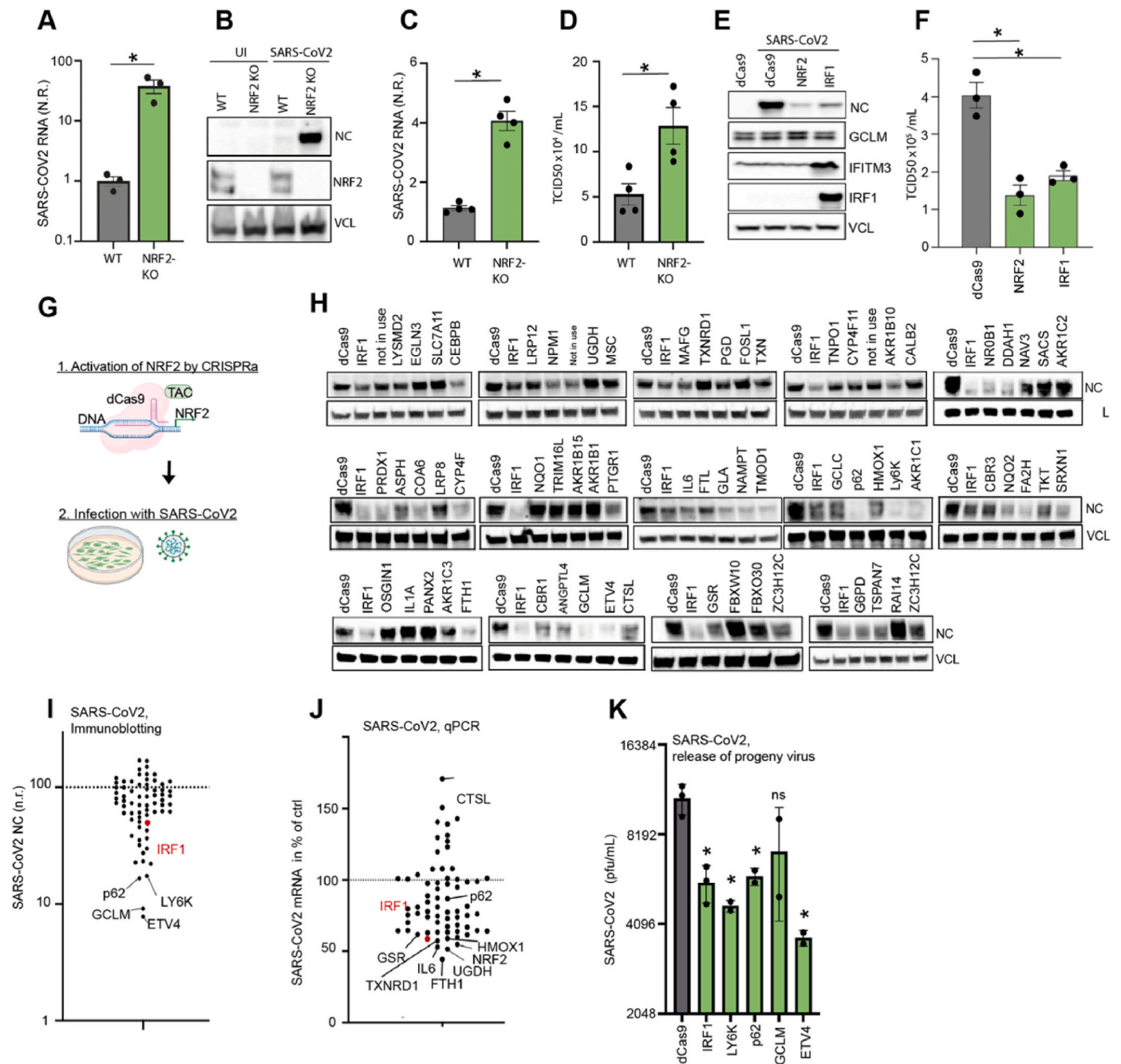


Fig. 1. NRF2 restricts SARS-CoV-2 replication

(A-B) SARS-CoV-2 permissive 786-O cell clone deficient in NRF2 (NRF2-KO) and control cells were infected with SARS-CoV-2 at MOI 0.01 for 24 h. Cell lysates were then prepared for qPCR for viral RNA or for immunoblotting against viral protein NC, NRF2, and loading control VCL (Vinculin). (C-D) SARS-CoV-2 permissive 786-O cells were used for CRISPR-Cas9-mediated elimination of NRF2. Cells were then infected with SARS-CoV-2 at MOI 0.01 for 24 h before cell lysates were prepared for qPCR and cell supernatants harvested for TCID50 analysis. (E-F) SARS-CoV-2 permissive Huh-7 cells were treated with dCas9 alone or dCas9 along with sgRNAs targeting the promoter region of NRF2 or IRF1. Cells were then infected with SARS-CoV-2 at MOI 0.01 for 24 h before cell lysates were prepared for immunoblotting and cell supernatants harvested for TCID50. (G) Graphic representation of CRISPRa for NRF2-driven genes. (H-J) CRISPRa was used to induce endogenous expression of indicated NRF2-driven genes in SARS-CoV-2 permissive Huh-7 cells. Cells were then infected with SARS-CoV-2 at MOI 0.01 for 8 h before lysates were collected for qPCR or immunoblotting. (H) Displays immunoblots for viral protein NC and loading control VCL. (I) Displays expression levels of NC normalized to VCL expression levels for each tested gene based on densitometric quantification of immunoblots. (J) Displays mean SARS-CoV-2 RNA levels by qPCR normalized to beta actin expression levels for each gene tested from two independent experiments. (K) TCID50 was established from cell supernatants harvested from Huh-7 cells where indicated genes were induced by CRISPRa before infection with SARS-CoV-2 at MOI 0.02 for 8 h. (A-F) Are representative of two independent experiments. Each data point represents one biological replicate with bars indicating mean $\pm$ s.e.m. (H-I) represents data from one experiment. (K) Each data point indicates mean of three biological replicates. Bars indicate mean $\pm$ s.e.m. of two or three independent experiments. p-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating $p < 0.05$.

