

1 **Elevated temperature inhibits SARS-CoV-2 replication in**
2 **respiratory epithelium independently of the induction of IFN-**
3 **mediated innate immune defences**

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19 **Running title:** Elevated temperature restricts SARS-CoV-2 replication

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22 cells, COVID-19, temperature, immune regulation, RNA-Seq

23 **Abstract**

24 The pandemic spread of SARS-CoV-2, the etiological agent of COVID-19, represents a
25 significant and ongoing international health crisis. A key symptom of SARS-CoV-2 infection
26 is the onset of fever, with a hyperthermic temperature range of 38 to 41°C. Fever is an
27 evolutionarily conserved host response to microbial infection and inflammation that can
28 influence the outcome of viral pathogenicity and regulation of host innate and adaptive
29 immune responses. However, it remains to be determined what effect elevated temperature
30 has on SARS-CoV-2 tropism and replication. Utilizing a 3D air-liquid interface (ALI) model
31 that closely mimics the natural tissue physiology and cellular tropism of SARS-CoV-2
32 infection in the respiratory airway, we identify tissue temperature to play an important role in
33 the regulation of SARS-CoV-2 infection. We show that temperature elevation induces wide-
34 spread transcriptome changes that impact upon the regulation of multiple pathways, including
35 epigenetic regulation and lncRNA expression, without disruption of general cellular
36 transcription or the induction of interferon (IFN)-mediated antiviral immune defences.
37 Respiratory tissue incubated at temperatures $>37^{\circ}\text{C}$ remained permissive to SARS-CoV-2
38 infection but severely restricted the initiation of viral transcription, leading to significantly
39 reduced levels of intraepithelial viral RNA accumulation and apical shedding of infectious
40 virus. To our knowledge, we present the first evidence that febrile temperatures associated
41 with COVID-19 inhibit SARS-CoV-2 replication. Our data identify an important role for
42 temperature elevation in the epithelial restriction of SARS-CoV-2 that occurs independently
43 of the induction of canonical IFN-mediated antiviral immune defences and interferon-
44 stimulated gene (ISG) expression.

45

46 **Introduction**

47 The pandemic spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2,
48 SCV2; (1-3)) is an ongoing international health crisis with over 58 million infections and 1.3
49 million reported deaths worldwide to date (WHO, <https://covid19.who.int>; November 2020).
50 The spectrum of SCV2 related disease (COVID-19; coronavirus disease 2019) is highly
51 variable, ranging from asymptomatic viral shedding to acute respiratory distress syndrome
52 (ARDS), multi-organ failure, and death. Besides coughing, dyspnoea, and fatigue, fever (also
53 known as pyrexia) is one of the most frequent symptoms of SCV2 infection (4-8). Fever is an
54 evolutionarily conserved host response to microbial infection, which can influence the
55 regulation of host innate and adaptive immune responses (9, 10). Unlike hyperthermia or heat
56 stroke, fever represents a controlled shift in body temperature regulation induced by the
57 expression of exogenous (microbial) and endogenous (host) pyrogenic regulatory factors,
58 including PAMPS (pathogen associated molecular patterns) and pro-inflammatory cytokines
59 (e.g. interleukin 6, IL-6) (9, 10). Body temperature naturally varies throughout the day, with
60 age, sex, and ethnic origin being contributing factors to body temperature regulation (9, 10).
61 In healthy middle-aged adults, fever is defined as a temperature range from 38 to 41°C (ΔT
62 $\sim 1-4^\circ\text{C}$ above baseline), with low (38 to 39°C), moderate (39.1 to 40°C), high (40.1 to
63 41.1°C), and hyperpyrexia ($>41.1^\circ\text{C}$) febrile temperature ranges (10). Temperature elevation
64 confers protection against a number of respiratory pathogens (9, 11), with antipyretic drug
65 treatment shown to increase the mortality rate of intensive care unit (ICU) patients infected
66 with influenza A virus (IAV) (12-14).

