

1 **Pharmacological inhibition of bromodomain and extra-terminal proteins induces NRF-2-**
2 **mediated inhibition of SARS-CoV-2 replication and is subject to viral antagonism**

3 Baxolele Mhlekele^{1,2}, Dylan Postmus^{1,2}, January Weiner 3rd², Saskia Stenzel^{1,2}, Francisco J.
4 Zapatero-Belinchón^{3,4,5}, Ruth Olmer⁶, Jenny Jansen^{1,2}, Anja Richter¹, Julian Heinze¹, Nicolas
5 Heinemann¹, Barbara Mühlemann¹, Simon Schroeder¹, Terry C. Jones^{1,7}, Marcel Alexander
6 Müller¹, Christian Drosten¹, Andreas Pich⁸, Volker Thiel^{9,10}, Ulrich Martin⁶, Daniela
7 Niemeyer¹, Gisa Gerold^{3,4,5}, Dieter Beule², Christine Goffinet^{1,2*}

8 ¹Institute of Virology, Charité - Universitätsmedizin Berlin, corporate member of Freie
9 Universität Berlin, Humboldt-Universität zu Berlin, Charitéplatz 1, 10117 Berlin, Germany

10 ²Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117,
11 Berlin, Germany

12 ³Department of Biochemistry, University of Veterinary Medicine Hannover, 30559 Hannover,
13 Germany

14 ⁴Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical
15 Infection Research; a joint venture between the Hannover Medical School and the Helmholtz
16 Centre for Infection Research, 30625 Hannover, Germany

17 ⁵Department of Clinical Microbiology, Virology & Wallenberg Centre for Molecular Medicine
18 (WCMM), Umeå University, SE-90185 Umeå, Sweden

19 ⁶Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Department
20 of Cardiothoracic, Transplantation and Vascular Surgery, REBIRTH - Center for Translational
21 Regenerative Medicine, Biomedical Research in Endstage and Obstructive Lung Disease
22 Hannover (BREATH), German Center for Lung Research (DZL), Hannover Medical School,
23 30625 Hannover, Germany

24 ⁷Centre for Pathogen Evolution, Department of Zoology, University of Cambridge, Downing
25 St., Cambridge, CB2 3EJ, U.K.

26 ⁸Institute of Toxicology, Hannover Medical School, Core Facility Proteomics, 30629
27 Hannover, Germany

28 ⁹Institute of Virology and Immunology (IVI), University of Bern, 3001 Bern, Switzerland

29 ¹⁰Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern,
30 3001 Bern, Switzerland

31 *Corresponding author:

32 Christine Goffinet

33 Institute of Virology, Charité Campus Mitte, Charité - Universitätsmedizin Berlin, corporate
34 member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Charitéplatz 1, 10117
35 Berlin, Germany

36 Phone: +49 30 450 525 489

37 Email: christine.goffinet@charite.de

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39 BETs, iBETs, SARS-CoV-2, VOCs, NRF-2, antagonism

40 **ABSTRACT**

41 Inhibitors of bromodomain and extra-terminal proteins (iBETs), including JQ-1, have
42 been suggested as potential therapeutics against SARS-CoV-2 infection. However, molecular
43 mechanisms underlying JQ-1-induced antiviral activity and its susceptibility to viral
44 antagonism remain incompletely understood. iBET treatment transiently inhibited infection by
45 SARS-CoV-2 variants and SARS-CoV, but not MERS-CoV. Our functional assays confirmed
46 JQ-1-mediated downregulation of ACE2 expression and multi-omics analysis uncovered
47 induction of an antiviral NRF-2-mediated cytoprotective response as an additional antiviral
48 component of JQ-1 treatment. Serial passaging of SARS-CoV-2 in the presence of JQ-1
49 resulted in predominance of ORF6-deficient variants. JQ-1 antiviral activity was transient in
50 human bronchial airway epithelial cells (hBAECs) treated prior to infection and absent when
51 administered therapeutically. We propose that JQ-1 exerts pleiotropic effects that collectively
52 induce a transient antiviral state that is ultimately nullified by an established SARS-CoV-2
53 infection, raising questions on their clinical suitability in the context of COVID-19.

54 **INTRODUCTION**

55 There is an unmet need for novel effective therapies against the evolving variants of
56 severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of the
57 coronavirus disease-2019 (COVID-19) pandemic. The hallmark of severe SARS-CoV-2
58 infections is an excessive inflammatory response resulting in tissue damage and multiorgan
59 failure (Bülow Anderberg et al., 2021). Therefore, therapeutic avenues that simultaneously
60 dampen SARS-CoV-2 replication and antagonise its pathophysiological effects are utmostly
61 desired.

62 A group of small molecular inhibitors of the bromodomain and extra-terminal (BET)
63 proteins (iBETs) has been suggested to hold potential for the realisation of such therapies
64 (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022; Vann et al., 2022). The BET
65 protein family is made up of four multifaceted, ubiquitously expressed and evolutionarily
66 conserved proteins: bromodomain-containing protein (BRD) BRD2, BRD3, BRD4, and BRTD
67 (Lara-Ureña and García-Domínguez, 2021). They act as epigenetic readers and transcriptional
68 co-activators by binding to the acetylated lysine residues in histones in the chromatin and
69 recruit the cellular transcriptional machinery to drive transcription of their target genes (Cheung
70 et al., 2021; Suarez-Alvarez et al., 2017). Alternatively, BET proteins can act as transcriptional
71 co-repressors by interacting with their cellular partners to form repressor complexes, which
72 suppress inappropriate transcriptional programs to maintain tissue homeostasis (Cheung et al.,
73 2021).

74 The ability of BET proteins to regulate transcription has made them prime targets for
75 several diseases that hijack the cellular transcriptional machinery, including SARS-CoV-2
76 infection (Chen et al., 2022; De Rijck et al., 2013; Gordon et al., 2020; Shorstova et al., 2021;
77 Wu et al., 2016). iBETs evict BET proteins from histones and their non-histone binding
78 partners (Stathis and Bertoni, 2018). Therefore, clinical development of iBETs, most of which
79 are in different phases of clinical trials in the context of cancer therapy and other pathologies
80 (Hajmirza et al., 2018; Shorstova et al., 2021), may provide a pharmacological tool for
81 therapeutic intervention against several diseases driven by the BET protein-mediated
82 transcriptional programs.

83 BRD2 co-activates expression of ACE2 (Samelson et al., 2022), the main receptor for
84 SARS-CoV-2 entry into target cells (Hoffmann et al., 2020). Consequently, treatment of target
85 cells with iBETs downregulates ACE2 expression and inhibits SARS-CoV-2 replication and
86 inflammatory responses (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022).
87 Additionally, BET proteins co-activate the interferon (IFN) (Patel et al., 2013) and NF- κ B

88 (Huang et al., 2009) signalling pathways, which are the main drivers of the inflammatory
89 responses in COVID-19 patients (Lee et al., 2020; Ramasamy and Subbian, 2021).

90 Despite the growing literature on the anti-SARS-CoV-2 activity of iBET candidates
91 (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022; Vann et al., 2022), key questions
92 remain regarding the genome-wide epigenetic alterations orchestrating iBET-mediated
93 transcriptional responses that underlie their anti-SARS-CoV-2 activity. The missing
94 information about the susceptibility of iBET-mediated antiviral activity to SARS-CoV-2
95 subversion is hampering their potential as the next generation of prophylactics in the context of
96 COVID-19. Here, we sought to address these key questions by conducting an in-depth
97 functional and multi-omics analysis of the signalling alterations that underlie JQ-1-mediated
98 anti-SARS-CoV-2 activity.

99 **MATERIALS & METHODS**

100 **Chemicals and inhibitors**

101 (+)-JQ-1 (Cat no. SML1524-5MG) was purchased from Sigma. ABBV-075 (Cat no.
102 S8400) (ClinicalTrials.gov Identifier: [NCT02391480](#)), OTX015 (Cat no. S7360)
103 (ClinicalTrials.gov Identifier: [NCT02698176](#)), ARV-825 (Cat no. S8297) and ML385 (Cat no.
104 S8790) were purchased from Selleckchem. Dimethyl sulfoxide (DMSO) (Item no. 10127403)
105 was purchased from Thermo Fisher Scientific.

106 **Cell lines**

107 Calu-3 (ATCC HTB-55), CaCo-2 (ATCC HTB-37), Vero E6 (ATCC CRL-1586) and
108 parental HEK293T cells (ATCC CRL-3216) were cultured in Dulbecco's Modified Eagle's
109 Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml
110 Penicillin-Streptomycin and 2mM L-glutamine (Gibco, UK) (hereafter referred to as 10%
111 DMEM) at 37°C/5% CO₂. Primary bronchial airway epithelial cells were cultured in air-liquid
112 interface (ALI) mode in the provided ALI medium at 37 °C/5% CO₂. Unless stated otherwise,
113 all cell cultures were maintained at 37°C/5% CO₂.

114 **Human bronchial airway epithelial cells (Air-liquid interface cultures)**

115 Human bronchial airway epithelial cells (hBAECs) were isolated from explanted lungs
116 obtained from the Hannover Lung Transplant Program after patients' informed consent, ethical
117 vote 2923-2015. For isolation of hBAECs, human bronchial tissue was cut into small pieces in
118 Hank's buffer (Thermo Fisher Scientific) containing 0.18% protease XIV and incubated for
119 two hours at 37°C. After thorough pipetting with a 25/50 ml serological pipette, cell solution

120 was filtered through a 100 μ m cell strainer (Corning) to remove clumps and 10 ml RPMI
121 supplemented with 10% FCS (Thermo Fisher Scientific) was added. After centrifugation for 10
122 min at 500g and 4°C, supernatant was removed and cells were resuspended in SAEGMTM
123 (PromoCell) + Primocin (InvivoGen) + Penicillin-Streptomycin (P/S) (Sigma-Aldrich). For
124 ALI cultures, 200,000 hBAECs were seeded onto PureCol- (Advanced BioMatrix) coated 12-
125 well inserts (Greiner Bio-One) in SAEGMTM + Primocin + P/S. 48 hours post seeding, culture
126 medium in apical and basal chambers was changed to PneumaCult-ALI medium (STEMCELL
127 Technologies). Air lift was performed 48 hours later by gently removing medium from the
128 apical chamber. Homogenous distributed cilia were visible three weeks after air lift and inserts
129 were used for infections.

130 **Cell viability assays**

131 Calu-3 cells (6×10^5 cells/ml) seeded overnight in 96-well plates were treated with serial
132 dilutions (prepared in culture medium) of the iBETs or corresponding DMSO controls for 72
133 hours; with PBS wash, medium change and fresh drug administration after every 24 hours.
134 Post-treatment, cells were subjected to viability assays using the CellTiter-Glo Luminescent
135 Cell Viability Assay Kit (Promega, Germany) for Calu-3 cells and CellTiter-Glo 3-D Cell
136 Viability Assay Kit (Promega, Germany) for hBAECs according to the manufacturer's
137 protocol. The raw data from the test samples were background subtracted, normalised to naïve
138 cells, and analysed using GraphPad Prism v9 (LaJolla, CA, USA) as previously described
139 (Mhlekode et al., 2021).

140 **Virus production**

141 SARS-CoV-2 (passage (P) 1, BetaCoV/Munich/BavPat1/2020|EPI_ISL_406862)
142 (Wölfel et al., 2020), SARS-CoV (P1, HKU-39849 Hong Kong) (Lau et al., 2005; Zeng et al.,
143 2003), and MERS-CoV (P2, EMC/2012) (Zaki et al., 2012) stocks were propagated in Vero E6
144 cells to generate P2, P2, and P3 stocks respectively. The virus stock production and quality
145 control by Next Generation Sequencing were conducted as previously described (Niemeyer et
146 al., 2021). All infectious pathogens were handled under biosafety level three (BSL-3)
147 conditions with respiratory personal protection equipment.

148 **Virus infection of Calu-3 cells**

149 Calu-3 cells (6×10^5 cells/ml) seeded overnight in 12-well plates were treated with serial
150 dilutions of iBETs or corresponding DMSO controls for 48 hours; with PBS wash, medium
151 change and fresh drug administration every 24 hours. Post-treatment, cells were inoculated with

152 viruses (SARS-CoV and SARS-CoV-2, MOI 0.1; and MERS-CoV, MOI 0.0005) and incubated
153 for one hour. After infection, cells were washed with PBS, supplied with fresh medium and
154 iBETs and incubated for 24 hours. The next day, 50 μ l of supernatant was collected into 300 μ l
155 of RAV1 lysis buffer (Machery-Nagel, Germany) for viral RNA extraction and 100 μ l into 100
156 μ l of 0.5% gelatine medium for plaque assays. Cells were washed with PBS, trypsinized and
157 reconstituted in culture medium, from which 50 μ l was collected into 300 μ l of RAV1 lysis
158 buffer (Machery-Nagel, Germany) to isolate cell-associated RNA. RNA samples were stored at
159 -20°C and plaque assay samples at -80°C until sample processing.