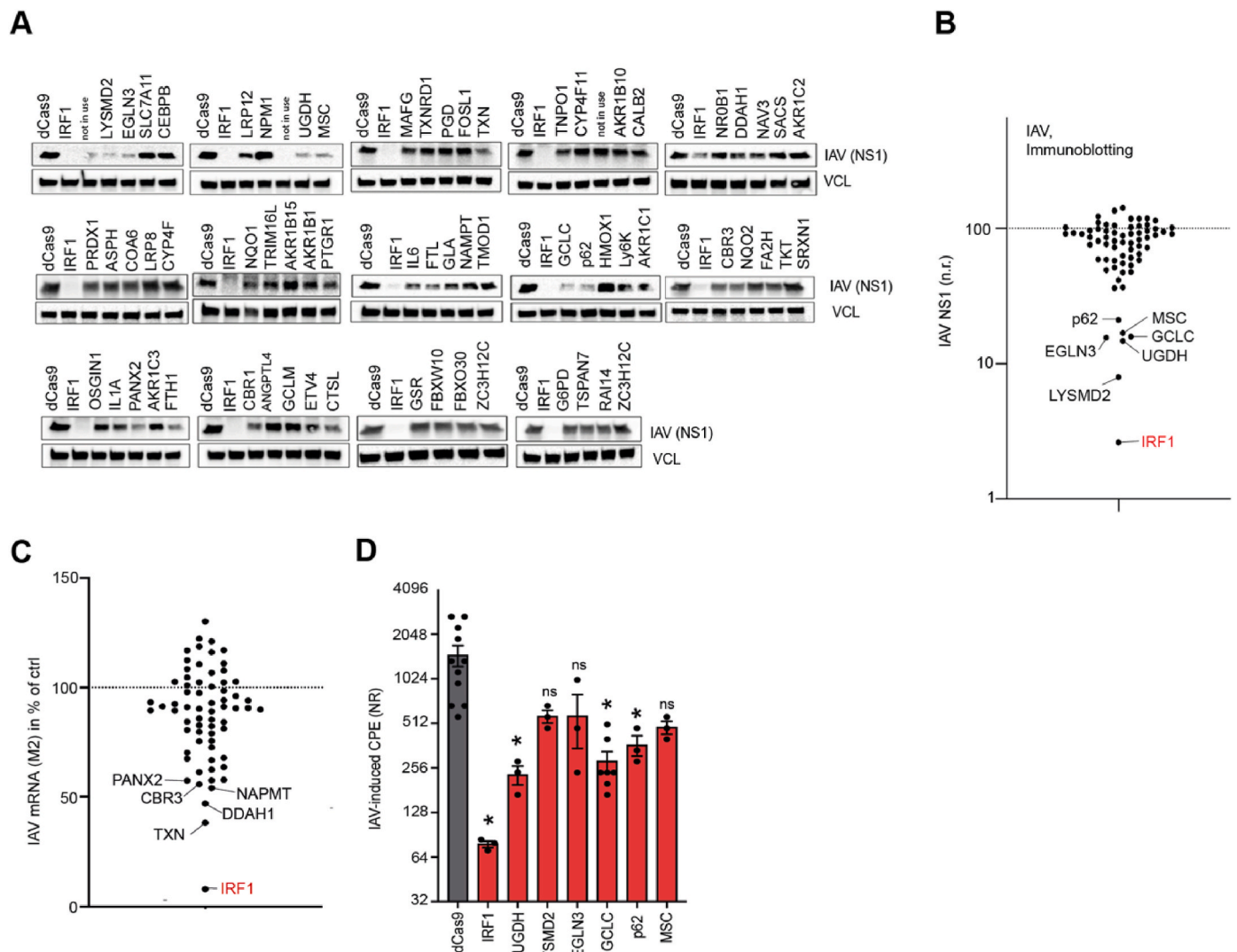


Fig. 2. Several NRF2-inducible genes restrict IAV replication

(A–C) CRISPRa was used to induce endogenous expression of indicated NRF2-driven genes in IAV permissive Huh-7 cells. Cells were then infected with IAV at MOI 0.5 for 7h before lysates were collected for qPCR or immunoblotting. (A) Displays immunoblots for viral protein NS1 and loading control VCL and B displays expression levels of NS1 normalized to VCL expression levels for each tested gene based on densitometric quantification of immunoblots. (C) Displays mean IAV M2 RNA levels by qPCR normalized to beta actin expression levels for each gene tested. (D) CPE was established in Huh-7 cells where indicated genes were induced by CRISPRa before infection with IAV. (A–B) Represents data from one experiment. (C) Each data point represents mean of two independent experiments. (D) Each data point indicates mean of three biological replicates. Bars indicate mean $\pm$ s.e.m. of three independent experiments. p-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating $p < 0.05$.

SARS-CoV-2, the replication of IAV was very potently reduced when IRF1 was overexpressed. When assessing viral replication at the RNA level using qPCR, IRF1 again demonstrated potent anti-viral effects, while a modest anti-viral effect was exclusively observed after CRISPRa-mediated induction of *thioredoxin* (*TXN*), *dimethylarginine dimethylaminohydrolase 1* (*DDAH1*), *carbonyl reductase 3* (*CBR3*), *nicotinamide phosphoribosyltransferase* (*NAMPT*), and *pannexin 2* (*PANX2*) (Extended data Figs. 3B and 2C). Of these, all except *NAMPT* also revealed a reduction in viral protein expression upon immunoblotting analysis, suggesting that the effect on viral RNA was sufficient to also reduce viral protein expression (Fig. 2A–C).

We then evaluated the genes with the strongest anti-viral effect at the protein level for their potential effect on reducing IAV-induced cytopathology. Here, *UGDH*, *GCLC*, and *p62* displayed significant cytoprotective effects, while *LYSDM2*, *EGLN3*, and *MSC* trended toward being protective but without being significant (Fig. 2D). Thus, we concluded that a subset of NRF2-inducible genes can restrict IAV replication.

2.3. NRF2-driven anti-viral genes are highly expressed in human airway epithelium

As the primary site of viral replication of both SARS-CoV-2 and IAV is the human airways, we wanted to assess if the identified NRF2-driven anti-viral genes are expressed in this tissue, and if so, whether they are still under the control of NRF2. We therefore used ChIP-seq analysis of stratified cultures of primary human airway epithelial cells (HAE-cultures) [32,33] to determine if the top anti-viral genes identified directly associate with NRF2 at the chromatin level in mature primary human airway epithelium (HAE) (Fig. 3A). We detected NRF2 binding peaks upstream of *FTH1* and *ETV4*, inside *p62* and *UGDH*, and in the presumed promoter regions of *GCLC* and *GCLM*. By contrast, NRF2 did not appear to associate with *LY6K* in this tissue (Fig. 3C). Interestingly, the NRF2-driven anti-viral genes were highly expressed in this tissue compared to the expression of beta-actin (*BACT*) and are therefore present at the site of infection at high levels (Fig. 3C). Interestingly,

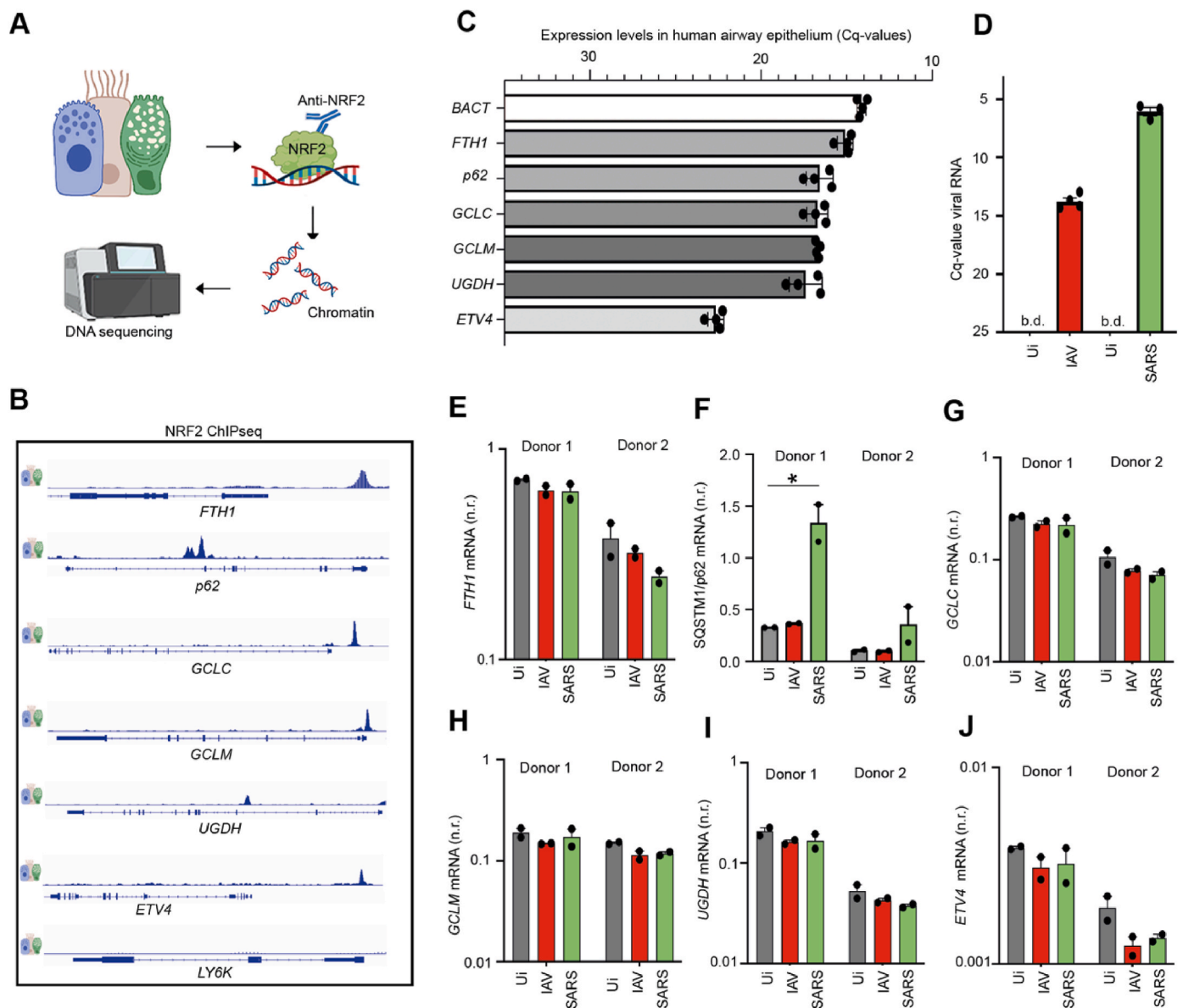


Fig. 3. NRF2-driven anti-viral genes are highly expressed in human airway epithelium