67 With respect to COVID-19, up to 90% of hospitalized patients show symptoms of
68 fever (4-8). The majority of patients display low (44%) to moderate (13 to 34%) grade fever
69 (4, 5). COVID-19 ICU patients also show a $\sim 10\%$ higher prevalence of fever relative to non-
70 ICU patients with milder disease manifestations (6, 15), suggesting fever to play a role during

71 COVID-19 disease progression. *In vitro* studies have shown that SCV2 replicates more
72 efficiently at lower temperatures associated with the upper respiratory airway (33°C), which
73 correlates with an overall weaker interferon (IFN)-mediated antiviral immune response
74 relative to core body temperatures observed in the lower respiratory airway (37°C) (16).
75 These data suggest that tissue temperature could be a significant determinant of SCV2
76 tropism and immune regulation. However, it remains to be determined what effect
77 temperature elevation above 37°C has on SCV2 infection. We therefore set out to determine
78 the net effect of elevated temperature on SCV2 infection within respiratory epithelial tissue.

79 Utilizing a three-dimensional (3D) respiratory model that closely mimics the tissue
80 physiology and cellular tropism of SCV2 infection observed in the respiratory airway of
81 COVID-19 patients (16-24), we demonstrate that elevated temperature ($\geq 39^{\circ}\text{C}$) restricts the
82 replication and propagation of SCV2 in respiratory epithelium independently of the induction
83 of type-I (IFN β) and type-III (IFN λ) IFN-mediated immune defences. We show that
84 respiratory epithelium remains permissive to SCV2 infection at temperatures up to 40°C, but
85 restricts viral transcription leading to significantly reduced levels of viral RNA (vRNA)
86 accumulation and apical shedding of infectious virus. Importantly, we identify temperature to
87 play an important role in the differential regulation of epithelial host responses to SCV2
88 infection, including epigenetic, long non-coding RNA (lncRNA), and immunity-related
89 pathways. Collectively, our data identify an important role for tissue temperature in the
90 epithelial restriction of SCV2 replication in respiratory tissue independently of the induction
91 of type-I and type-III IFN-mediated antiviral immune defences.

92

93

94 **Results**

95 **Differentiation of primary human bronchial epithelial cells into ciliated respiratory**

96 **epithelium supports SARS-CoV-2 replication *in vitro*.** In order to establish a respiratory

97 model suitable for studying the effect of temperature on SCV2 replication, we differentiated

98 primary human bronchiolar epithelial (HBE) cells (isolated from a male Caucasian donor

99 aged 63 years) into pseudostratified respiratory epithelium. Haematoxylin and Eosin (H&E)

100 and immunohistochemistry (IHC) staining of respiratory airway cultures demonstrated that

101 these tissues to contain a mixture of epithelial and goblet cells, with significant levels of

102 apical ciliation and expression of ACE2 (Figure 1A), the principal surface receptor for SCV2

103 and major determinant of tissue tropism (25-28). Infection of respiratory airway cultures with

104 SCV2 (strain England 2; 10^4 PFU/tissue) at 37°C demonstrated that these tissues support

105 SCV2 infection and replication, with intraepithelial and apical vRNA accumulation

106 detectable by *in situ* hybridization by 96 h post-infection (Figure 1A). Notably, we observed

107 discrete clusters of SCV2 RNA accumulation within the respiratory epithelium, indicative of

108 SCV2 cell-type specific tropism and/or localized patterns of immune restriction (see below;

109 (29, 30)). The overall morphology of the respiratory epithelium remained largely intact, with

110 little to no discernible shedding of the ciliated surface epithelium over the time course of

111 infection (Figure 1A). Measurement of genome copies (RT-qPCR) and infectious virus

112 (TCID₅₀) within apical washes collected over time (24 to 144 h) demonstrated the linear

113 phase of virus shedding to occur between 48 and 96 h, with peak titres at 120 h post-infection

114 (Figure 1B to E).