160 **Virus infection of human bronchial airway epithelial cells (hBAECs)**

161 JQ-1 or DMSO was added into the culture medium (PneumaCult-ALI) in the basal
162 compartment of cells cultured in transwells and incubated for 48 hours, followed by medium
163 exchange and new drug administration after 24 hours as mentioned above. Post-treatment, cells
164 were washed three times with pre-warmed PBS to remove mucus from the apical compartment
165 and inoculated with SARS-CoV-2 (2×10^4 PFU diluted in OptiPRO medium), followed by
166 incubation for 90 minutes. The inoculum was then aspirated and cells washed with PBS three
167 times. The transwells were then transferred into new plates with the drug-containing culture
168 medium. To harvest samples for zero time point, 200 μ l of culture medium was immediately
169 added onto the cells in the apical compartment and incubated for 15 min to elute the virus
170 particles. Following incubation, 50 μ l of virus eluate was collected into 300 μ l RAV1 lysis
171 buffer (Machery-Nagel, Germany) for viral RNA extraction and 100 μ l into 100 μ l of 0.5%
172 gelatine medium for plaque assays, after which the eluate residues were aspirated from the
173 apical compartment and followed by incubation of cells. This harvesting procedure was
174 repeated every 24 hours for kinetic experiments. The samples were stored at -20°C and -80°C ,
175 respectively, until processing.

176 **Quantitative real-time PCR**

177 Viral RNA from the supernatant was extracted using the NucleoSpin RNA Virus
178 Isolation Kit (Machery-Nagel, Germany) according to the manufacturer's protocol. To
179 quantify viral RNA copies, a 12.5 μ l reaction/well was prepared in 4titude® 96-Well Semi-
180 Skirted PCR Plates for LC480 (Roche) using a SuperScript III One-Step RT-PCR Kit
181 (Invitrogen, Germany). Reverse transcription of the viral RNA and amplification of the cDNA
182 was conducted in a LightCycler 480 System (Roche, Germany) using the following protocol:
183 55°C , 10 min; 95°C , 3 min; 95°C , 15 sec; 58°C , 30 sec and 40°C , 30 sec for 45 cycles and

184 qPCR primers against SARS-CoV and SARS-CoV-2 E gene and probes (Corman et al., 2020).
185 The absolute quantification of the viral RNA copies was calculated by a standard curve method
186 using viral RNA standards (Corman et al., 2020).

187 **Plaque titration assays**

188 Seeded Vero E6 cells (4×10^5 cell/ml) were left overnight in 24-well plates. The virus-
189 containing samples (50 μ l) were serially diluted in 450 μ l aliquots of serum-free Opti-Pro
190 medium (Gibco, UK), after which 200 μ l from each dilution were added in duplicates to the
191 cells, followed by one hour incubation. Post-infection, viral inoculum was aspirated from the
192 cells, cells washed with PBS, and overlaid with 2.4% Avicel (FMC BioPolymers, Germany)
193 mixed with 2xDMEM (1:1 ratio), followed by incubation for 72 hours. The overlay was
194 aspirated from the cells, after which they were fixed for 30 min with 6% formaldehyde and
195 stained with crystal violet solution (0.2% crystal violet, 2% ethanol and 10% formaldehyde) for
196 20 min. Plaque Forming Units (PFU) were determined from at least two dilutions for which
197 distinct plaques were detectable and expressed as PFU/ml.

198 **Flow cytometry**

199 JQ-1 and DMSO-treated Calu-3 cells were infected with GFP-tagged SARS-CoV-2
200 (synSARS-CoV-2-GFP-P2A-ORF7a clone #41, (Thi Nhu Thao et al., 2020) (MOI 0.25)
201 following the above-mentioned protocol. Subsequently, cells were trypsinized, PBS-washed
202 and fixed for 90 minutes with 4% PFA. GFP signal was quantified by flow cytometry in
203 FACSCelesta (BD Bioscience, Germany) and analysed by FlowJo v10.8 (Tree Star, Ashland,
204 Oregon, USA).

205 **Immunoblotting**

206 Calu-3 cells were lysed in 60 μ l RIPA Lysis Buffer (Thermo Fisher Scientific,
207 Germany) supplied with 1 % Protease Inhibitor Cocktail Set III (Merck Chemicals, Germany)
208 for 30 min at 4 °C. Subsequently, cell lysates were pelleted and protein concentration in
209 supernatant was determined by BCA protein assay (ThermoFisher Scientific). 20 μ g total
210 protein from each sample was mixed with 4x Laemmli buffer, which was supplemented with
211 10% beta-mercaptoethanol, then boiled for ten minutes at 95°C to ensure protein denaturation.
212 Proteins were resolved on 6% SDS-PAGE gel and transferred to a nitrocellulose membrane
213 (0.45 μ m pore size, GE Healthcare) by Trans-Blot Turbo system (BioRad). Membranes were
214 blocked with 5% dried milk in 0.1% PBS-Tween (0.9% NaCl, 10 mM Tris-HCl [pH 7.5], 0.1%
215 Tween 20) for 30 min at room temperature. Blocked membranes were incubated with the

216 primary antibodies against ACE2 (#AF933, R&D Systems) and beta-actin (#A5316, Sigma-
217 Aldrich). Secondary antibodies conjugated with horseradish peroxidase (HRP) were used for
218 chemiluminescence-based detection by Fusion Fx7 (Peqlab Biotechnologie GmbH). Detection
219 was performed using SuperSignal™ West Femto substrate (ThermoFisher Scientific).

220 **Production of pseudotyped lentivirus particles and transduction assays**

221 SARS-CoV-2-S- and VSV-G-pseudotyped lentiviral particles (Hu et al., 2020) were
222 produced by calcium phosphate-based transfection of HEK293T cells with the packaging
223 plasmid pCMV ΔR8.91 (Zufferey et al., 1997), the lentiviral transfer plasmid pCSII-EF-
224 luciferase (Agarwal et al., 2006), and pCMV-VSV-G (Stewart et al., 2003) using the CalPhos
225 Mammalian Transfection Kit (Takara Bio Company, Germany) according to the manufacturer's
226 protocol. Dose-dependent treatment of Calu-3 cells with JQ-1, followed by transduction with
227 pseudovirus particles in 96-well plates was conducted under BSL-2 conditions, after which the
228 infection efficiency was analysed luminometrically in Synergy™ HTX Multi-Mode Microplate
229 Reader (BioTek Instruments, Inc.) using the Luciferase Assay System (Promega, Germany)
230 according to the manufacturer's protocol.

231 **ATAC-seq**

232 Bulk ATAC-seq and RNA-seq were conducted in parallel from the identical infection
233 experiment. Calu-3 cells were treated with JQ-1 and DMSO, respectively, for 24 hours,
234 infected with SARS-CoV-2 (MOI 0.1) under continuous treatment for another 24 hours.
235 Uninfected but treated, and naïve Calu-3 cells were used as reference. Post-infection, ATAC-
236 seq libraries were prepared from 50,000 cells per replicate using Illumina Tagment DNA
237 Enzyme and Buffer Kit (Illumina 20034197) according to the Omi-ATAC-seq protocol (Corces
238 et al., 2017) with the following minor optimizations. Briefly, 5 µl from the partially amplified
239 barcoded fragments was subjected to SYBR Green qPCR in a LightCycler 480 System (Roche,
240 Germany) using the FastStart Essential DNA Green Master Mix (Roche, Germany) according
241 to the manufacturer's protocol. Amplification was conducted for 20 cycles using a universal
242 forward primer and the sample-specific barcoded reverse primers (Buenrostro et al., 2013).
243 Amplification curves from SYBR Green qPCR were generated and used to determine the
244 number of cycles that give 1/3 of the maximum fluorescence. Final library preparation was
245 conducted by conventional PCR for 8-12 cycles. The amplified libraries were subjected to a
246 single left-sided bead purification using the AMPure XP magnetic beads (Beckman Coulter,
247 Germany). Libraries were sequenced on SP lane of NovaSeq 6000 System (Illumina) at the

248 MDC/BIH Genomic Core Facility to generate 40 million 75-nucleotide paired-end reads per
249 sample.

250 **RNA-seq**

251 Total RNA was isolated from 3×10^5 cells per replicate using Direct-Zol RNA Miniprep
252 Kit (Zymo Research, USA) according to the manufacturer's protocol and shipped in dry ice to
253 the MDC/BIH Genomic Core Facility for quality assessment using TapeStation (Agilent
254 Technologies). Libraries were prepared using TrueSeq Stranded mRNA kit to generate
255 Illumina-compatible libraries by following the manufacturer's protocol (Illumina). Libraries
256 were sequenced on SP lane of NovaSeq 6000 System (Illumina) to generate 40 million 75-
257 nucleotide paired-end reads per sample.

258 **Liquid chromatography tandem mass spectrometry (LC-MS/MS)**

259 Treatment of Calu-3 cells and infection with SARS-CoV-2 were performed following
260 the above-mentioned protocol. Treated but uninfected and naïve cells served as references.
261 Preparation of cell lysate samples and protein quantification were conducted as mentioned
262 above, from which 50 μ g of proteins per sample were prepared and resolved on 7.5% linear
263 SDS-PAGE gels as described elsewhere (Mhlekode et al., 2021). The in-gel protein digestion
264 and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) experiments
265 were performed as previously described (Jochim et al., 2011).

266 **Bioinformatics analysis**

267 For RNA-seq, reads were aligned to the human genome version GRCh38 and counted
268 using STAR (Dobin et al., 2013). Differential expression analysis was conducted using
269 DESeq2 version 1.30 (Love et al., 2014). Transcription factor analysis was performed using the
270 R package Dorothea (Garcia-Alonso et al., 2019) For ATAC-seq, reads were aligned to the
271 human genome GRCh38, after which the peaks were called using MACS2 v. 2.2.7.1 (Zhang et
272 al., 2008). For differential TF binding analysis, the R package DiffBind v. 3.0.15 (Stark and
273 Brown) was used. Motif search analysis was performed using the MEME suite (Bailey et al.,
274 2015) and DREME (<https://meme-suite.org/meme/doc/dreme.html>). Gene set enrichment
275 analysis was performed with the R package cluster Profiler v. 3.18 (Yu et al., 2012). All results
276 were corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini and
277 Hochberg, 1995). Genes were annotated as involved in IFN signalling, the cell cycle, or
278 autophagy by referring to the Reactome Interferon Signalling geneset (R-HSA-913531), the
279 Reactome Cell Cycle geneset (R-HSA-1640170), and the KEGG Autophagy - animal geneset

280 (hsa04140), respectively. Genes identified as targets for NRF2 signalling were identified based
281 on a previous report (Olagnier et al., 2020). LC-MS/MS data analysis was conducted as
282 previously described (Mhlekode et al., 2021).

283 **Statistical analysis**

284 If not stated otherwise, bars show the arithmetic mean of indicated amount of
285 repetitions. Error bars indicate standard error of the mean (SEM) from the indicated amount of
286 individual experiments. Statistical significance was calculated by performing a Student's t-test
287 using GraphPad Prism. *P* values ≤ 0.05 were considered significant: $\square 0.05$ (*), $\square 0.0021$ (**),
288 $\square 0.0002$ (***), $\square 0.0001$ (****) and n.s. = not significant (≥ 0.05).

289 **Data and code availability**

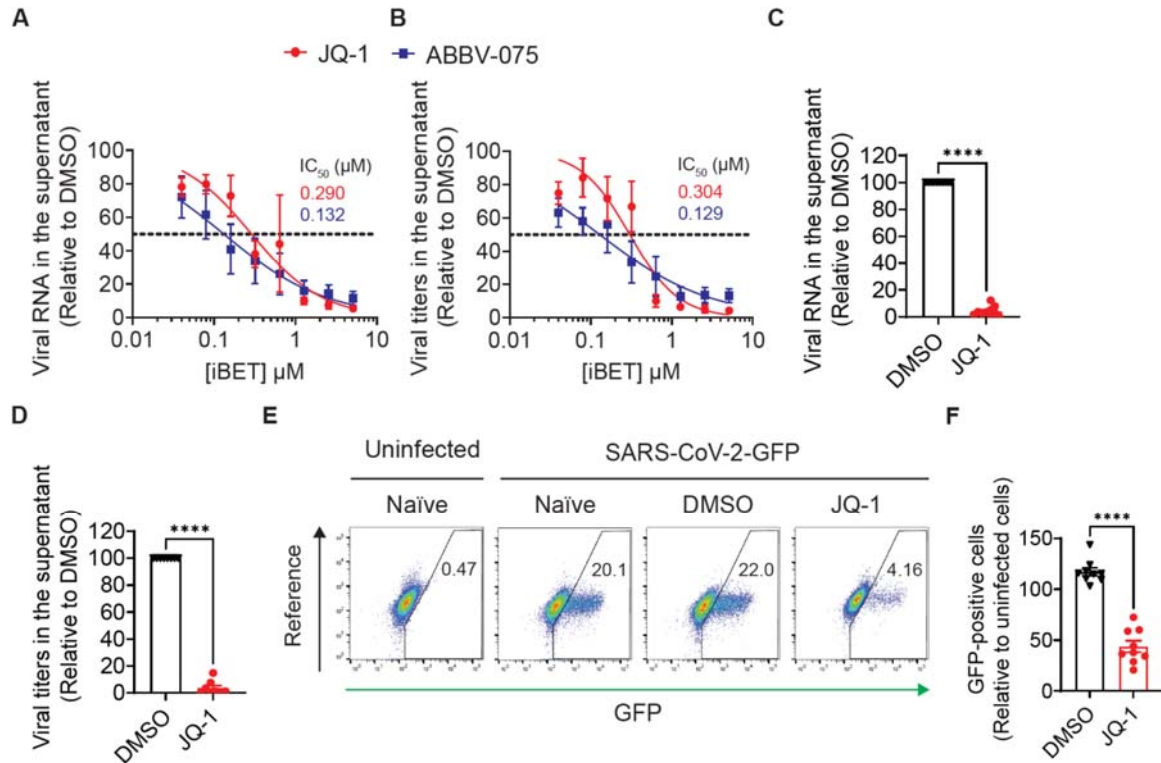
290 All code used to perform the analysis of the RNAseq and ATACseq data will be
291 available at https://github.com/GoffinetLab/SARS-CoV-2_JQ1-Antiviral-Study. Raw and
292 processed OMICs data will be deposited on the GEO database.