(A) Graphic representation of NRF2 ChIP-seq in primary human epithelium cultured in the HAE-ALI system. (B) Display of NRF2 peaks identified in HAE-ALI cultures for the indicated genes. (C) Expression levels displayed as Cq-values for the indicated genes by qPCR in HAE-ALI cultures. (D–J) HAE-ALI cultures were established from two healthy donors. Cells were then infected with either IAV at MOI 2.5 for 24 h or SARS-CoV-2 at MOI 0.05 for 48 h before cells were harvested for qPCR analysis for the indicated hosts and viral RNA. (B) Displays data from one of two independent healthy donors. (C–J) Represents data from two independent donors. Each data point indicates a biological sample from one donor. Bars indicate mean of data points with error bars indicated $\pm$ s.e.m. *p*-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating *p* < 0.05.

except for *p62*, infecting HAE-cultures with SARS-CoV-2 or IAV did not seem to further enhance the expression levels of the examined genes in this tissue, suggesting that these restriction factors are constitutively present to prevent viral replication in human airway epithelium (Fig. 3D–J).

2.4. CRISPR activation of NRF2-inducible genes inhibit replication of HSV1 and VACV

NRF2 agonists also inhibit the replication of DNA viruses such as HSV1 and VACV [13]. We therefore used the CRISPRa approach to identify which NRF2-driven effector genes can restrict replication of these viruses. For HSV1, we used the immortalized human keratinocyte cell line HaCat. Cells were infected following CRISPRa-induction of NRF2-driven genes, and cell lysates and cell supernatants were collected

20 h post infection (approximately one replication cycle). Over-expression of *NAD(P)H:quinone oxidoreductase 1* (*NQO1*) almost completely inhibited HSV1 replication measured by immunoblotting for the viral protein ICP5 (Fig. 4A–B). In addition, potent anti-viral effects of *cytochrome C oxidase assembly factor 6* (*COA6*), *aldo-keto reductase 1C3* (*AKR1C3*), and *glutathione reductase* (*GSR*) were observed. CRISPRa of *p62*, which restricted SARS-CoV-2 and IAV, also significantly reduced HSV1 replication, albeit not as potently (Fig. 4A–B). At the RNA level, *NQO1*, *FOS-like antigen 1* (*FOSL1*), *LY6K*, *thioredoxin* (*TXN*), *tropomodulin 1* (*TMOD1*), *EGLN3*, and *DDAH1*, all robustly reduced viral RNA expression levels (Fig. 4C). Of these, *NQO1* was the only that matched the effect observed at the protein level. We then tested the most potent anti-viral genes, as well as NRF2 (*NFE2L2*), for their effect on release of progeny HSV1 particles by TCID50 assay. Here, CRISPRa of *NRF2*, *p62*, *AKR1C3*, and *COA6* all significantly reduced virus release, while *GSR*

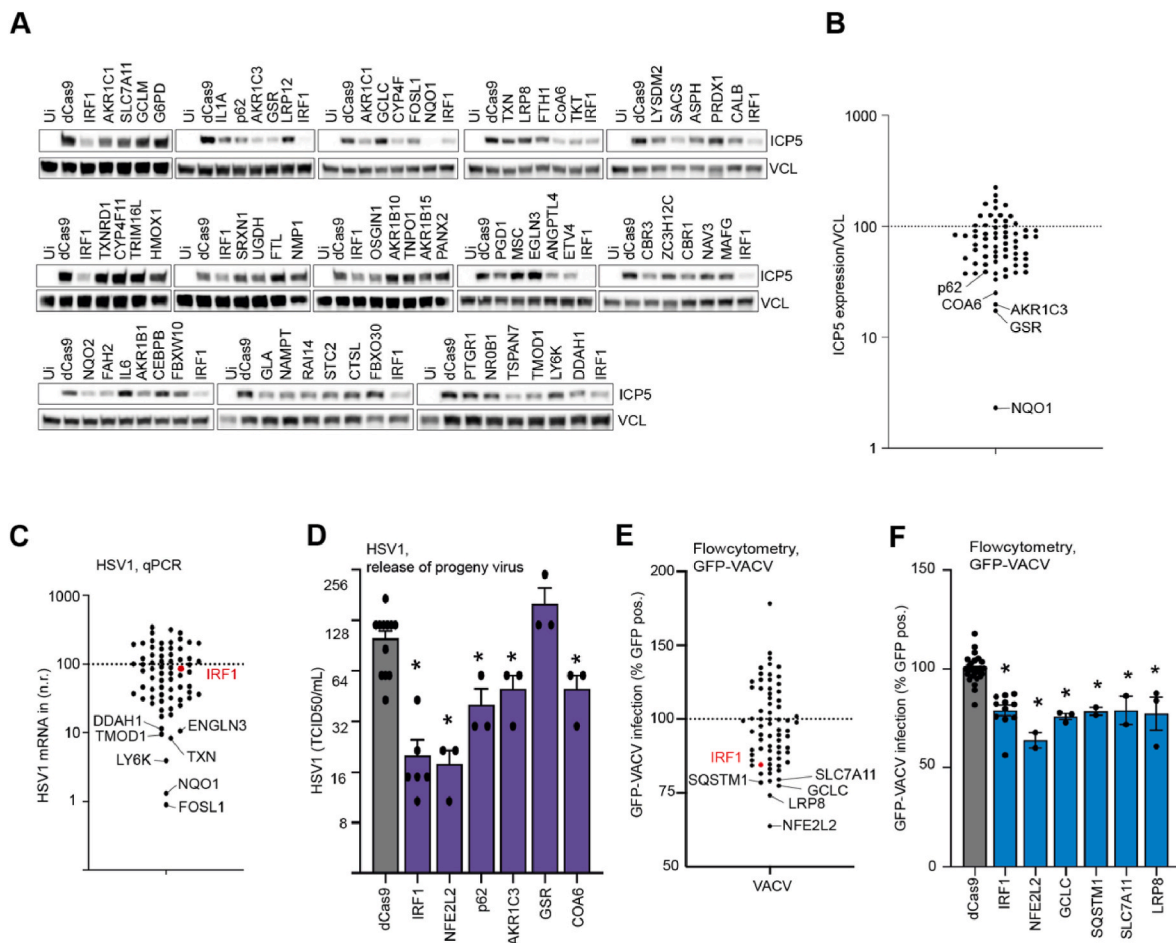


Fig. 4. CRISPR activation of NRF2-inducible genes inhibits replication of HSV1 and VACV

(A–D) CRISPRa was used to induce endogenous expression of indicated NRF2-driven genes in HSV1 permissive HaCaT cells. Cells were then infected with HSV1 at MOI 0.2 for 20 h before lysates and supernatants were collected for immunoblotting (A–B), qPCR (C), and plaque assay (D). (A) Displays immunoblots for viral protein ICP5 and loading control VCL and B displays expression levels of ICP5 normalized to VCL expression levels for each tested gene based on densitometric quantification of immunoblots. (C) Displays median HSV1 RNA levels by qPCR normalized to beta actin expression levels for each gene tested. (D) Plaque forming units were established from cell supernatants harvested from HaCaT cells where indicated genes were induced by CRISPRa before infection with HSV1. (E–F) HaCaT cells were infected with a GFP-expressing VACV variant after induction of NRF2-driven genes by CRISPRa. Cells were then analyzed for GFP expression by flow cytometry. (A–B) Represents data from one experiment. (C) Each data point represents mean of two independent experiments. (D) Data is representative of two independent experiments with each data point representing one biological replicate. (E) Data points represent the mean of two biological replicates from one experiment. (F) Is a graphical representation of data from selected genes analyzed in (E), (D), and (F) *p*-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating *p* < 0.05.

did not (Fig. 4D).