115 In order to determine which cellular pathways were modulated in response to SCV2

116 infection, we performed RNA-Seq analysis on RNA extracted from mock-treated or SCV2-

117 infected respiratory cultures incubated at 37°C for 72 h, a time point in the linear phase of

118 viral shedding (Figure 1C to E). Out of the 787 differentially expressed genes (DEGs) (Figure

119 2A, Supplemental File S1; $p < 0.05$, ≥ 1.5 or ≤ -1.5 log₂ Fold Change [log₂ FC]), pathway
120 analysis identified DEG enrichment in immune system and cytokine related pathways to be
121 significantly upregulated in SCV2-infected relative to mocked-treated tissues (Figure 2B, C
122 $p < 0.0001$). Type-I (IFN β , *IFNB1*) and type-III (IFN λ , *IFNLI-3*) IFNs, along with a subset of
123 interferon stimulated genes (ISGs; e.g. *BST2*, *ISG15*, *Mx1*, and *ZBP1* amongst others), and
124 other pro-inflammatory cytokines (*IL6* and *IL32*) were upregulated (Figure 2D). Indirect
125 immunofluorescence staining of tissue sections demonstrated that induced ISG expression
126 coincided with productively infected areas of the respiratory epithelium (Figure 2E, Mx1),
127 identifying localized areas of immune regulation within infected tissue. Our findings are
128 consistent with previous reports showing that SCV2 infection induces an IFN-mediated
129 antiviral immune response upon infection of epithelial tissue (16, 18-21, 30-32).

130

131 **Respiratory airway cultures induce a heat stress response at elevated temperature.** We
132 next investigated the influence of temperature on our respiratory epithelium model. Mock
133 treated respiratory cultures were incubated at 37, 39, or 40°C (representative of core body
134 temperature and low to moderate grade febrile temperatures, respectively) for 72 h prior to
135 tissue fixation or RNA extraction and RNA-Seq. H&E staining demonstrated no obvious
136 morphological changes to the respiratory epithelium upon incubation at elevated temperature
137 (Figure 3A), although an increase in epithelium thickness was detected relative to tissues
138 incubated at 37°C (Figure 3B; 39°C $p < 0.0001$, 40°C $p < 0.0001$). RNA-Seq analysis
139 demonstrated no difference in the expression level of ACE2 (Figure 3C; $p = 0.4001$) or a
140 reference set of genes known to be constitutively expressed across a wide range of cell types
141 and tissues (Figure 3D; 24 genes, $p = 0.9106$; (33-35)). These data indicate that cellular
142 transcription remains largely unperturbed at elevated temperatures up to 40°C. Out of the 650
143 DEGs identified (Figure 3E, Supplemental File S2; $p < 0.05$, ≥ 1.5 or ≤ -1.5 log₂ FC), pathway

144 analysis identified DEG enrichment in multiple pathways in tissues incubated at 40°C
145 relative to 37°C, including meiotic recombination, DNA methylation, rRNA expression, and
146 collagen metabolism (Figure 3F). DEG enrichment was also observed in cellular pathways
147 relating to heat stress (Figure 3F black arrow, 3G; R-HSA-3371556) and cellular response to
148 external stimuli (Figure 3G; R-HSA-8953897). Analysis of complete gene sets associated
149 with the cellular response to heat stress (Figure 3H, $p=0.0013$) or cellular response to heat
150 (Figure S1, $p=0.0018$; GO:0034605) identified that these pathways were upregulated in
151 respiratory tissues incubated at 40°C relative to 37°C (Supplemental File S2). We conclude
152 that our respiratory tissue model induces a heat stress response at elevated temperature
153 without visible damage to the epithelium or induction of IFN-mediated antiviral immune
154 defences.