293 **RESULTS**

294 **Prophylactic administration of inhibitors of bromodomain and extra-terminal proteins** 295 **(iBET) inhibit SARS-CoV-2 infection in lung epithelial Calu-3 cells**

296 Inhibition of SARS-CoV-2 infection by several iBET compounds has been reported in different
297 infection models (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022). To gain more
298 insights into their antiviral potency in the context of SARS-CoV-2 infection, we compared
299 antiviral activity of four iBETs, three (JQ-1, ABBV-075, and OTX-015) of which compete with
300 the acetylated molecules for binding to the bromodomains of the BET proteins, and one (ARV-
301 825) that targets BET proteins (dBET) carrying proteolysis-targeting chimera (PROTAC)
302 sequences to proteasomal degradation. iBET treatment of Calu-3 cells prior to SARS-CoV-2
303 infection led to a dose-dependent reduction of viral genomic RNA quantities (Fig. 1A and Sup.
304 Fig. 1C) and infectious titers (Fig. 1B and Sup. Fig. 1D) in the supernatant in the absence of
305 detectable cell toxicity (Sup. Figs. 1A and 1E). Of the investigated iBET candidates, JQ-1 (IC_{50}
306 = 0.290 μ M) and ABBV-075 (IC_{50} =0.132 μ M) were more potent than OTX-015 (IC_{50} = 3.553
307 μ M) and ARV-825 (IC_{50} = 1.431 μ M) against SARS-CoV-2 infection in Calu-3 cells.
308 Prophylactic administration of JQ-1 to hBAECs reduced SARS-CoV-2 viral RNA quantities by
309 24.8-fold (Fig. 1C) and infectious titers by 28.2-fold (Fig. 1D) without detectable toxicity (Sup.
310 Fig. 1B), demonstrating the relevance of iBET-mediated anti-SARS-CoV-2 activity in a
311 physiologically relevant primary cell model (Samelson et al., 2022). JQ-1 treatment rendered

312 Calu-3 cells 2.7-fold less susceptible to infection by recombinant GFP-expressing SARS-CoV-
313 2, reflecting the consistency of JQ-1-mediated anti-SARS-CoV-2 activity across different
314 readouts (Fig. 1E-F).



315 **Figure 1. Prophylactic administration of inhibitors of bromodomain and extra-terminal**
316 **proteins (iBET) inhibit SARS-CoV-2 infection in lung epithelial Calu-3 cells.**
317

318 (A-B) Calu-3 cells were pretreated with indicated concentrations of iBETs for 48 hours,
319 followed by infection with SARS-CoV-2 (MOI 0.1) under continuous presence of the drug. At
320 24 hours post infection, relative (A) SARS-CoV-2 RNA and (B) infectious titers in
321 supernatants were quantified from three independent experiments.

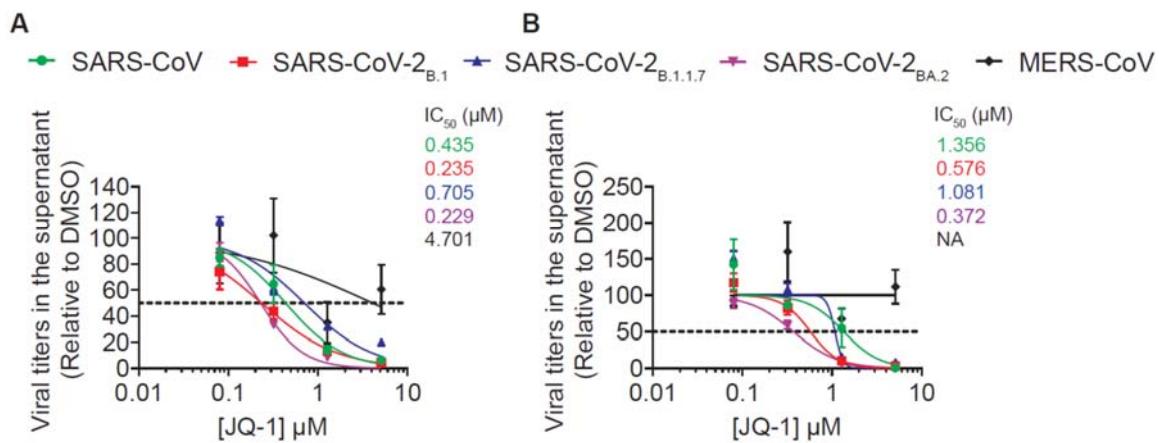
322 (C-D) hBAECs were pretreated with JQ-1 (2.56 μM) for 48 hours, followed by infection with
323 SARS-CoV-2 (2×10^4 PFUs) under continuous presence of the drug. At 48 hours post-infection,
324 (C) abundance of viral RNA from duplicates of five independent experiments and (D)
325 infectivity from duplicates of four independent experiments were quantified in supernatants.
326 Data shown are relative to DMSO treatment.

327 (E-F) Calu-3 cells were pretreated with JQ-1 for 48 hours prior to infection with a recombinant,
328 GFP-expressing SARS-CoV-2 (MOI 0.25). Shown are (E) representative FACS dot plots and
329 (F) quantification of percentage of GFP-positive cells relative to DMSO treatment at 24 hours
330 post infection, from three independent experiments with triplicates each. Unpaired

331 nonparametric Student *t*-test was used to compare the means between the experimental groups.
332 Error bars represent the arithmetic mean±SEM.

333 **JQ-1 exhibits a subgenera-specific antiviral activity among *Betacoronaviridae* in lung** 334 **epithelial Calu-3 cells**

335 To investigate whether the iBET-mediated anti-SARS-CoV-2 activity extends beyond SARS-
336 CoV-2 parental strains, which have been the main focus of the previous studies (Gilham et al.,
337 2021; Mills et al., 2021; Samelson et al., 2022), we compared the antiviral potency of JQ-1 in
338 Calu-3 cells infected with a panel of β -coronaviruses. JQ-1 potently and dose-dependently
339 inhibited infection by SARS-CoV, SARS-CoV-2_{B.1}, SARS-CoV-2_{B.1.1.7} and SARS-CoV-2_{BA.2},
340 in contrast to MERS-CoV, as indicated by the reduction of viral RNA copies (Fig. 2A) and
341 infectious titers (Fig. 2B) in culture supernatants. These data suggest that JQ-1 exhibits a
342 subgenera-specific antiviral activity, which is directed towards infection by *Sarbecoviruses* and
343 not *Merbecoviruses* to which MERS-CoV belongs.



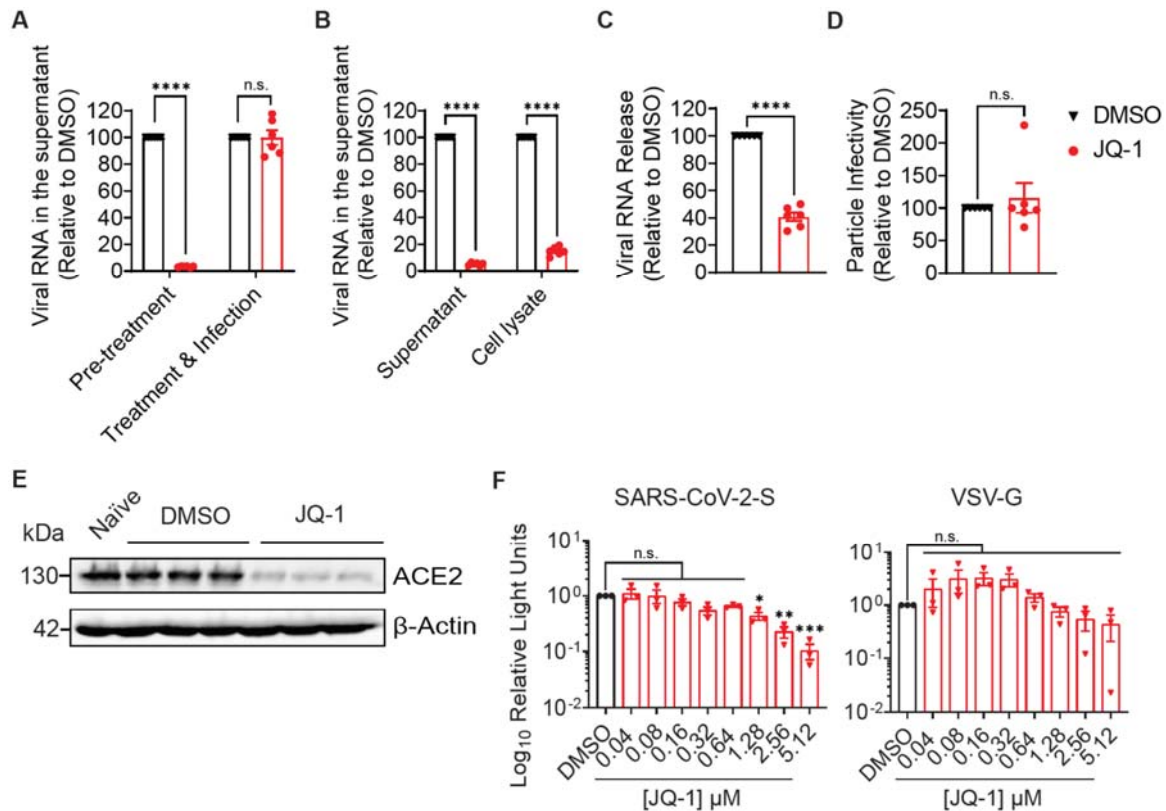
344 **Figure 2. JQ-1 exhibits a subgenera-specific activity among *Betacoronaviridae* in lung**
345 **epithelial Calu-3 cells.**
346

347 Calu-3 cells were pretreated with indicated concentrations of JQ-1 prior to infection with
348 indicated viruses. Shown are DMSO-normalised (A) viral RNA abundance and (B) infectivity
349 in supernatants harvested at 24 h.p.i. with *Sarbecoviruses* (MOI 0.1) and *Merbecovirus* (MOI
350 0.0001). Error bars represent the mean±SEM.

351 **JQ-1 exhibits a cell-directed anti-SARS-CoV-2 activity in lung epithelial Calu-3 cells**

352 Next, we compared the effect of JQ-1-mediated anti-SARS-CoV-2 activity when administered
353 prophylactically and at the time point of infection. Focusing on a single round of infection, we
354 found that JQ-1 inhibited SARS-CoV-2 replication when administered prophylactically in
355 Calu-3 cells, while it failed to inhibit infection when administered to cells during infection (Fig.

356 3A). PCR-based quantification of viral RNA copies showed that JQ-1-mediated reduction of
 357 viral RNA copies in the supernatant of infected Calu-3 cells is accompanied by, and probably a
 358 consequence of, reduced quantities of cell-associated viral RNA (Fig. 3B). The ratio of
 359 extracellular to total viral RNA indicated that JQ-1 impaired release of SARS-CoV-2 virions
 360 (Fig. 3C), without *per se* reducing the specific particle infectivity of secreted particles (Fig.
 361 3D). In accordance with previous reports (Gilham et al., 2021; Mills et al., 2021; Samelson et
 362 al., 2022), JQ-1 treatment reduced steady-state levels of ACE2 expression (Fig. 3E), an
 363 observation that was accompanied by reduced susceptibility of Calu-3 cells to transduction with
 364 SARS-CoV-2 spike, but not VSV-G-pseudotyped lentiviral particles (Sup. Fig. 3F). Together,
 365 these data suggest that JQ-1 exhibits a cell-directed anti-SARS-CoV-2 activity involving, but
 366 likely not restricted to, downregulation of ACE2 expression.



367
 368 **Figure 3. JQ-1 exhibits a cell-directed anti-SARS-CoV-2 activity in lung epithelial Calu-3**
 369 **cells**

370 (A) Calu-3 cells were either pre-treated with JQ-1 (2.56 μ M) for 48 hours, followed by SARS-
 371 CoV-2 infection (MOI 0.1) and continued treatment (Pre-treatment), or treatment was initiated
 372 at the time point of infection (Treatment & Infection). 24 hours post-infection, relative viral
 373 RNA abundance in supernatants was quantified.

374 **(B-D)** The relative efficiency of different steps of the replication cycle were quantified
375 following JQ-1 treatment and infection of Calu-3 cells, including **(B)** viral RNA abundance in
376 supernatants and cell lysates, **(C)** particle release and **(D)** specific particle infectivity. The error
377 bars represent the arithmetic mean \pm SEM from duplicates of three independent experiments.
378 Viral release was calculated by dividing the concentration of viral RNA in the supernatant by
379 the sum of the concentrations of the viral RNA in the supernatant and cell lysate. Particle
380 infectivity was calculated by dividing the viral titers by the corresponding viral RNA
381 concentration in the supernatant.