While HSV1 replicates in the nucleus, VACV replicates in the cytosol. We were therefore interested to test if some NRF2-driven genes could also restrict this virus. To this end, we used a GFP-expressing variant of VACV and used this to HaCaT cells after gene induction by CRISPRa. VACV replication was then assessed by flow cytometry by comparing the number of infected cells between control and CRISPRa-induced HaCaT cells. Using this setup, we identified *low-density lipoprotein receptor-related protein 8 (LRP8)*, *GCLC*, *p62*, and *solute carrier 7A11 (SLC7A11, a.k.a. xCT)* as potential restriction factors for VACV (Fig. 4E–F). These results suggested that NRF2-driven genes can also restrict DNA viruses from different virus families.

Together, the experiments allowed us to identify a network of 32 NRF2-driven genes with potential to restrict viral replication in one or more of these viruses (Table 1).

2.5. p62 is a restriction factor for SARS-CoV-2, IAV, and HSV1

In contrast to the rest of the NRF2-driven genes examined by CRISPRa, only overexpression of p62 seemed to restrict all the tested

viruses, albeit only a modest effect was observed for HSV1. To validate p62 as an important anti-viral restriction factor, we first depleted p62 using siRNA before infecting cells with SARS-CoV-2. Reduced p62 levels led to increased viral replication measured by NC immunoblotting (Fig. 5A and Extended Fig. 6A and B), viral RNA qPCR (Fig. 5B–C), and TCID₅₀ of the released progeny virus (Fig. 5D). Similarly, p62 knock-down by siRNA increased IAV replication assessed by measuring NS1 expression using immunoblotting (Fig. 5E and Extended Fig. 6C and D) and viral RNA with qPCR (Fig. 5F–G). This also seemed to be the case for HSV1, as p62 depletion led to a clear increase in the expression of HSV1 proteins ICP5 and ICP27 (Fig. 5H and Extended Fig. 6E and F), viral RNA (Fig. 5I–J), and close to one log increase in progeny viral particle release (Fig. 5K). Thus, these experiments showed that p62 is a broad acting anti-viral restriction factor.

2.6. p62 restricts viral replication in a manner independent of canonical autophagy

p62 is one of the main selective autophagy receptors [26,27] and plays a key role in immunity by for example targeting cytoplasmic

Table 1

List of observed antiviral effects by gene through CRISPRa.

	RNA	Protein	TCID50	RNA	Protein	CPE	RNA	Protein	TCID50	GFP
SQSTM1	-	+++	+	-	++	++	-	++	++	++
UGDH	+++	-		+	+++	++	++	-		-
GCLC	-	++		+	+++	++	-	-		++
FTH1	+++	++		++	+		++	+		+
AKR1C1	++	++		-	++		-	+		-
ETV4	-	+++	+	-	++		-	+		-
NQO2	+	-		+	++		+	+++		+
LY6K	-	+++	+	-	++		+++	-		-
GCLM	-	+++	-	-	-		-	+		++
MAFG	++	+		-	-		++	+		+
TKT	+	+		-	-		-	+++		++
OSGIN1	++	-		-	+		-	+		++
RAI14	++	-		++	-		++	++		-
FTL	+	-		-	++		++	-		-
HMOX1	++	++		-	-		-	-		+
LRP12	+	-		-	+		++	++		-
TXN	-	+		++	+		+++	-		-
NQO1	++	-		-	-		+++	+++		-
PRDX1	-	++		+	+		-	-		-
CBR1	-	+		-	++		++	-		-
GSR	++	+		-	-		++	++	-	-
TNPO1	+	-		+	+		-	-		-
CBR3	+	-		++	+		-	-		-
AKR1B15	++	-		+	-		-	-		-
CYP4F3	+	++		-	-		-	+		-
TXNRD1	+++	-		-	-		-	-		+
G6PD	++	++		-	-		-	-		-
LYSMD2	-	-		+	+++	-	++	-		-
ENGLN3	-	-		+	+++	-	+++	++		-
MSC	++	-		+	+++	-	++	++		+
AKR1C3	-	-		-	-		-	-	++	-
COA6	-	-		-	-		-	-	+	-

pathogens and their components to lysosomal degradation [34]. For this reason, we examined whether the anti-viral function of p62 towards SARS-CoV-2 was mediated through autophagy. For that reason, we first treated hACE2-expressing A549 cells (A549-hACE2) with either control siRNA or siRNA targeting p62 RNA either in the presence or absence of the autophagy-inhibitor bafilomycin A1 (Baf1A). Interestingly, when infecting these cells with SARS-CoV-2, Baf1A did not seem to affect the anti-viral property of p62 as its inhibition still promoted viral replication (Fig. 5L and Extended Fig. 6G and H). In addition, the anti-viral effect of inducing p62 through CRISPRa was not noticeably affected by Baf1A (Fig. 5M and Extended Fig. 6I and J). These results suggested that p62 might affect replication of SARS-CoV-2 in a manner independent of canonical autophagy. To test more specifically, we used A549-hACE2 cells lacking the autophagy-related protein 5 (ATG5), which is essential for the progression of canonical autophagy [35]. Again, inhibition of p62 promoted viral replication in the ATG5-KO cells similarly as in control cells (Fig. 5N and Extended Fig. 6K–M).

We then wanted to test if the antiviral effect of p62 against IAV and HSV1 was also independent of canonical autophagy. ATG16L1 is a core component of both bulk and selective autophagy and therefore essential for these processes [36,37]. We therefore treated WT and ATG16L1-deficient (ATG16L1-KO) A549 cells with IAV. Deletion of ATG16L1 affected viral replication as expression of NS1 was increased in ATG16L1-KO cells in comparison with the WT control suggesting that autophagy participates in the restriction of IAV replication. However, the targeting of p62 by siRNA promoted replication of IAV in both WT and ATG16L1-KO cells to a similar degree (Fig. 5O and Extended Fig. 6N–P) suggesting that the effect was independent of canonical autophagy as well. For HSV1, we used HaCaT cells with highly reduced ATG5 expression (ATG5-KO) generated by CRISPR Cas9 targeting. Here, ATG5 deficiency reduced viral replication suggesting that this virus relies on a functional autophagy machinery for optimal replication. Importantly, reducing expression of p62 promoted HSV1 infection equally well in WT and ATG5-KO cells as assessed by ICP5

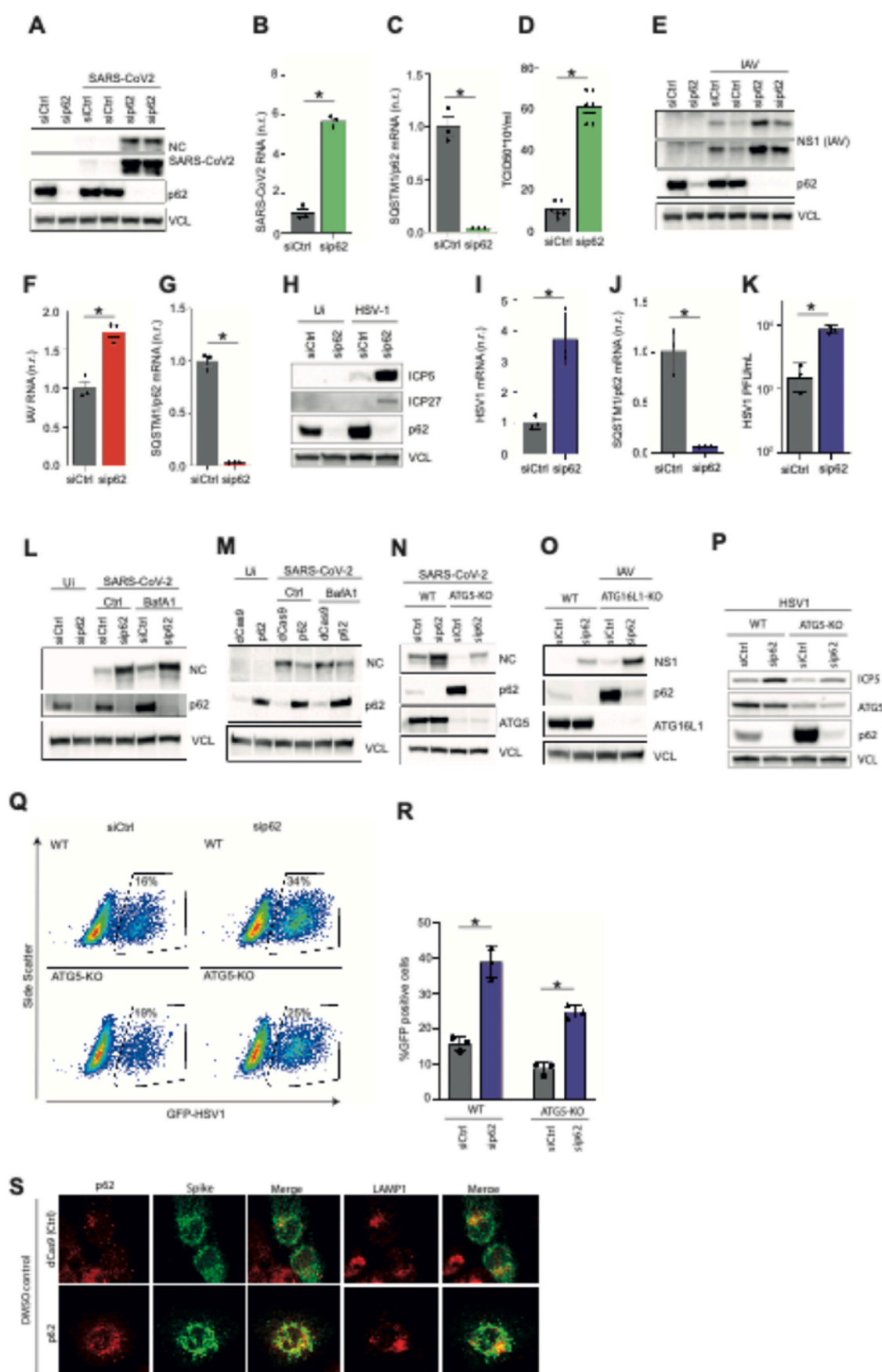
immunoblotting (Fig. 5P and Extended Fig. 6Q and R). Similarly, when using a GFP-expressing HSV1 variant, the number of GFP-positive cells was equally increased in WT and in ATG5-KO cells by flow cytometry in cells where p62 was targeted by siRNA (Fig. 5Q–R).

We then sought to also investigate the potential autophagy-independent action of p62 in infected cells in another manner. Therefore, we used CRISPRa to induce endogenous overexpression of p62 in SARS-CoV-2 permissive Huh-7 cells, infected them with SARS-CoV-2, and investigated potential colocalization of p62 and the SARS-CoV-2 spike (S) protein using confocal microscopy. We observed some colocalization between p62 and the SARS-CoV-2 S protein in the dCas9 condition, which increased upon CRISPRa of p62 (Fig. 5S). We also stained for the late endosome and lysosomal marker LAMP1. Likewise, we observed some colocalization between LAMP1 and the SARS-CoV-2 S protein in the dCas9 condition, which augmented upon CRISPRa of p62 (Fig. 5S). Importantly, we could observe colocalization between p62 and the SARS-CoV-2 Spike protein (S) in LAMP1-positive areas, especially in the CRISPRa p62 condition (Fig. 5S).

That the antiviral action of p62 is insensitive to BafA1 and independent of important autophagy genes, but that CRISPRa of p62 increased colocalization of both p62 and S, and S and LAMP1, suggests that p62 works antivirally independently of canonical autophagy, but that the mechanism could still involve sequestering of viral proteins to LAMP1-positive cellular compartments.

3. Discussion

Several studies have explored the anti-viral potential of NRF2 agonists that operate through KEAP1 inhibition. However, subsequent investigation reported that these agonists also induced anti-viral resistance through NRF2-independent mechanisms [13,18,19], calling into question whether NRF2 has any direct anti-viral properties. In this report, we show that NRF2 provides resistance to infection with SARS-CoV-2. Subsequently, we used a focused CRISPRa screen to



(caption on next page)

Fig. 5. p62 is a broadly effective restriction factor working in a manner independent of canonical autophagy

(A–D) SARS-CoV-2 permissive A549-hACE2 cells were treated with siRNA as indicated before infection with SARS-CoV-2 at MOI 0.01 for 24 h. Cell lysates and supernatants were then collected for analysis by immunoblotting (A), qPCR (B–C), and TCID50 (D). (E–G) IAV permissive A549 cells were treated with siRNA as indicated before infection with IAV at MOI 0.3 for 6 h. Cell lysates were then collected for analysis by immunoblotting (E) and qPCR (F–G). (H–K) HSV1 permissive HaCaT cells were treated with siRNA as indicated before infection with HSV1 at MOI 0.2 for 20 h. Cell lysates were collected for analysis by immunoblotting for viral proteins ICP5 and ICP27 as well as LC3 and VCL as loading control (H) and for qPCR (I–J) while cell culture supernatants were collected for plaque assay (K). (L) SARS-CoV-2 permissive A549-hACE2 cells were treated with siRNA as indicated before infection with SARS-CoV-2 at MOI 0.01 for 24 h and bafilomycin A1 treatment 3 h before harvest for immunoblotting of cell lysates. (M) CRISPRa was used to induce endogenous expression of p62 in SARS-CoV-2 permissive Huh-7 cells. Cells were then infected with SARS-CoV-2 at MOI 0.01 for 24 h and treated with bafilomycin A1 3 h before harvest for immunoblotting of cell lysates. (N) ATG5-deficient SARS-CoV-2 permissive A549-hACE2 and control cells were established by CRISPR-Cas9 and treated with siRNA as indicated before infection with SARS-CoV-2 at MOI 0.01 for 24 h. Cell lysates were then prepared for immunoblotting. (O) IAV permissive A549 cells deficient in ATG16L (ATG16L-KO) and control cells were treated with siRNA as indicated before infection with IAV at MOI 0.3 for 6 h. Cell lysates were then prepared for immunoblotting. (P–R) HSV1 permissive HaCaT cell clone deficient in ATG5 (ATG5-KO) and control cells were treated with siRNA as indicated before infection with HSV1 at MOI 0.2 for 20 h. Cell lysates were collected for analysis by immunoblotting and cells were harvested for flow cytometry. (P) Displays immunoblots for viral protein ICP5, ATG5, and VCL as loading control. (Q) Displays a scatter plot of one biological replicate. (S) CRISPRa was used to induce endogenous expression of p62 in SARS-CoV-2 permissive Huh-7 cells. Cells were then infected with SARS-CoV-2 at MOI 10 for 8 h before they were fixed and stained with antibodies against p62, SARS-CoV-2 Spike, LAMP1, and DAPI for confocal microscopy. (A–K) Experiments are representative of at least two independent experiments with each data point indicating one biological replicate. Bars indicate mean $\pm$ s.e.m. *p*-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating *p* < 0.05. (L–S) represents data from one experiment. (S) Displays representative images. (R) Each data point represents one biological replicate with bars indicating mean $\pm$ s.e.m. *p*-values were established using Graphpad Prism 10 using the Student's *t*-test with “*” indicating *p* < 0.05.

identify a diverse range of antiviral NRF2-inducible effectors. These effectors constitute a previously unappreciated network of anti-viral genes that can restrict the replication of not only SARS-CoV-2, but also IAV, HSV1, and VACV. Importantly, we identified p62 as a broadly acting effector effective across virus families.