382 **(E)** Immunoblot analysis of ACE2 expression in JQ-1-treated Calu-3 cells

383 **(F)** Luminometric quantification of transduction efficiency of JQ-1-treated Calu-3 cells using
384 lentiviral pseudoparticles decorated with SARS-CoV-2-S and VSV-G proteins. Error bars
385 represent the mean \pm SEM from three independent experiments. Unpaired nonparametric
386 Student *t*-test was used to compare the means between the experimental groups.

387 **SARS-CoV-2 infection and JQ-1 treatment modulate the chromatin regulatory landscape** 388 **in lung epithelial Calu-3 cells**

389 To investigate the chromatin regulatory landscape that associates with JQ-1-mediated SARS-
390 CoV-2 inhibition, we subjected Calu-3 cells to bulk ATAC-seq. Principal component analysis
391 (PCA) showed clustering of samples according to their experimental groups, reflecting
392 comparable chromatin profiles between individual samples in each group (Sup. Fig. 2A). JQ-1
393 treatment imposed larger changes than SARS-CoV-2 infection. Samples from uninfected
394 DMSO-treated cells closely clustered with samples from naïve cells, suggesting that DMSO
395 treatment induced minimal changes to the host chromatin profile. Compared to the control
396 groups, JQ-1 administration to Calu-3 cells increased accessibility to the transcriptional start
397 sites (TSS) irrespective of the infection status, followed by SARS-CoV-2 infection, as indicated
398 by the increase in the density of peaks mapping to the TSSs (Fig. 4A), illustrating the
399 differential ability of JQ-1 treatment and SARS-CoV-2 infection to remodel chromatin
400 accessibility to the TSSs. Annotation of the accessible peaks to the genomic features in the
401 chromatin showed a large coverage for promoter sequences located within one kb from the TSS
402 regions across all experimental groups (Fig. 4B). However, irrespective of the infection status,
403 JQ-1 treatment shifted a proportion of the accessible regions away from the promoter
404 sequences located within one kb from the TSS regions to the introns and distal intergenic
405 regions. This suggests that regulation of transcription under JQ-1 treatment is driven by a more
406 evenly-distributed accessibility of genomic features compared to SARS-CoV-2 infection or

407 absence of JQ-1, where transcriptional regulation is predominantly driven by the proximal
408 regulatory cis-elements in the promoters. Hierarchical clustering of significantly regulated
409 peaks in each experimental group revealed distinct chromatin accessibility profiles, modulated
410 distinctly by either SARS-CoV-2 infection or JQ-1 treatment, with some commonly
411 upregulated peaks resulting from both SARS-CoV-2 infection and JQ-1 treatment, respectively
412 (Fig. 4C).

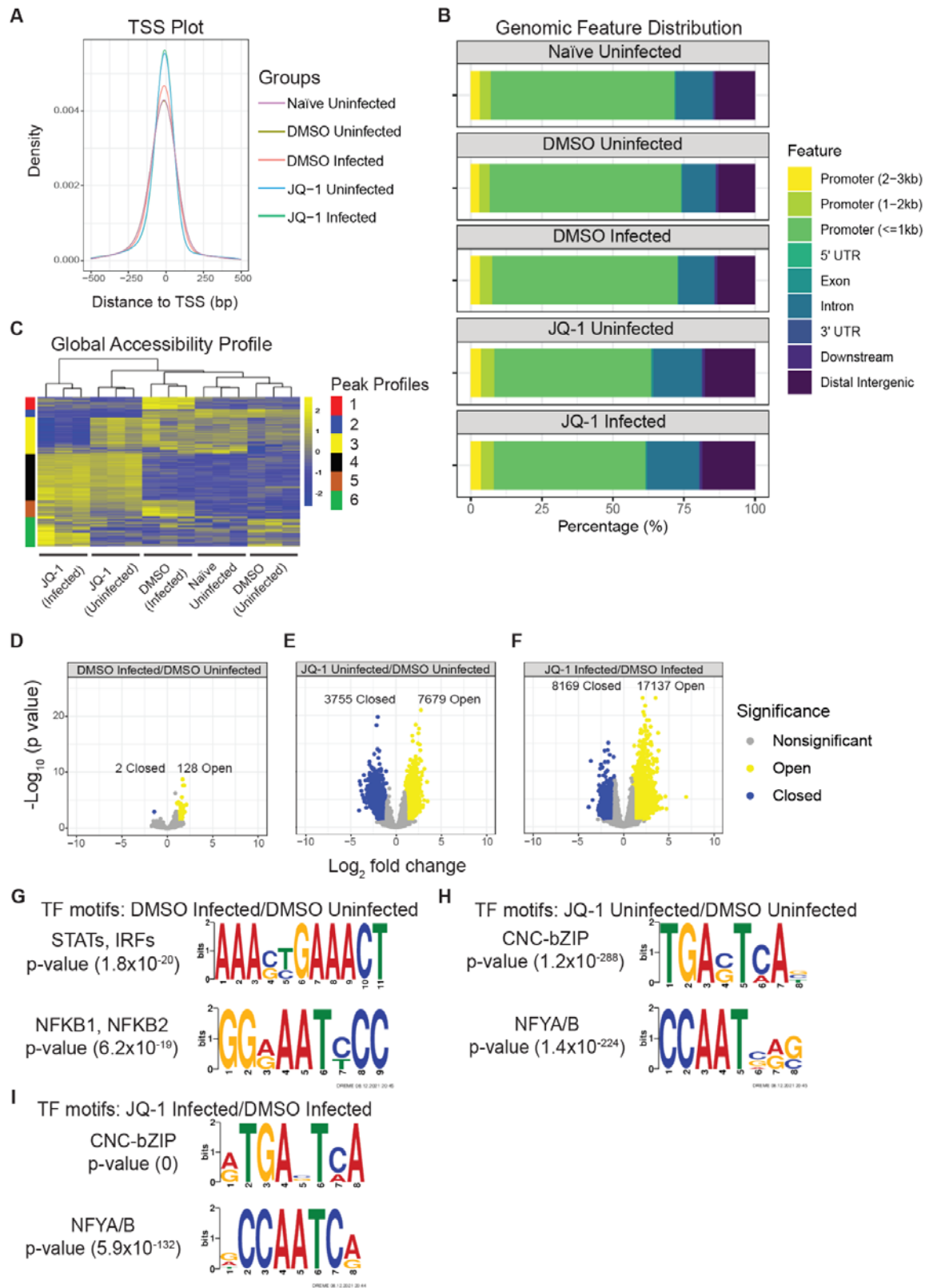
413 Volcano plots of the ATAC-seq data revealed that SARS-CoV-2 infection only mildly
414 modulated the chromatin accessibility profile (significant changes in 130 ATAC-seq peaks, 128
415 of which were accessible while only 2 were inaccessible) (Fig. 4D). In contrast, JQ-1 treatment
416 drastically altered the chromatin accessibility landscape (Fig. 4E, significant changes of 11,434
417 ATAC-seq peaks, 7679 of which were accessible while 3,755 were inaccessible). Strikingly,
418 combination of JQ-1 treatment and infection induced 2.2-fold increased change to the
419 accessibility of genomic regions when compared to JQ-1 treatment only (Fig. 4F, significant
420 changes in 25,306 ATAC-seq peaks, whereby 17,137 were accessible while 8,169 were
421 inaccessible), implying that changes induced by JQ-1 pretreatment are quantitatively superior
422 to those induced by infection.

423 As expected (Wilk et al., 2021; You et al., 2021), compared to uninfected cells,
424 pathways that drive innate immune responses were enriched in the accessible ATAC-seq peaks
425 from SARS-CoV-2-infected cells (Sup. Fig 2B). Accessible ATAC-seq peaks from JQ-1-
426 treated cells showed no significant association with any particular biological pathway when
427 compared to DMSO-treated cells (Sup. Fig 2B-C), suggesting that JQ-1 induced a rather global
428 change rather than alterations of specific biological processes. On the other hand, when
429 compared to infected cells, accessible ATAC-seq peaks from infected cells in the presence of
430 JQ-1 were mostly enriched with ribosome biogenesis and RNA processing pathways (Sup. Fig
431 2B), including RNA splicing reported to be inhibited by SARS-CoV-2 (Banerjee et al., 2020).
432 Of note, nonsense-mediated mRNA decay, a cellular RNA surveillance pathway that exhibits a
433 broad antiviral activity (Balistreri et al., 2014; Wada et al., 2018), was among the highly
434 enriched pathways in the accessible peaks (Sup. Fig 2B). Conversely, pathways associated with
435 sensory perception of smell were downregulated in the accessible peaks from SARS-CoV-2-
436 infected cells, both in the presence of DMSO and JQ-1 (Sup. Fig 2C), but not by JQ-1 *per se*,
437 suggesting an inability of JQ-1 to hamper SARS-CoV-2-mediated downregulation of the smell
438 receptor signalling pathway. Furthermore, accessible peaks from JQ-1-treated infected cells
439 were associated with downregulation of the neuropeptide signalling pathway (Sup. Fig 2C), as
440 opposed to infection or JQ-1 treatment, suggesting JQ-1's potential to interfere with neuronal

441 communication specifically in the infection context. Together, these data show that SARS-
442 CoV-2- and JQ-1-mediated modulation of the chromatin accessibility landscape occurs at
443 multiple levels, with differing magnitudes and breadth.

444 We next searched for transcription factor (TF) binding motifs that were significantly
445 enriched in the accessible ATAC-seq peaks using the DREME algorithm, which is designed to
446 find short and multiple nonredundant binding motifs of eukaryotic TFs and calculate their
447 statistical significance (Bailey, 2011). In infected cells, accessible peaks were enriched for
448 binding motifs for inflammatory TF families, including Signal Transducer and Activator of
449 Transcription (STAT), Interferon Regulatory Factors (IRF) and Nuclear Factor-kappa B
450 (NFkB), which drive IFN signalling and induction of pro-inflammatory cytokines, respectively
451 (Park and Iwasaki, 2020) (Fig. 4G and Sup. Table 1A), suggesting increased accessibility of
452 STAT-, IRF- and NFkB-binding sites to induce IFN response pathways and production of pro-
453 inflammatory cytokines (Bülow Anderberg et al., 2021; Ramasamy and Subbian, 2021). Unlike
454 in SARS-CoV-2 infection, the accessible peaks in JQ-1-treated cells showed significant
455 enrichment of several binding motifs of a wide variety of TF families, suggesting a broader
456 modulation of gene expression by JQ-1 over SARS-CoV-2 infection (Sup. Table 1B-C).
457 Independent of infection, among many others, peaks from JQ-1-treated cells were enriched
458 with binding motifs for TF families such as CNC-bZIP and NF-Y (Fig. 4H-I, Sup. Table 1B-
459 C), which induce antiviral cellular cytoprotective responses (He et al., 2020; Olganier et al.,
460 2020) and more accessible chromatin (Oldfield et al., 2014, 2019), respectively. Detection of
461 these enrichments, independent of SARS-CoV-2 infection, argues for a dominant role of JQ-1
462 treatment on transcription modulation via the CNC-bZIP and NFY TF families. Secondly,
463 motifs annotated to a superfamily of steroid-induced nuclear receptor (NR) TFs, whose
464 signalling drives cellular RNA splicing processes (Auboeuf et al., 2004; Elhasnaoui et al.,
465 2021), were also enriched in JQ-1-treated groups irrespective of the infection status (Sup. Table
466 1B-C). However, JQ-1 treatment in the presence of SARS-CoV-2 infection (Sup. Table 1C)
467 displayed significant enrichment with TF binding motifs for a wide range of TF families
468 compared to the absence of infection (Sup. Table 1B), suggesting that SARS-CoV-2 infection
469 influences JQ-1-mediated TF profile and likely fine-tunes the JQ-1-mediated transcriptome.
470 Interestingly, compared to uninfected cells, peaks from uninfected JQ-1-treated cells were
471 significantly enriched with a binding motif for NFkB-1/NFkB-2 TFs (Sup. Table 1B), despite
472 JQ-1 being an inhibitor of the NFkB-1-mediated canonical pathway that induces cytokine
473 production (Samelson et al., 2022). Together, these data illustrate the genome-wide TF binding

474 profiles that drive the transcriptional programs governing SARS-CoV-2 infection- and JQ-1-
475 mediated changes.



476

477

Figure 4. SARS-CoV-2 infection and JQ-1 treatment modulate the chromatin regulatory

478

landscape in lung epithelial Calu-3 cells.