The NRF2-driven anti-viral defence seems to be independent of canonical anti-viral effectors, including the IFNs and pro-inflammatory cytokines. Indeed, many reports have documented that NRF2 even works to limit these responses [8,21,38–41]. For the human host, launching protective responses to infection based on pro-inflammatory cytokines and IFNs is clearly an effective anti-viral strategy. The approach, however, comes with significant collateral damage in the form of immunopathology [42]. Constant engagement of these canonical systems would therefore lead to significant loss of fitness due to reduced functionality of the implicated organ or tissue. We instead suggest that the abundant expression of NRF2-driven anti-viral genes at steady-state at epithelial surfaces, as revealed by our analysis of human airway epithelium, is a strategy that protects the human host from infection in non-inflammatory manners.

The NRF2 system seems in several ways similar to the IFN-system, where a diverse range of effector ISGs limit viral replication with each effector displaying distinct preferences between viruses and cellular systems [20,43]. In addition, the median amplitude of the overexpressed NRF2 effectors is not unlike what has previously been observed in a large ISG overexpression screen [20]. One important difference is that while the IFN-system seems to rely on induction downstream of cellular sensors of viral replication, NRF2-driven effectors appear to be highly expressed at homeostasis at epithelial surfaces including the airways. Here, NRF2-driven genes could potentially prevent viral replication while avoiding hyperinflammation and tissue pathology. IFN-inducing sensors of microbial DNA or RNA would be engaged in those instances where the NRF2 system fails to limit viral replication, IFN-inducing sensors of microbial RNA and DNA to engage in effective, but tissue-disrupting, canonical antiviral responses.

That NRF2 plays a role in the anti-viral response is supported by reports demonstrating that SARS-CoV-2 and other viruses seem to specifically target NRF2 expression [44–46].

Identification of p62 as a broadly acting anti-viral factor and the discovery that it operates in an autophagy-independent manner were surprising. Especially since we observed clear colocalization of viral proteins with LAMP1. Importantly, LAMP1 is also a marker of microautophagy [47], and if viral proteins are degraded through this mechanism, then that would explain the accumulation of viral S-protein in LAMP1-positive areas. Besides autophagy, p62 is involved in many other cellular processes, of which several could potentially be utilized to block viral replication. p62 is important for the sensing of amino acids

by the mTORC1 complex and downstream activation of S6K1 and 4EBP1 to control translation [48].

It is unclear how increased translation of mRNA and anabolism in general could inhibit viral replication, but our observation that p62 seems to affect viral protein, and not viral RNA, suggests that it reduces the translation of viral proteins. However, pharmaceutical inhibition of mTOR using rapamycin analogs, a.k.a. rapalogs, makes human epithelial cells and mice more, not less, susceptible to SARS-CoV-2 infection [49]. This makes it unlikely that p62 inhibits viral replication through activation of mTOR. Importantly, a recent report has demonstrated that another autophagy-related gene, RB1CC1/FIP200 also limits SARS-CoV-2 replication in a manner independent of canonical autophagy [50]. However, in this case FIP200 affected viral RNA levels which was not the case for p62 in our hands.

p62 is also involved in oxidative stress responses where it seems to be part of a positive feedback loop as p62 phosphorylation induces activation of NRF2 through direct binding of KEAP1 [51]. This feedback loop could perhaps reinforce the induction of other anti-viral genes operating downstream of NRF2 activation.

The identification of p62 as a broad anti-viral restriction factor opens new potential avenues for therapeutic intervention in viral infections. Development of small molecule modifiers could be a strategy to enhance p62 activity or expression to improve the host's ability to combat various viral pathogens, including those responsible for respiratory infections. Although no such modifiers are currently commercially available, naturally occurring spermidine could potentially serve as a platform for the development of such modifiers. Although it is still unclear if p62 is a direct target of spermidine Yuan et al., have demonstrated that treatment with spermidine can ameliorate cell senescence in a p62-dependent manner [52]. Directly targeting NRF2 might prove more challenging. NRF2 agonists such as 4-OI or DMF do induce potent anti-viral responses *in vitro*, but NRF2 has also been demonstrated to limit important anti-viral IFN-responses *in vitro* as well as *in vivo* [8,53].

With this work, we demonstrated that NRF2 restricts SARS-CoV2 and that NRF2 controls an unappreciated network of anti-viral genes that restrict replication across virus families. Importantly, these restriction factors seem to be highly expressed at epithelial surfaces, which are preferred points of entry for many human pathogenic viruses.

4. Methods and materials

4.1. Cell culture

786-O NRF2-KO and control cells were purchased from ATCC. Human lung carcinoma A-549 cells (ATCC-CCL-185) and Vero African green monkey kidney cells (ATTC-CCL-81) were purchased from LGC.

Human epithelial liver cancer Huh-7 cells and immortalized human HaCaT keratinocytes were kindly provided by Søren R. Paludan (Aarhus University, Denmark). The HaCaT STAT1 KO cells and the HaCaT ATG5 KO cells were established as previously described [13,54]. Human lung carcinoma A-549 cells expressing hACE2 were kindly provided by David Olganier (Aarhus University, Denmark). A-549 ATG16L1 KO cells were kindly provided by Fulvio Reggiori (Aarhus University, Denmark) and generated identically as previously described in U2OS cells [55] with the sgRNAs listed in [Extended Data Table 1](#).

Vero E6 cells expressing hTMPRSS2 were kindly provided by Makoto Takeda (University of Tokyo, Japan) [56]. MDCK cells were kindly provided by Silke Stertz (Universität Zürich, Schweiz). 786-O cells were cultured in RPMI-1640 Medium (R8758, Sigma-Aldrich) and the remaining cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (D6429, Sigma-Aldrich). All cell culture medium was supplemented with 10% heat-inactivated fetal bovine serum (F9665, Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (10378-016, Gibco). Hereafter denoted complete culture medium. Vero E6 cells expressing hTMPRSS2 cells were cultured in complete culture medium in the presence of 10 µg/ml blasticidin (ant-bl-05, Invivogen). All cell lines were grown at 37 °C 5% CO₂ and regularly tested for mycoplasma contamination by sequencing (GATC Biotech, Germany).

4.2. Generation of gene deficient cells by CRISPR-Cas9 editing

The 786-O NRF2 KO and the A-549 hACE2 ATG5 KO cells that were established by CRISPR-Cas9 technology were generated using sgRNAs. The sgRNAs were purchased as chemically modified sgRNA with 2'-O-methyl at the three first and last bases and 3' phosphorothioate bonds between the first three and the last two bases (CRISPR Revolution sgRNA EZ Kit, Synthego). The gene editing was performed as follows: Ribonucleoprotein (RNP) complexes were formed at room temperature (RT) during a 15 min incubation of 6 µg of Cas9 protein (Integrated DNA Technologies, 1081059) and 3.2 µg sgRNA. 1x10⁵ PBS-washed cells were resuspended in 20 µl OPTI-MEM (31985062, Gibco), mixed with the RNP complexes, and electroporated in the SG Cell Line 4D-Nucleofector X 20 µl format (V4XC-3032, Lonza) using the Primary cell P3, CM-138 program on the 4D-Nucleofector Core and X units (AAF-1003B and AAF-1003X, Lonza). After electroporation, cells were transferred from the electroporation strip wells to cell culture well plates. Cells were cultured for at least 96 h before virus infection. The sequences of the used sgRNAs are listed in [Extended Data Table 1](#).