479 (A) TSS plot depicting the density and distribution of accessible ATAC-seq peaks around
480 transcription start sites within a window of -500 to 500 bp.
481 (B) Analysis of the genomic features annotated to accessible ATAC-seq peaks in the
482 chromatin. (C) Heatmap hierarchical clustering of differentially accessible ATAC-seq peaks
483 scaled as z-score across rows.
484 Volcano plots showing relative \log_2FC and statistical significance of differentially regulated
485 ATAC-seq peaks in (D) DMSO-treated infected versus DMSO-treated uninfected, (E) JQ-1-
486 treated uninfected versus DMSO-treated, uninfected and (F) JQ-1-treated infected versus
487 DMSO-treated, infected contrasts. Significantly (FDR of ≤ 0.05) regulated peaks are indicated.
488 TF motif enrichment analysis from the accessible ATAC-seq peaks in (G) DMSO-treated,
489 infected versus DMSO-treated uninfected, (H) JQ-1-treated uninfected versus DMSO-treated,
490 uninfected and (I) JQ-1-treated infected versus DMSO-treated, infected contrasts. The TF
491 motifs were identified using the DREME algorithm, where the height of the letters represents
492 the frequency of each base in the motif.

493 **SARS-CoV-2- and JQ-1-mediated modulations of the chromatin accessibility landscape**
494 **underlie the transcriptomic and proteomic changes in lung epithelial Calu-3 cells**

495 Next, we subjected the samples to bulk RNA-seq and mass spectrometry to establish their
496 transcriptomic and proteomic profiles, respectively, including the downstream effects of SARS-
497 CoV-2- and JQ-1-mediated modulations on the chromatin accessibility landscape. PCA of the
498 RNA-seq data revealed that the samples clustered according to their experimental groups,
499 reflecting similar transcriptomic profiles between replicates in each group (Sup. Fig. 3A). As in
500 ATAC-seq (Sup. Fig. 2A), JQ-1 treatment induced more important changes than SARS-CoV-2
501 infection. Samples from naïve and uninfected DMSO-treated cells clustered close to each other,
502 with minimal effect of DMSO treatment on the cellular transcriptome (Sup. Fig. 3A).

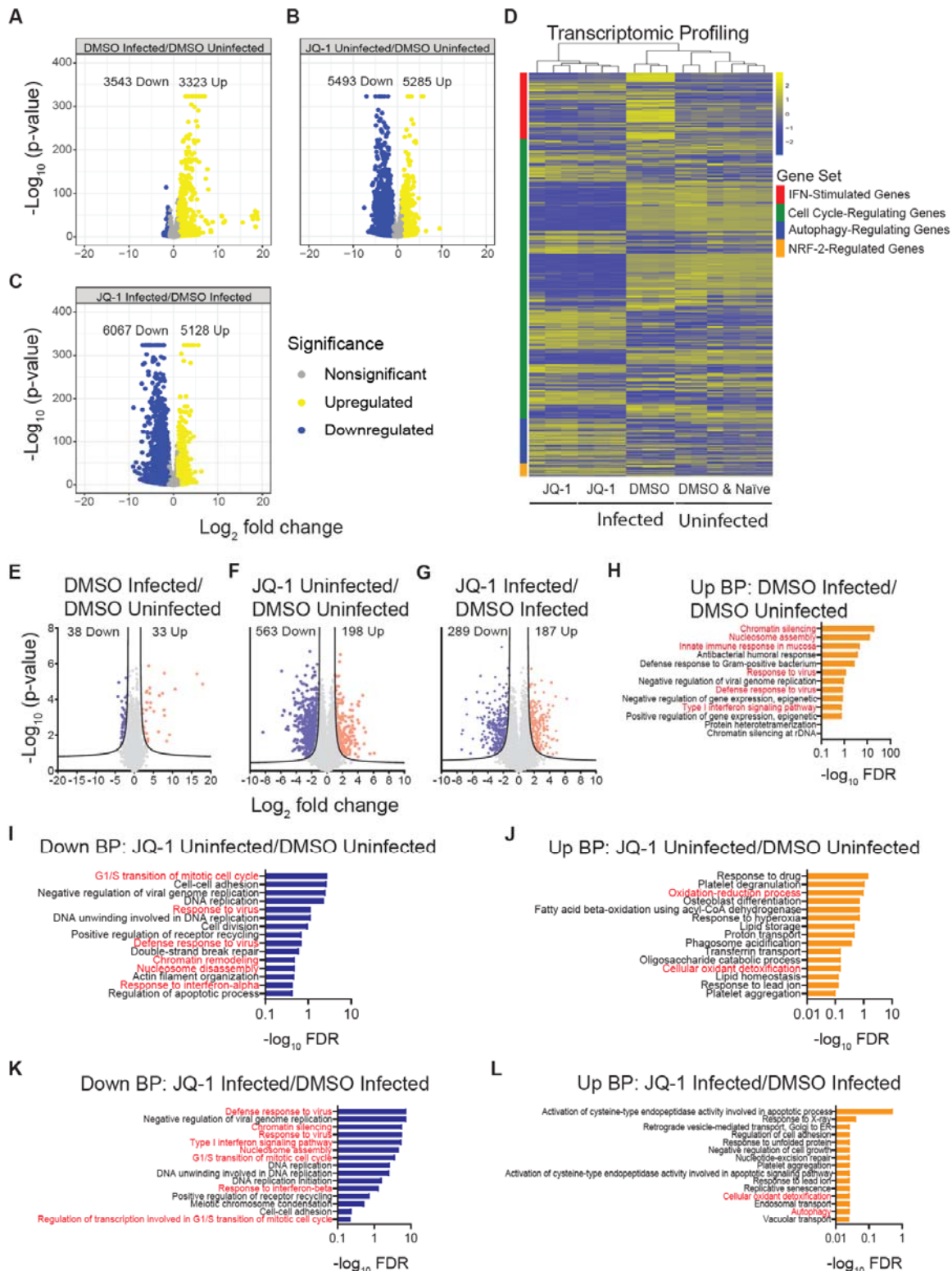
503 SARS-CoV-2 infection induced significant changes to the expression of 6866 genes, of
504 which 3323 were upregulated and 3543 downregulated (Fig. 5A). In the absence of infection,
505 JQ-1 treatment induced significant changes to the expression of 10,778 genes, of which 5285
506 were upregulated and 5493 were downregulated (Fig. 5B). On the other hand, when compared
507 to infection only, JQ-1 treatment of infected cells induced significant changes to the expression
508 of 11,195 genes, with 5128 upregulated and 6067 downregulated (Fig. 5C). Again, these data
509 suggest that JQ-1 treatment modulates the host transcriptome to a larger extent compared to
510 SARS-CoV-2 infection.

511 Hierarchical clustering of genes driving selected biological pathways revealed that
512 SARS-CoV-2 infection induced upregulation of IFN-stimulated genes (Fig. 5D). JQ-1-treated
513 groups, irrespective of their infection status, shared similar transcriptomic signatures for the
514 displayed pathways, as did the uninfected DMSO-treated and naïve groups. Compared to other
515 groups, JQ-1 treatment altered the cell cycle transcriptomic profile and upregulated genes
516 driving autophagy and NRF-2-mediated cellular cytoprotective response, irrespective of the
517 infection status (Fig. 5D). As expected, IFN and cytokine-mediated signalling pathways were
518 upregulated in SARS-CoV-2-infected samples, while pathways driving oxidative
519 phosphorylation were among the top downregulated pathways (Sup. Fig. 3B). Conversely, JQ-1
520 treatment in the presence of infection upregulated the pathways driving oxidative
521 phosphorylation and downregulated the IFN and cytokine signalling pathways (Sup. Fig. 3B).
522 These JQ-1-driven changes were less pronounced or even absent in the absence of infection
523 (Sup. Fig. 3B). Nucleosome assembly, organisation, chromatin assembly, and other pathways
524 associated with regulation of gene transcription were equally enriched across all analysed
525 contrasts (Sup. Fig. 3B).

526 To generate proteomic profiles, we resolved proteins from the whole cell lysate on
527 linear SDS-PAGE gels, visualised the proteins with Bio-Safe Coomassie Blue stain (Sup. Fig.
528 3C) and subjected them to liquid chromatography coupled with tandem mass spectrometry
529 (LC-MS/MS) as described elsewhere (Mhlekude et al., 2021). PCA from the proteomic data
530 demonstrated sample clustering according to their experimental groups, reflecting similar
531 proteomic profiles between individual samples in each group (Sup. Fig. 3D). SARS-CoV-2
532 infection induced differential abundance of 71 proteins, with 33 upregulated and 38
533 downregulated (Fig. 5E). In the absence of infection, JQ-1 treatment induced differential
534 regulation of 761 proteins, 189 of which were upregulated and 563 downregulated (Fig. 5F).
535 JQ-1 treatment in the presence of SARS-CoV-2 infection induced differential regulation of 476
536 proteins, with 187 upregulated and 289 downregulated (Fig. 5G).

537 GO term analysis of the biological processes revealed that IFN and innate immune
538 signalling driven by the STATs, IRFs, and NF- κ B-1 transcription factors were among the
539 upregulated pathways in SARS-CoV-2-infected samples, as compared to uninfected samples
540 (Fig. 5H). Furthermore, chromatin silencing and nucleosome assembly were the top two
541 highly-enriched pathways. Irrespective of the infection status, IFN signalling, chromatin
542 silencing, nucleosome assembly, and G1/S transition of cell cycle were among the
543 downregulated pathways in JQ-1-treated groups (Fig. 5 I-K). Paradoxically, nucleosome
544 assembly and chromatin organisation-related pathways were upregulated at the transcriptomic

545 levels between the JQ-1-treated groups irrespective of the infection status (Sup. Fig. 5B), but
546 downregulated at the proteomic level (Fig. 5I-K). On the other hand, irrespective of the
547 infection status, the NRF-2-driven oxidant detoxification pathway that forms part of the cellular
548 antiviral cytoprotective response was among the upregulated pathways in the JQ-1-treated
549 groups (Fig. 5J-L). Moreover, the autophagy pathway, which is reduced by SARS-CoV-2
550 (Gassen et al., 2021), was upregulated in the JQ-1-treated group in the presence of infection
551 (Fig. 5L). Together, the transcriptomic and proteomic changes largely recapitulate the
552 modulated biological pathways, which are driven by transcription factors whose binding motifs
553 are enriched as determined by ATAC-seq.



554

555

Figure 5. SARS-CoV-2 infection- and JQ-1-mediated modulations of the chromatin

556

accessibility landscape underlie the transcriptomic and proteomic profiles in lung

557

epithelial Calu-3 cells.

558 Volcano plots showing relative \log_2FC and statistical significance of differentially expressed
559 genes in (A) DMSO-treated infected versus DMSO-treated uninfected, (B) JQ-1-treated
560 infected versus DMSO-treated infected and (C) JQ-1-treated uninfected versus DMSO-treated
561 uninfected contrasts. The number of significant (FDR of ≤ 0.05) DRGs are indicated.

562 (D) Heatmap of DRGs across four biological pathways (IFN-stimulated genes, cell cycle-
563 regulating genes, autophagy-regulating genes, and NRF-2-regulated genes) scaled as z-score
564 across rows.

565 Volcano plots showing relative \log_2FC and statistical significance of differentially abundant
566 proteins in (E) DMSO-treated infected versus DMSO-treated uninfected, (F) JQ-1-treated
567 uninfected versus DMSO-treated uninfected and (G) JQ-1-treated infected versus DMSO-
568 treated, infected contrasts. The number of significantly (FDR ≤ 0.05) regulated proteins is
569 indicated.

570 Top enriched (FDR ≤ 0.05) GO Biological Process terms of upregulated proteins in (H) DMSO-
571 treated infected versus DMSO-treated, uninfected, and differentially regulated proteins in (I-J)
572 JQ-1-treated, infected versus DMSO-treated infected and (K-L) JQ-1-treated uninfected versus
573 DMSO-treated uninfected contrasts.

574 **JQ-1 treatment antagonises SARS-CoV-2-mediated suppression of the antiviral NRF-2-** 575 **mediated cytoprotective response**

576 Despite the growing literature on the anti-SARS-CoV-2 activity of JQ-1 and other iBETs
577 (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022; Vann et al., 2022), TFs
578 orchestrating transcriptional responses in the context of JQ-1-mediated anti-SARS-CoV-2
579 activity remain to be investigated. For this purpose, we performed global TF activity profiling
580 using the bulk RNA-seq dataset, which was collected in parallel with ATAC-seq data to
581 identify TFs with significantly regulated gene module scores across our experimental groups.
582 SARS-CoV-2 infection of Calu-3 cells induced activity of the inflammatory TF families
583 (STATs, IRFs, and NF κ B-1) (Fig. 6A), whose signalling is associated with severe COVID-19
584 cases (Lam et al., 2021). JQ-1 treatment in the presence of SARS-CoV-2 infection suppressed
585 the activity of these inflammatory TF families, suggesting an anti-inflammatory effect of JQ-1
586 (Fig. 6A). Interestingly, while SARS-CoV-2 infection induced NF κ B-1-driven canonical
587 signalling pathway, it strongly suppressed NF κ B-2-driven noncanonical signalling pathway,
588 reflecting a more precise SARS-CoV-2-mediated regulation of NF κ B signalling pathways.

589 Irrespective of the infection status, JQ-1 treatment suppressed activity of the TF
590 families that regulate cell cycle and proliferation (E2F & MYB), whose synergistic signalling is

591 associated with severe lung injury in COVID-19 cases (Lam et al., 2021). This is in line with
592 JQ-1-mediated dysregulation of cell cycle-regulating genes (Fig. 5D) and downregulation of
593 pathways driving G1/S transition of the mitotic cell cycle captured in the proteomic data in
594 (Figs. 5 I-K). Furthermore, JQ-1 also induced the transcriptional activities of nuclear factor
595 (erythroid-derived-2)-like-2 (NFE2L2)/(NRF-2) and androgen receptor (AR), which drive a
596 cytoprotective response (He et al., 2020) and RNA alternative splicing (Rana et al., 2021; Shah
597 et al., 2020), respectively (Fig. 6A). It is noteworthy that pharmacological induction of NRF-2
598 signalling by 4-OI and DMF inhibits SARS-CoV-2 replication *in vitro* (Olagnier et al., 2020).
599 NRF-2 and AR belong to CNC-bZIP and steroid nuclear receptor (NR) families of TFs,
600 respectively; whose binding motifs were significantly enriched in the accessible ATAC-seq
601 peaks from JQ-1-treated cells independent of the infection status (Sup. Table 1B-C). Together,
602 these data suggest that the chromatin regulatory regions captured in our ATAC-seq data were
603 not only accessible, but also transcriptionally active.

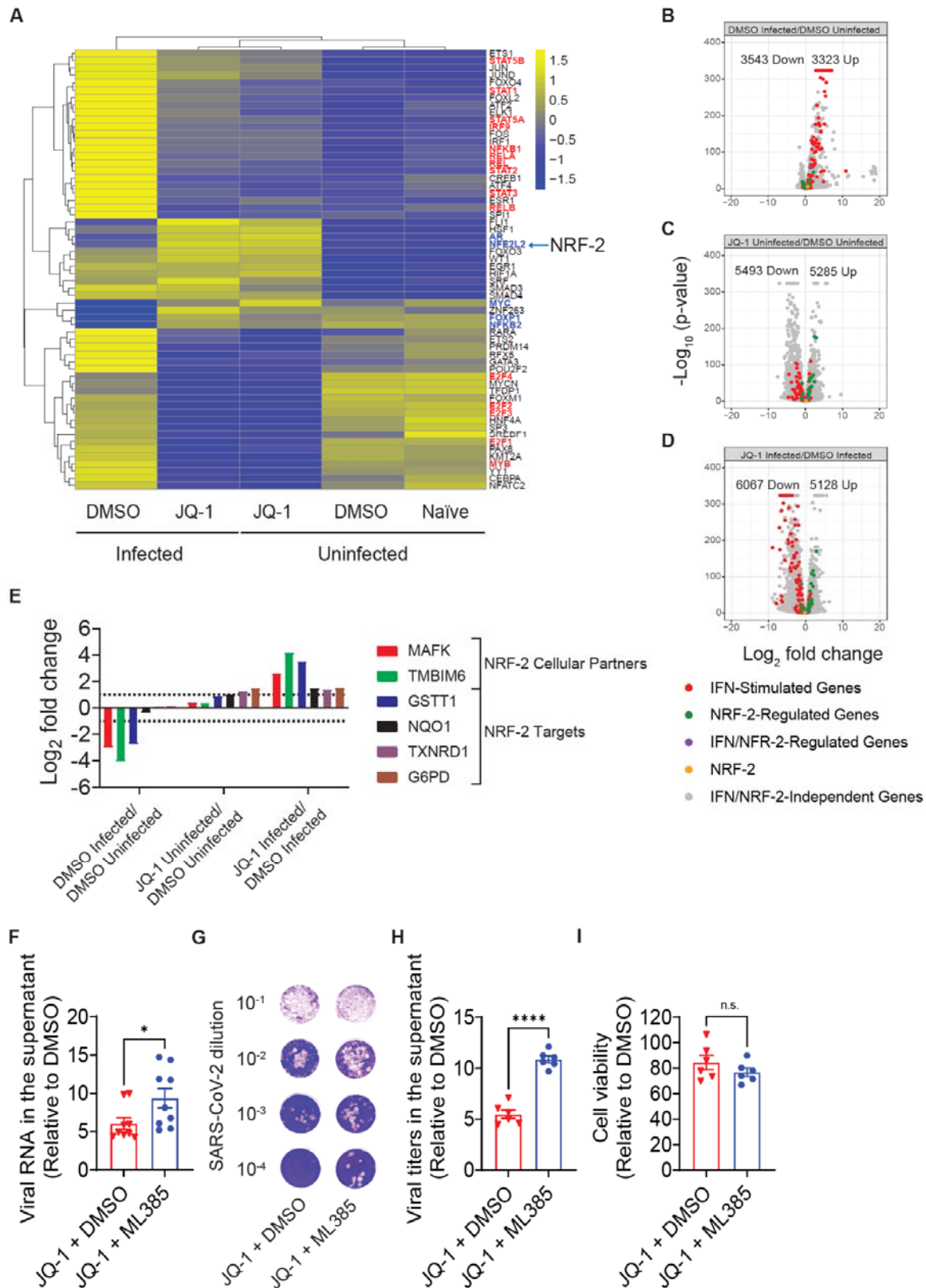
604 To discern between SARS-CoV-2-mediated upregulation of IFN signalling, inhibition
605 of NRF-2 signalling, and JQ-1-mediated antagonistic effects in these processes, we generated
606 volcano plots to analyse the \log_2 FCs of STAT/IRF and NRF-2 target genes. As expected, IFN-
607 stimulated genes (ISGs) were upregulated in the context of SARS-CoV-2 infection (Fig. 6B).
608 SARS-CoV-2 infection showed minimal changes on the expression of NRF-2 target genes and
609 no effect on NRF-2 expression *per se* (Fig. 6B). JQ-1 treatment induced the downregulation of
610 ISGs in the absence (Fig. 6C) and presence (Fig. 6D) of SARS-CoV-2 infection. Irrespective of
611 the infection status, JQ-1 treatment induced an upregulation of the expression of NRF-2 target
612 genes (Fig. 6C-D). Like SARS-CoV-2 infection (Fig. 6B), JQ-1 treatment did not significantly
613 change expression of NRF-2 itself, independent of the infection status (Fig. 6C-D). These data
614 suggest that both SARS-CoV-2 and JQ-1-mediated modulations of NRF-2 signalling do not
615 significantly alter NRF-2 expression *per se*, but likely modulate the expression of its signalling
616 cofactors.

617 NRF-2 induces gene expression by binding to the *cis*-acting antioxidant response
618 elements (ARE) on the promoters of its target genes as a heterodimer with the small
619 musculoaponeurotic fibrosarcoma (MAF) transcription factors, which act as its indispensable
620 cofactors (Itoh et al., 1997; Katsuoka and Yamamoto, 2016). Furthermore, transmembrane B
621 cell lymphoma 2-associated X protein (BAX) inhibitor motif-containing 6 (TM6) protects
622 the host against oxidative stress by inducing NRF-2 signalling (Kim et al., 2020; Lee et al.,
623 2007). Interestingly, the proteomic data showed significant reduction of \log_2 FCs for MAFK
624 and TM6, and NRF-2 target proteins in SARS-CoV-2-infected Calu-3 cells (Fig. 6E). JQ-1

625 treatment antagonised these SARS-CoV-2-mediated changes, with more pronounced inhibition
626 in the context of infection (Fig. 6E). These data suggest that SARS-CoV-2 suppresses NRF-2
627 signalling by downregulating its indispensable signalling cofactors.

628 Further log₂FC analysis revealed that SARS-CoV-2 infection induced a dramatic
629 upregulation of COQ6 expression (Sup. Fig. 4A), which drives the production of coenzyme
630 Q10 to shuttle electrons between complexes I, II and III; and associated dehydrogenase enzymes
631 in the mitochondrial electron transport chain (Acosta Lopez et al., 2019; Wang and Hekimi,
632 2016). These data hint at the need for a ubiquinone biosynthetic pathway in SARS-CoV-2
633 pathogenesis, despite the defective oxidative phosphorylation (Sup. Fig. 3B). Moreover, SARS-
634 CoV-2 infection downregulated COX16 that serves as an indispensable component of
635 cytochrome-c-oxidase (complex IV) in the electron transport chain (Wintjes et al., 2021) and
636 upregulated the chromatin silencing histone variants (H2AFY, H2AFV and H2AFZ) (Sup. Fig.
637 4A), which likely drive the SARS-CoV-2-mediated upregulation of chromatin silencing and
638 nucleosome assembly pathways (Fig. 5H). More importantly, SARS-CoV-2 downregulated the
639 expression of YTHDF2, a methyl-6-adenosine (m⁶A) reader of the RNA molecules in the host
640 that binds to and degrade m⁶A-bearing host (Du et al., 2016) and viral (Gokhale et al., 2016;
641 Gonzales-van Horn and Sarnow, 2017; Lichinchi et al., 2016) RNA molecules to prevent the
642 expression of aberrant cellular RNA and inhibit viral replication, respectively. JQ-1 displayed
643 an antagonistic effect against the above-mentioned SARS-CoV-2-mediated proteomic
644 modulations (Sup. Fig. 4A).

645 NRF-2 signalling, which is induced by JQ-1 treatment in Calu-3 cells (Fig. 6A), inhibits
646 SARS-CoV-2 infection when induced by 4-OI and DMF (Olagnier et al., 2020). This prompted
647 us to investigate whether pharmacological inhibition of NRF-2 signalling antagonises JQ-1-
648 mediated anti-SARS-CoV-2 activity. Interestingly, co-treating Calu-3 cells with JQ-1 and
649 ML385, a specific inhibitor of NRF-2 (Singh et al., 2016), prior to infection, led to a significant
650 increase in viral RNA copies (Fig. 6F) and infectious titers (Fig. 6G-H) in the supernatant
651 compared to JQ-1 and DMSO co-treated cells, in the absence of detectable cell toxicity (Fig.
652 7I). These data suggest that JQ-1 inhibition of SARS-CoV-2 infection in Calu-3 cells involves
653 efficient NRF-2 signalling. Together, these data shed light on JQ-1-mediated multi-pronged
654 approach underlying its anti-SARS-CoV-2 activity.



655

656

Figure 6. JQ-1 treatment antagonises SARS-CoV-2-mediated suppression of the antiviral

657

NRF-2-mediated cytoprotective response

658 (A) Scaled TF activity scores in different treatments for RNAseq data, based on the Dorothea
659 database.

660 Volcano plots showing relative \log_2 FC and statistical significance of differentially expressed
661 genes involved in IFN and NRF-2 signalling in (B) DMSO-treated infected versus DMSO-
662 treated uninfected, (C) JQ-1-treated infected versus DMSO-treated infected and (D) JQ-1-
663 treated uninfected versus DMSO-treated uninfected contrasts.

664 (E) \log_2 FC analysis of differentially expressed proteins implicated in NRF-2 signalling. The
665 bars indicate relative \log_2 FC in expression between indicated contrasts and proteins with a
666 relative \log_2 FC of >1 and a $FDR \leq 0.05$ were considered significant.

667 Relative quantification of (F) viral RNA copies and (G-H) infectivity from SARS-CoV-2
668 infected Calu-3 cells treated as indicated. The data were expressed as the normalised
669 percentage infection relative to DMSO only-treated cells. Error bars show the SEM from
670 triplicates of three and two independent experiments respectively.

671 (I) Cell viability analysis in Calu-3 cells treated as indicated. The graphs show background-
672 subtracted and normalised data relative to DMSO only-treated cells. Error bars show the SEM
673 from triplicates of two independent experiments.