5. Human airway epithelial (HAE) air-liquid interface cultures

Cells were collected, cultured, differentiated, maintained, infected with virus and harvested from membranes as previously described [57].

5.1. NRF2 chromatin immunoprecipitation sequencing (ChIP-Seq) of HAE-ALI cells

HAE-ALI cells were fixated directly on the membranes and processed according to the Epigenetic Services ChIP Fixation Protocol (Active Motif). Approximately, 2 × 10⁶ cells were harvested on day 0, 9, and 42 for each of the two donors for each ChIP-seq sample. The ChIP-seq was performed by Active Motif using an anti-NRF2 antibody (sc-13032, Santa Cruz). All ChIP-seq data are made available in Supplementary Information 1.

5.2. CRISPR activation (CRISPRa)

Cells were detached, washed with PBS and resuspended in Opti-MEM (31985062, Gibco). We used 2 × 10⁵ Huh7 or 4 × 10⁵ HaCaT cells for each replicate of qPCR and 4 × 10⁵ Huh-7 or HaCaT cells for each immunoblotting analysis. Then, cells were electroporated with dSpCas9-

VPR mRNA (95 µg/ml) and indicated sgRNAs (50 µg/ml) in the SG Cell Line 4D-Nucleofector X 20 µl format (V4XC-3032, Lonza) using the Primary cell P3, CM-138 program on the 4D-Nucleofector Core and X units (AAF-1003B and AAF-1003X, Lonza). After electroporation, cells were transferred from the electroporation strip wells to cell culture well plates containing complete culture medium. Cells were incubated for 24 h before virus infection or harvest.

The dSpCas9-VPR mRNA was *in vitro* transcribed from a *SapI*-digested plasmid kindly provided by Rasmus O. Bak (Aarhus University, Denmark) [58] according to the manufacturer's instructions with the following deviations using the MEGascript tT7 Transcription Kit (AMB13345, Invitrogen). Final concentration of GTP was 1.5 mM. We used pseudouridine-5'-triphosphate (B7972, ApexBio) instead of UTP. We included the m₂^{7,3'}-⁰GP₃G ARCA Cap Analog (NU-855L, Jena Bioscience) in the reaction in a final concentration of 6 mM. We purified the *in vitro* transcribed RNA using the RNA Clean & Concentrator-25 kit (R1018, Zymo Research).

Three sgRNAs were chosen per gene of interest from the human Calabrese CRISPR activation library set A and B [59]. The sgRNAs were purchased as chemically modified sgRNA from Synthego (CRISPR-Revolution sgRNA EZ Kit, Synthego). SgRNA sequences are listed in [Extended Data Table 2](#). The CRISPRa-induced gene activation levels were measured by qPCR.

5.3. Short-interfering RNA (siRNA)-mediated knockdown

For immunoblotting analysis, 2.5 × 10⁵ cells were seeded per 6-well in 2 ml complete culture medium. The following day, cells were transfected using lipofectamine RNAiMAX transfection reagent (13778075, Invitrogen) according to the manufacturer's protocol with the following deviations. We used 40 nM siRNA and 8 µl of transfection reagent per well and incubated the mix of diluted siRNA and diluted transfection reagent for 20 min. The cells were infected with virus 72 h post-transfection. For qPCR analysis, half the number of cells, media and reagents were used in a 12-well. We used siRNA targeting p62 (sc-29679, Santa Cruz Biotechnology) and control siRNA (sc-37007, Santa Cruz Biotechnology).

5.4. Viruses

We propagated a plaque-purified IAV from a purchased influenza A/PR/8/34 (H1N1) strain (10100374, Charles River) as follows. A confluent cell layer of MDCK cells in a 12-well culture plate was washed in serum-free cell culture media and infected for 1 h with serial dilutions of IAV in PBSi (0.3% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% Ca²⁺/Mg²⁺). The virus inoculum was replaced with a 1 mL/well overlay consisting of MEM, agarose, and essential supplements. A 50 ml overlay was prepared by mixing a RT mixture of 25 ml 2X MEM, 9 ml sterile H₂O, 500 µl DEAE-Dextran (1%), 750 µl NaHCO₃ (sodiumbicarbonat) (7.5%), and 25 µl TPCK Trypsin with 17.5 ml hot purified agar melted in sterile H₂O (2%). A 500 ml 2xMEM solution was prepared from 100 ml 10x MEM, 10 ml of 200 mM glutamine, 10 ml Pen/Strep, 20 ml of 7.5% sodiumbicarbonate, 10 ml of 1 M Hepes, 21 ml of 10% BSA (0.2 µm filtered in water) and 350 ml sterile UF H₂O. After 48 h, a 70% confluent layer of MDCK cells in a T175 cell culture flask was washed with PBS and infected for 1 h with 3-4 picked plaques in a total volume of 5 ml PBSi. The virus inoculum was removed, and the cell layer was washed twice with PBS before adding 25 ml of OPTIMEM supplemented with 1 µg/ml TPCK trypsin. Virus was harvested 48 h post-infection as follows. The supernatant was centrifuged 5 min at 300×g to remove the cells. The supernatant was subsequently centrifuged at 4500×g for 60 min at 4 °C. The supernatant containing the IAV stock was aliquoted and stored at -80 °C until use. The titer was determined by plaque assay.

We used the Syn SARS-CoV-2 eGFP Clone 6.3 (SARS-CoV-2) [60] and the SARS-CoV-2 FR-4286 (SARS-CoV-2 FR-4286) kindly provided by

Professor Georg Kochs (University of Freiburg, Germany) and the HSV-1 KOS strain expressing GFP (HSV-1), kindly provided by Søren R. Paludan (Aarhus University, Denmark). We propagated the SARS-CoV-2 viruses in Vero E6 cells expressing hTMPRSS2 [61] and the HSV-1 in Vero cells. A near-confluent cell layer was infected at MOI 0.05 (SARS-CoV-2) or 0.02 (HSV-1) in DMEM supplemented with 2% heat-inactivated fetal bovine serum (F9665, Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (10378-016, Gibco). Virus was harvested at 70% CPE at ~72 h (SARS-CoV-2) or ~48 h (HSV-1) post-infection as follows. The supernatant was centrifuged for 5 min at 300×g (SARS-CoV-2) or the culture flasks were frozen at -80 °C and then thawed and centrifuged for 1 h at 4500×g to remove cell debris (HSV-1). The viruses were concentrated using (UFC910024, Millipore Sigma), aliquoted and stored at -80 °C. The titer was determined by TCID₅₀ (SARS-CoV-2) or plaque assay (HSV-1). We used vTag2GFP, a recombinant vaccinia virus (VACV) expressing GFP under the control of an early/late synthetic VACV promoter, kindly provided by Dr. Rafael Blasco (INIA, Spain). Tag2GFP stocks were generated in BSC-1 cells and semi-purified by ultracentrifugation through a 36% sucrose cushion. BSC-1 cells were maintained in 10% FBS containing media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

5.5. Virus infections

HAE-ALI cells were infected with the virus diluted in DMEM (11880036, Gibco). IAV infections were performed in PBSi on PBS-washed cell layer. SARS-CoV2 and HSV1 were added into the culture medium of the seeded cells.

5.6. VACV infection assays

2x10 [5] HaCaT STAT1 KO cells/sample were CRISPR activated as described above and seeded in 12-well plates. Next day, cells were infected with VACV vTag2GFP (MOI: 1 pfu/cell) and examined for GFP expression at 16 h post-infection by flow cytometry. Briefly, infected or mock-infected cells were harvested after trypsin-EDTA incubation and washed with PBS before fixation with paraformaldehyde 4% in PBS for 12 min. Cells were finally washed with PBS and analyzed in a FACS-Calibur flow cytometer (BD Sciences). Fluorescence levels were relativized to infection of electroporated cells with no sgRNAs. Every gene was analyzed in duplicate in at least two experiments. Total RNA was extracted using RNeasy plus (Qiagen).

5.7. Determination of TCID₅₀

The indicated genes were induced in 4×10^5 Huh-7 (SARS-CoV-2) or 5×10^5 HaCaT (HSV1) cells by CRISPRa as described above. After electroporation, approximately 1×10^5 Huh7 or 1.5×10^5 HaCaT cells were seeded per 48-well in 250 µl complete culture media in each of three 48-wells. 24 h later, the media was changed, and cells were infected for 2 h with SARS-CoV-2 GFP at MOI 0.02 or for 1 h with HSV-1 at MOI 0.2. The virus inoculum was then removed, and cells were washed in PBS and finally incubated in 300 µl complete culture media for additional 6 h (SARS-CoV-2) or 23h (HSV1). The TCID₅₀ analysis was carried out after a total of 8 h (SARS-CoV-2) or 24 h (HSV1). That is, 10 µl (SARS-CoV-2) or 50 µl (HSV1) undiluted or 2-fold diluted supernatant were added to Vero E6 TMPRSS2 (SARS-CoV-2) or Vero (HSV1) cells that were seeded the previous day 2×10^4 cells per 96-well in 90 µl (SARS-CoV-2) or 50 µl (HSV1) DMEM supplemented with 2% heat-inactivated fetal bovine serum (F9665, Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (10378-016, Gibco). Cells were incubated for 72 h (SARS-CoV-2) or 48-72 h (HSV1) before the CPE was assessed by light microscopy. Each supernatant dilution was examined in quadruplicates. The TCID₅₀/ml was calculated using the Reed-Muench method.

5.8. Cytopathogenic effect CPE assay

The indicated genes were induced in 6×10^5 Huh-7 cells by CRISPRa as described above. After electroporation, 2×10^4 cells were seeded per 96-well in 50 µl complete culture media in each of 3x10 96-wells and PBS was added to adjacent wells. Next day, cells were washed carefully three times with serum-free culture media before infection in triplicates with IAV (150 HAU per well) in serum-free culture media in a two-fold dilution series. The virus inoculum was removed after 1 h and cells were left in DMEM supplemented with 2% heat-inactivated fetal bovine serum (F9665, Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (10378-016, Gibco) for 72 h until CPE was assessed by light microscopy.

5.9. BafA1 treatment

3h before infected cells were harvested, BafA1 or vehicle (DMSO) was added into the cell culture media in a concentration of 200 nM.

5.10. Immunoblotting

Immunoblotting was performed as previously described [57] with the following modifications. Between 30 and 60 µg were loaded per well depending on the cell type. The images were acquired using the Azure 300 (Azure Biosystems). The used primary antibodies are listed in [Extended Data Table 3](#).

5.11. Real-time quantitative PCR (qPCR)

Host, SARS-CoV-2, IAV and HSV-1 gene expression levels were determined by qPCR. The RNA was isolated and the qPCR was setup and run as previously described [57] with the deviations below. [Extended Data Table 4](#) lists the assay IDs of the TaqMan gene expression assays (4331182, Thermo Scientific) targeting the host genes and HSV-1. IAV segment 7 (M) mRNA was detected using a custom-made TaqMan gene expression assay (4332078, Thermo Scientific) with a probe provided by the vendor and following forward (ATTTCCTATGAGACCGATGCT) and reverse (AGGATGGGGCTGTGACC) primers [62]. [Extended Data Table 5](#) lists the forward primer, reverse primer and probe (SarsCov19, LGC Biosearch) used to quantify the SARS-CoV-2 mRNA. For the qPCR reaction of SARS-CoV-2 mRNA we used 5 µl 2x TaqMan RT-PCR mix, 0.2 µl 40x TaqMan RT enzyme mix, 0.5 µl 10 µM forward primer F2, 0.7 µl 10 µM reverse primer R2, 0.2 µl 10 µM probe, RNA template and H₂O in a total volume of 10 µl. The qPCR setups measuring SARS-CoV-2 mRNA were run with the following conditions: 30 min 48 °C, 15 min 95 °C and 40 cycles of 15 s 95 °C and 1 min 60 °C.

5.12. Confocal microscopy

Huh7 cells were grown on coverslips, infected, and treated as indicated before being fixed with 4% formaldehyde (28908, Pierce) in PBS for 15 min at RT. Cells were washed in PBS 3 times before they were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 5 min at RT. After blocking in PBS containing 5% BSA (A7906, Sigma-Aldrich) and 10% goat serum for 1 h at RT, cells were incubated with primary antibodies diluted in PBS containing 5% BSA for 1 h at RT. Cells were washed in PBS 3 times before they were incubated with Alexa Fluor-conjugated secondary antibodies diluted 1:500 in PBS containing 5% BSA for 1 h at RT and, lastly, cell nuclei were stained with DAPI (D9542, Sigma-Aldrich, 1 µg/ml) for 10 min at RT. Coverslips were washed in PBS 3 times before they were mounted on slides using ProLong Glass Antifade Mountant (P36982, Invitrogen). Images were acquired on (Pln Apo Objective 40x/0.95NA Air) (405 nm, 488 nm, 561 nm, and 640 nm lasers) in a Zeiss LSM 800 Airyscan, laser scanning confocal, and processed with the ImageJ 2.0 software. The primary antibodies used were anti-p62/SQSTM1 (71001, Progen, 1:200), anti-SARS-CoV-2 S

(GTX632604, GeneTex, 1:300), and anti-LAMP-1 (9091, Cell Signalling Technology, 1:400). The secondary antibodies used were (A32723, A-21450, and A11011, Invitrogen).

5.13. Flow cytometry

To determine the proportion of HSV1-infected cells of WT and ATG5-KO HaCat treated with control or p62 siRNA, the GFP expression was detected 20 hpi. in 3 biological replicates using flow cytometry. Briefly, the cells were harvested and stained with ZombieNIR Fixable Viability Kit (Biolegend, 423105). Cells were washed and fixed using 2 % formaldehyde (Pierce, 28908) for 15 min at RT. Cells were resuspended in PBS with 1 % BSA and analyzed on NovoCyte Quanteon 4025 flow cytometer equipped with four lasers (405 nm, 488 nm, 561 nm and 637 nm) and 25 fluorescence detectors (Agilent, Santa Clara, CA). For analysis, cells were gated using the following gating strategy: total cells (FSC-H/SSC-H); single cells (FSC-A/FSC-H); single cells (SSC-A/SSC-H); viable cells (R780 filter/SSC-A), and finally GFP-positive cells (B530 filter/SSC-A) (Extended Data Fig. 5). In total, 100,000 cells alive were collected. Data were analyzed using FlowJo (version 10.10.0, Tree Star Inc., USA).

5.14. Selection of NRF2-driven genes of interest

Genes to be included in the screen were chosen using the following criteria: A minimum peak height of 15 was used for the NRF2 ChIPSeq dataset. For the RNAseq performed on HaCaT cells treated with 4OI, we used a 2-fold increase in expression. For the RNAseq performed on A549 cells treated with siRNA targeting NRF2, we included genes with a cutoff of 2.5-fold decrease in expression as compared with controls. A few genes, e.g., *ETV4*, *RAI14*, and *IL-6*, were included despite not meeting the inclusion criteria in one of the RNAseq due to a simple failure of detection in the analysis sample. The datasets from the RNA and ChIP sequencing of A549, as well as the one from the RNA sequencing of 4-OI-treated HaCaT cells, have been described previously [8,13].

5.15. Ethics and inclusion

We have appreciated local support from non-authors in the acknowledgement section. No human samples or animal experiments were included in this study.

5.16. Statistical analysis

A description of statistical work and parameters can be found in each figure legend. Graphs and statistics were computed using the Graph Pad Prism 9 software. *P*-values are indicated in the figures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2026.104135>.

Data availability

Data will be made available on request.

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