674 Unpaired nonparametric Student *t*-test was used to compare the means between experimental
675 groups.

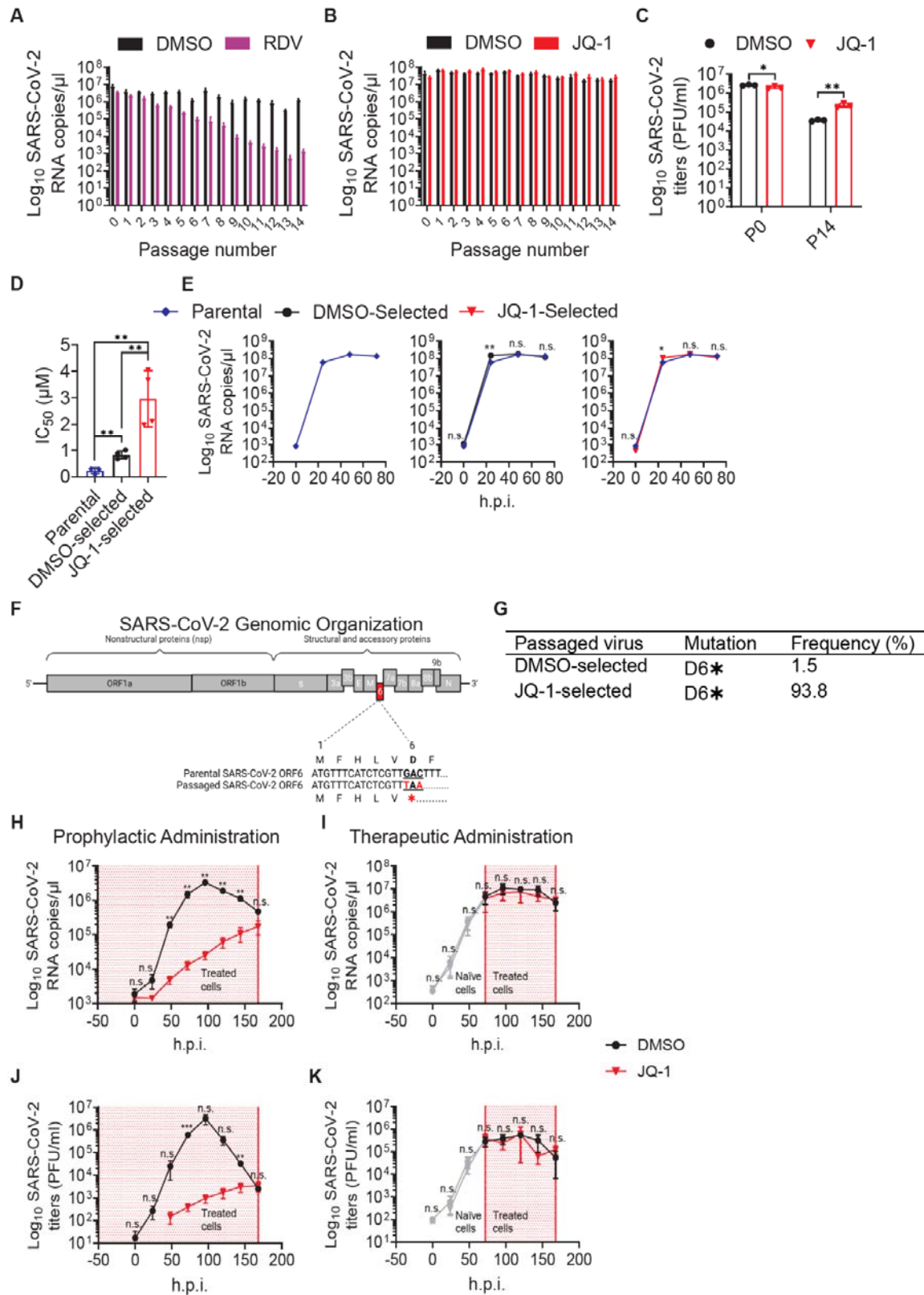
676 **SARS-CoV-2 subverts JQ-1-mediated antiviral state in lung epithelial Calu-3 and human** 677 **bronchial airway epithelial cells**

678 SARS-CoV-2 can adapt to and acquire resistance against potent virus-directed antivirals such
679 as remdesivir (RDV) *in vitro* (Stevens et al., 2022; Szemiel et al., 2021), a replication
680 bottleneck that induces the emergence SARS-CoV-2 variants *in vivo* (Heyer et al., 2022). To
681 investigate whether SARS-CoV-2 can acquire resistance to iBETs, we serially passaged it in
682 Calu-3 cells under escalating two-fold concentrations of JQ-1 (Sup. Fig. 5A), along with RDV
683 as a reference. SARS-CoV-2 serial passaging under increasing two-fold concentrations of RDV
684 led to a gradual reduction of viral RNA copies in the supernatant, with a potential resistance
685 phenotype emerging at the earliest at passage 14 (Fig. 7A). In contrast, SARS-CoV-2 serial
686 passaging under escalating two-fold concentrations of JQ-1 displayed unrelenting high
687 concentrations of viral RNA copies from passage two until passage 14. These data suggest a
688 transient nature of JQ-1-mediated anti-SARS-CoV-2 activity and early adaptation of SARS-
689 CoV-2 to JQ-1 (Fig. 7B). Quantification of viral titers from passages one and 14 revealed that
690 SARS-CoV-2 virions were sensitive to JQ-1 treatment at the beginning of the passaging

691 experiments, and acquired resistance and replication fitness to JQ-1 during serial passaging
692 (Fig. 7C).

693 To determine the extent to which SARS-CoV-2 passaging under JQ-1 led to the
694 acquisition of resistance, we investigated the sensitivity of DMSO- and JQ-1-selected SARS-
695 CoV-2 virions to JQ-1 in Calu-3 cells by quantifying the viral RNA copies in the supernatant
696 and calculating IC_{50} concentrations. Compared to parental SARS-CoV-2, virions that were
697 serially passaged under JQ-1 exhibited reduced sensitivity to JQ-1 treatment in Calu-3 cells
698 (Fig. 7D), which was more pronounced (12-fold) compared to DMSO-selected virions (4-fold)
699 (Sup. Fig. 5B). Viral growth kinetics in Calu-3 cells showed that DMSO- and JQ-1-selected
700 SARS-CoV-2 virions may have acquired some degree of replication fitness compared to the
701 parental SARS-CoV-2 virions (Fig. 7E). Next-Generation Sequencing (NGS) revealed virus
702 sequences encoding a premature stop codon at position six of ORF6 in the genome from virions
703 passaged in the presence of JQ-1, as opposed to parental virus (Fig. 7F). This premature stop
704 codon was present far more frequently in JQ-1-selected virions (93.8% of reads covering this
705 region) than DMSO-selected virions (1.5% of reads covering this region) (Fig. 7G).

706 Finally, we performed kinetic, multi-cycle infection experiments in hBAECs to
707 compare JQ-1-mediated anti-SARS-CoV-2 activity in the contexts of prophylactic and
708 therapeutic treatment. In the set-up mimicking prophylactic administration, we started
709 treatment of hBAECs with DMSO or JQ-1 48 hours prior to SARS-CoV-2 infection (Sup. Fig.
710 5C). For therapeutic administration, we infected naïve hBAECs and started treatment 48 hours
711 post-infection (Sup. Fig. 5D). JQ-1 treatment was continued for the duration of the entire
712 experiment. Prophylactic administration of JQ-1 resulted in antiviral activity that was more
713 pronounced earlier post infection, which however vanished over time, as indicated by the
714 gradual increase in viral RNA copies (Fig. 7H) and infectious titers (Fig. 7J). Strikingly,
715 administration of JQ-1 post establishment of infection failed to curb SARS-CoV-2 replication,
716 as indicated by similar viral RNA copies (Fig. 7I) and infectious titers (Fig. 7I) between
717 DMSO- and JQ-1-treated groups. Together, these data suggest that JQ-1 treatment results in a
718 transient antiviral state, which however can be subverted by SARS-CoV-2.



719

720

Figure 7. SARS-CoV-2 infection subverts JQ-1-mediated antiviral state in lung epithelial

721

Calu-3 and human bronchial airway epithelial cells (hBAECs).

722 Quantification of SARS-CoV-2 RNA (copies/ μ l) from triplicates of 15 serial passages under
723 two-fold escalating concentrations of (A) remdesivir and (B) JQ-1, with DMSO as a mock.

724 (C) Quantification of SARS-CoV-2 titers (PFU/ml) in triplicates from P1 (JQ-1 0.32 μ M) and
725 P14 (JQ-1 5.12 μ M) passages. The means between experimental groups were compared using
726 nonparametric Student *t*-test.

727 (D) Quantification of IC₅₀ values from JQ-1 dose-dependent inhibition curves determined in
728 JQ-1-treated Calu-3 cells infected with parental and passaged SARS-CoV-2 virions (MOI 0.1).
729 The error bars represent the mean \pm SEM from four independent experiments and the means
730 between experimental groups were compared using nonparametric Student *t*-test.

731 (E) Viral growth kinetics in DMSO-treated Calu-3 cells showing viral RNA quantities from
732 parental and passaged (P14) SARS-CoV-2 virions (MOI 0.1) from three independent
733 experiments. The means between experimental groups were compared using nonparametric
734 multiple *t*-test.

735 (F) Schematic diagram of the SARS-CoV-2 genome depicting the mutation creating a
736 premature stop codon at position six of ORF6 among the passaged (P14) virions.

737 (G) Quantification of the ORF6 D6* mutation frequency from sequences from
738 passaged (P14) SARS-CoV-2 virions.

739 Virus growth kinetics in DMSO and JQ-1 (2.56 μ M)-treated hBAECs depicting SARS-CoV-2
740 RNA quantities (copies/ μ l) in the contexts of (H) prophylactic and (I) therapeutic drug
741 administration from three independent experiments.

742 Virus growth kinetics in DMSO and JQ-1 (2.56 μ M)-treated hBAECs depicting SARS-CoV-2
743 titers (PFU/ml) in the contexts of (J) prophylactic and (K) therapeutic drug administration from
744 three independent experiments.

745 The area shaded in red indicates the time period of drug administration and the grey lines in the
746 context of therapeutic administration indicate infection before drug administration. The means
747 between experimental groups were compared using nonparametric multiple *t*-test. Error bars
748 represent the mean \pm SEM.

749 **DISCUSSION**

750 Prior studies attributed the iBET-mediated anti-SARS-CoV-2 activity to the
751 downregulation of cellular ACE2 expression (Gilham et al., 2021; Mills et al., 2021; Samelson
752 et al., 2022), but failed to capture the pleiotropic nature of the antiviral state induced by these

753 compounds and their susceptibility to SARS-CoV-2 subversion. Here, we show that JQ-1-
754 mediated anti-SARS-CoV-2 activity goes beyond the downregulation of ACE2 expression by
755 inducing a vast transcriptional remodulation, including the mounting of antiviral NRF-2-
756 mediated responses. Furthermore, we show that JQ-1-mediated antiviral activity is transient
757 when administered prophylactically and nullified by SARS-CoV-2 when administered
758 therapeutically following an established infection, suggesting effective viral antagonistic
759 strategies.

760 In agreement with prior studies in Calu-3 cells (Gilham et al., 2021; Mills et al., 2021;
761 Samelson et al., 2022; Vann et al., 2022), iBETs displayed an antiviral activity against SARS-
762 CoV-2 infection in lung epithelial Calu-3 cells and hBAECs when administered
763 prophylactically, suggesting that the iBET-mediated anti-SARS-CoV-2 activity is readily
764 translated from cell lines *in vitro* to clinically relevant human-derived infection models. The
765 antiviral activity of JQ-1 and its analogues has largely been reported in the context of SARS-
766 CoV-2 parental strains (Gilham et al., 2021; Mills et al., 2021; Samelson et al., 2022) and VOC
767 Delta (Vann et al., 2022). Our data showing sensitivity of SARS-CoV-2 and SARS-CoV, but
768 not MERS-CoV, to JQ-1 suggest that MERS-CoV replication, and likely that of other
769 *Merbecoviruses*, is independent of BET proteins. This difference can potentially be attributed
770 to the interactions between SARS-CoV-2-E and cellular BET proteins (Chen et al., 2022; Vann
771 et al., 2022), through a conserved histone motif present in SARS-CoV-E and SARS-CoV-2-E
772 proteins (Gordon et al., 2020; Schoeman and Fielding, 2020), which is lacking in MERS-CoV-
773 E protein (Schoeman and Fielding, 2020).

774 The regulatory landscape orchestrating the iBET-mediated transcriptional responses to
775 SARS-CoV-2 infection has remained largely unexplored. Accessible peaks in SARS-CoV-2-
776 infected samples were enriched with binding motifs for the inflammatory TF family (STATs,
777 IRFs and NF κ B), which were significantly associated with the upregulation of IFN and
778 cytokine signalling pathways. On the other hand, accessible peaks from the same samples were
779 significantly associated with the downregulation of smell receptor signalling pathways,
780 reminiscent of the olfactory dysfunction reported in COVID-19 cases (Brann et al., 2020; de
781 Melo et al., 2021; Qiu et al., 2020). Significant association of accessible peaks from JQ-1-
782 treated samples in the presence of infection with the downregulation of the smell receptor
783 signalling pathways suggests that JQ-1 is unable to dampen SARS-CoV-2-mediated olfactory
784 dysfunction (Brann et al., 2020; de Melo et al., 2021; Qiu et al., 2020).

785 Similar to the ATAC-seq data, we captured massive differential regulation of genes and
786 proteins in JQ-1-treated samples, irrespective of infection, compared to SARS-CoV-2 infection.

787 Upregulated genes and proteins in SARS-CoV-2-infected samples were significantly associated
788 with the upregulation of IFN and cytokine signalling pathways. In line with the detected
789 enrichment of accessible binding motifs for the inflammatory TF family, supervised
790 hierarchical clustering further showed that SARS-CoV-2 infection induced the transcription of
791 IFN-stimulated genes. In accordance with reported SARS-CoV-2-mediated impairment of
792 mitochondrial biogenesis (Guarnieri et al., 2022), downregulated genes in SARS-CoV-2-
793 infected samples were significantly associated (among others) with the downregulation of
794 pathways driving mitochondrial oxidative phosphorylation (OXPHOS). The upregulation of
795 chromatin silencing and nucleosome assembly pathways at the proteomic level in SARS-CoV-
796 2-infected samples is consistent with SARS-CoV-2-ORF8-mediated chromatin compaction
797 (Kee et al., 2021) and may represent the epigenomic mechanism underlying SARS-CoV-2-
798 mediated host-shutoff (Finkel et al., 2021).

799 Interestingly, despite inducing a strong NFκB-1 TF activity that drives inflammatory
800 cytokine production (Ramasamy and Subbian, 2021), SARS-CoV-2 infection induced an
801 equally strong suppression of NFκB-2 TF activity, which regulates the maturation of antibody-
802 producing B cells and development of germinal centres (GC) in the lymph nodes (Caamaño et
803 al., 1998; Silva et al., 2016). Conversely, irrespective of infection, JQ-1 suppressed NFκB-1 TF
804 activity and induced NFκB-2 TF activity. It is noteworthy that inhibition of NFκB-1
805 transcriptional footprint inhibits SARS-CoV-2 replication (Nilsson-Payant et al., 2021). These
806 data reflect a carefully executed and precise regulation of NFκB-mediated signalling pathways
807 by SARS-CoV-2 infection and JQ-1 administration.

808 Association of the upregulated genes and proteins in JQ-1-treated samples with the
809 downregulation of IFN signalling and viral genome replication pathways validates JQ-1-
810 mediated suppression of innate immune responses (Chen et al., 2022; Mills et al., 2021;
811 Samelson et al., 2022) and antiviral activity (Gilham et al., 2021; Mills et al., 2021; Samelson
812 et al., 2022; Vann et al., 2022). SARS-CoV-2 infection limits induction of autophagy (Gassen
813 et al., 2021), and pharmacological induction of NRF-2, which is a cellular cytoprotective
814 response-inducing transcription factor that belongs to CNC-bZIP TF family, inhibits SARS-
815 CoV-2 replication (Olagnier et al., 2020). Of note, a binding motif for CNC-bZIP TF family
816 was among the top enriched TF motifs in accessible peaks from JQ-1-treated samples,
817 irrespective of infection.

818 Of particular interest, supervised hierarchical clustering revealed that JQ-1 treatment
819 upregulated the expression of autophagy-regulating and NRF-2 target genes, irrespective of
820 SARS-CoV-2 infection. In accordance, pathway enrichment analysis at the proteomic level

821 revealed upregulation of autophagy in JQ-1-treated samples in the presence of infection and
822 NRF-2-mediated cellular oxidant detoxification pathway irrespective of infection status. Motif
823 enrichment for the TFs belonging to CNC-bZIP and NFY families in the context of JQ-1
824 administration, irrespective of infection, revealed the chromatin regulatory landscape
825 underlying JQ-1-mediated induction of NRF-2 signalling (Lv et al., 2022), which exhibits an
826 anti-SARS-CoV-2 activity in the context of DMF- and 4-OI-mediated induction (Olagnier et
827 al., 2020). Moreover, signalling by the NFY TFs increases chromatin accessibility by
828 preventing nucleosome encroachment (Oldfield et al., 2014, 2019). Therefore, enrichment of
829 the binding motifs for the NFY TFs supports the highly accessible chromatin landscape
830 detected in the context of JQ-1 treatment. Together, these data show that JQ-1 induced the
831 activities of NRF-2, which drive the induction of antiviral cellular cytoprotective response
832 (Olagnier et al., 2020).

833 SARS-CoV-2 infection upregulated IFN-stimulated genes, but displayed minimal
834 alterations on the expression of NRF-2 target genes, without altering NRF-2 expression *per se*.
835 On the other hand, JQ-1 administration downregulated the expression of IFN-stimulated genes
836 and induced the expression of NRF-2 target genes irrespective of infection. However, like
837 SARS-CoV-2 infection, JQ-1 administration also did not alter NRF-2 expression, despite
838 significantly altering the expression of its target genes. These data suggest that differential
839 regulation of NRF-2 signalling by SARS-CoV-2 infection and JQ-1 administration does not
840 alter NRF-2 expression *per se*, but likely the expression of its signalling co-factors.

841 Accordingly, the proteomic data showed that SARS-CoV-2 infection induced
842 significant log₂FC downregulation of MAFK and TMBIM6, which act as indispensable co-
843 factors in the induction of NRF-2 signalling (Itoh et al., 1997; Katsuoka and Yamamoto, 2016;
844 Kim et al., 2020; Lee et al., 2007). Accompanying SARS-CoV-2-mediated downregulation of
845 NRF-2 signalling co-factors was the downregulation of NRF-2 target proteins. JQ-1
846 administration induced an upregulation of MAFK, TMBIM6, and NRF-2 target proteins, with a
847 more pronounced effect in the presence of infection. These data complement SARS-CoV-2-
848 and JQ-1-mediated differential regulation of NRF-2 TF activity and suggest that both SARS-
849 CoV-2 infection and JQ-1 administration regulate NRF-2 TF activity by modulating the
850 expression of its signalling co-factors and not NRF-2 expression *per se*. Suppression of NRF-2-
851 mediated cytoprotective response in biopsies from COVID-19 patients (Olagnier et al., 2020)
852 supports the biological plausibility of our data.

853 DMF- and 4-OI-mediated induction of NRF-2-driven cytoprotective protective response
854 with anti-SARS-CoV-2 activity (Olagnier et al., 2020) and known JQ-1-mediated induction of

855 NRF-2 signalling (Lv et al., 2022), prompted us to pursue the effect of JQ-1-mediated
856 induction of NRF-2 signalling in the context of JQ-1-mediated anti-SARS-CoV-2 activity.
857 Partial antagonism of JQ-1-mediated anti-SARS-CoV-2 activity by ML385, a specific inhibitor
858 of NRF-2 (Singh et al., 2016), suggest that induction of NRF-2 signalling by JQ-1
859 administration contributes to JQ-1-mediated anti-SARS-CoV-2 activity, which is consistent
860 with NRF-2-mediated anti-SARS-CoV-2 reported in the context DMF- and 4-OI-mediated
861 induction (Olagnier et al., 2020). The combination of JQ-1-mediated downregulation of ACE2
862 expression and induction of NRF-2 signalling shown in our study, suggest that JQ-1 exhibits a
863 pleiotropic anti-SARS-CoV-2 activity that affects multiple steps of the viral replication cycle.

864 How exactly JQ-1-mediated induction of NRF-2 signalling exerts an anti-SARS-CoV-2
865 activity remains unknown. Olagnier et al. proposed that the induction of hypoxia-inducible-
866 factor-1-alpha (HIF-1 α) gene expression program induced by 4-OI administration, which was
867 downregulated in biopsies from COVID-19 patients along with NRF-2 signalling pathway,
868 potentially contribute to NRF-2-mediated antiviral cytoprotective response (Olagnier et al.,
869 2020). In contrast, Codo. et al. reported that SARS-CoV-2-mediated induction of ROS
870 stabilises HIF-1 α to induce its gene expression program that promotes glycolysis to sustain
871 SARS-CoV-2 replication in monocytes (Codo et al., 2020). Consistent with the later report, we
872 found induction of HIF-1 α TF activity following SARS-CoV-2 infection in Calu-3 cells.

873 Furthermore, irrespective of infection, JQ-1 administration also induced HIF-1 α TF
874 activity. Downregulation of HIF-1 α TF activity in control groups suggest that both SARS-CoV-
875 2 infection and JQ-1 administration induce HIF-1 α signalling. This is consistent with the idea
876 of alternative JQ-1-driven HIF-1 α TF activity-inducing mechanisms that are independent of
877 SARS-CoV-2-mediated ROS production and argues against the potential effect of HIF-1 α -
878 mediated signalling in NRF-2-induced anti-SARS-CoV-2 activity proposed elsewhere
879 (Olagnier et al., 2020) . Alternatively, NRF-2 signalling induces the expression of a
880 cytoprotective enzyme called heme-oxygenase-1 (HO-1) (Olagnier et al., 2020; Wagener et al.,
881 2020), which catalyses the catabolism of heme into biliverdin, iron, and carbon monoxide (CO)
882 (Dunn et al., 2014). Interestingly, the products of HO-1-mediated heme catabolism exhibit a
883 broad-spectrum antiviral activity (Espinoza et al., 2017).

884 Of particular interest, prophylactic administration of free iron and biliverdin to Vero E6
885 cells prior to infection inhibited SARS-CoV-2 replication (Kim et al., 2021), In contrast,
886 despite binding to SARS-CoV-2-S protein with nanomolar affinity and dampening spike
887 interactions with neutralising antibodies, administration of 100 μ M biliverdin at the time of
888 infection did not inhibit infection of Vero E6 cells by SARS-CoV-2 (Rosa et al., 2021). These

889 studies (Kim et al., 2021; Rosa et al., 2021) suggest that like JQ-1, biliverdin-induced anti-
890 SARS-CoV-2 activity is directed at the host and not the infecting virions. This is consistent
891 with the maintenance of infection efficiency by the virions secreted under JQ-1 treatment.
892 Taken together, these studies highlight the mechanism underlying NRF-2-mediated anti-SARS-
893 CoV-2 activity, which itself remains to be fully unravelled.

894 Immune selection of SARS-CoV-2 has led to the emergence of VOCs bearing an
895 arsenal of mutations in the spike and other viral proteins, which confer immune evasion
896 capabilities and transmission superiority (Tuekprakhon et al., 2022; Willett et al., 2022).
897 SARS-CoV-2 adaptation to virus-directed antivirals such as remdesivir (RDV) has been
898 reported *in vitro* (Stevens et al., 2022; Szemiel et al., 2021) and *in vivo* (Heyer et al., 2022).
899 Here, as early as passage one (P1) under JQ-1 selection, SARS-CoV-2 RNA copies and
900 infectious titers increased in the supernatant and reached a plateau that was maintained across
901 14 passages despite the subsequent two-fold escalations of JQ-1 concentrations. Compared to
902 the parental strain, JQ-1-selected, and to a lesser extent also DMSO-selected, SARS-CoV-2
903 virions showed reduced sensitivity to JQ-1 treatment in Calu-3 cells. These data suggest that
904 passaging under DMSO, and even more under JQ-1, induced the emergence of SARS-CoV-2
905 variants with enhanced viral fitness and reduced sensitivity to JQ-1, unlike the acquisition of
906 resistance to RDV that led to viral replication defect *in vitro* (Stevens et al., 2022; Szemiel et
907 al., 2021).

908 NGS analysis of SARS-CoV-2 virions passaged under JQ-1 treatment revealed
909 acquisition of a premature stop codon corresponding to position six of ORF6, (ORF6^{D6STOP}
910 mutation), a mutation that was less pronounced in DMSO-selected virions. Like JQ-1, SARS-
911 CoV-2-ORF6 antagonises IFN signalling in infected cells (Miorin et al., 2020; Schroeder et al.,
912 2021; Xia et al., 2020). This IFN signalling-repressive effect of JQ-1 may have minimised the
913 need for ORF6-mediated repression of IFN signalling and promoted ORF6 trade-offs. This is
914 consistent with the early emergence of SARS-CoV-2 variants harbouring an in-frame deletion
915 in ORF6 following passaging in IFN-deficient Vero E6 cells (Riojas et al., 2020). In contrast,
916 replication of virions passaged under DMSO are expected to benefit from preserving the
917 ORF6-mediated antagonism of IFN signalling. Investigation of how the acquisition of
918 ORF6^{D6STOP} mutation mediates resistance to JQ-1-mediated antiviral activity and whether this
919 ORF6 trade-off constitutes an antagonistic pleiotropy by enhancing sensitivity to IFN treatment
920 is warranted. Together, these data suggest that acquisition of the ORF6^{D6STOP} mutation is not
921 uniquely induced by JQ-1 treatment, but gains more dominance after encountering an IFN-
922 deficient milieu induced by JQ-1 treatment.

923 The accumulating evidence from our study and previous studies (Gilham et al., 2021;
924 Mills et al., 2021; Samelson et al., 2022; Vann et al., 2022) show that iBETs exhibit an anti-
925 SARS-CoV-2 activity when administered prophylactically and not therapeutically (Chen et al.,
926 2022). Whether SARS-CoV-2 infection counteracts JQ-1-mediated infection barriers or
927 acquires alternative capabilities to infect and replicate in the host remains to be addressed in
928 future studies. Interestingly, in one study (Chen et al., 2022), administration of iBETs at the
929 time of infection, as opposed to pre-treatment, resulted in absence of an inhibitory effect and
930 even exacerbation of SARS-CoV-2 replication. In line with this report, JQ-1 displayed no
931 antiviral activity when administered in a therapeutic set-up in our study. The proviral effect of
932 therapeutically administered JQ-1 reported by (Chen et al., 2022), which is absent in our study,
933 may be related to different time points of sample harvest following infection (48 versus 24
934 hours, respectively). Since SARS-CoV-2 infection potently suppresses NRF-2-mediated
935 antiviral cytoprotection responses (Olagnier et al., 2020), subsequent and long-term JQ-1
936 administration may be unable to revert this effect, respectively, resulting in SARS-CoV-2-
937 mediated nullification of JQ-1-mediated anti-SARS-CoV-2 activity. Together, these data evoke
938 questions about the clinical suitability for both prophylactic and therapeutic administration of
939 JQ-1 (and likely other iBETs) in the context of COVID-19 and illuminate the potential hurdles
940 that iBETs will have to overcome in order to improve disease prognosis.

941

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952 **AUTHOR CONTRIBUTIONS**

953 B. Mhle. and C.G. conceived the study and designed the experiments. B. Mhle., S.Sten., J.J.,
954 A.R., J.H. and N.H. performed the experiments. R.O., S. Schr., U.M. and D.N. provided the
955 material. B. Mhle., D.P., J.M.W., F.Z., B. Mue., T.C.J. and C.G. analysed the data. B. Mhle.

956 and C.G. drafted the manuscript. M.A.M., C.D., A.P., V.T., D.N., G.S., D.B., and C.G.
957 supervised the research. All authors reviewed and edited the manuscript.

958 **CONFLICT OF INTERESTS**

959 The authors declare no conflict of interests

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