155

156 **Elevated temperature restricts the replication of SARS-CoV-2 in respiratory airway**
157 **cultures.** We next examined the effect of temperature on SCV2 replication in our 3D tissue
158 model. Respiratory cultures were incubated at 37, 39, or 40°C for 24 h prior to mock
159 treatment or SCV2 infection and continued incubation at their respective temperatures.
160 Measurement of genome copies and infectious virus within apical washes collected over time
161 (24 to 72 h) demonstrated that extracellular SCV2 titres were significantly decreased at both
162 39 and 40°C relative to 37°C (Figure 4A to D). RT-qPCR analysis of RNA isolated from
163 infected tissues at 72 h post-infection demonstrated significantly lower levels of intracellular
164 vRNA in tissues incubated at 39 or 40°C (Figure 4E; 39°C $p=0.0278$, 40°C $p=0.0033$). These
165 data indicate that respiratory tissue remains permissive to SCV2 infection (tissue entry) but
166 refractory to SCV2 replication at elevated temperatures $\geq 39^\circ\text{C}$. To our knowledge, these data
167 identify for the first-time a temperature-dependent growth defect in SCV2 replication at
168 elevated temperature ($>37^\circ\text{C}$).

169 In order to examine the underlying mechanism(s) of this intracellular restriction, we
170 compared the relative DEG profiles ($p < 0.05$, ≥ 1.5 or $\leq -1.5 \log_2$ FC) from mock-treated or
171 SCV2-infected tissues at 37 or 40°C (Figure 5, S2, S3, Supplemental Files S1 to S4). Distinct
172 patterns of DEG expression were detected between each paired condition analyzed (Figure
173 5A, B; Supplemental File S5), with clusters of gene commonality (Figure 5B, purple lines) or
174 shared gene pathway ontology (Figure 5B, blue lines). We also identified lncRNAs and
175 micro-RNAs (miRNAs) to be differentially expressed between temperature and SCV2
176 infection conditions (Figure S4; Supplemental File S6). Pathway analysis identified DEG
177 enrichment to be highly specific for each paired condition analyzed, with a clear enrichment
178 in immunity related pathways at 37°C relative to all other conditions (Figure 5C). These data
179 indicate that tissue temperature plays an important role in the differential regulation of
180 transcriptional host responses to SCV2 infection of epithelial tissue. To examine the
181 influence of temperature on immune regulation further, we directly compared the
182 transcriptome profiles from SCV2 infected tissues at 37 and 40°C. Out of the 859 DEGs
183 identified (Figure 6A; $p < 0.05$, ≥ 1.5 or $\leq -1.5 \log_2$ FC), pathway analysis identified immune
184 system and cytokine related pathways to be suppressed during SCV2 infection at 40°C
185 relative to 37°C (Figure 6B, C; Supplemental File S7), findings consistent with a failure of
186 SCV2 to induce the expression of type-I ($\text{INF}\beta$, *IFNB1*) or type-III ($\text{INF}\lambda$, *IFNL1-3*) IFNs at
187 40°C (Figure 6D). Analysis of this subset of differentially expressed immune genes identified
188 a decrease in gene expression during SCV2 infection at 40°C (mean \log_2 counts per million
189 [CPM] 1.147, SD 3.656) relative to mock treatment at 37°C (mean \log_2 CPM 1.578, SD
190 3.716; Figure 6C). These data suggest that SCV2 infection of respiratory tissue at 40°C is not
191 sufficient to induce a robust innate immune response, indicating that the observed
192 temperature restriction of SCV2 at elevated temperature occurs independently of IFN-
193 mediated antiviral immune defences.

194 In order to substantiate these findings, we examined the influence of temperature on
195 SCV2 replication in VeroE6 cells, a cell line derived from *Chlorocebus Sabaues* (African
196 green monkey) known to be permissive to SCV2 infection but defective in type-I IFN-
197 mediated immune defences (26, 36, 37). SCV2 replication was restricted at 40°C, but not
198 39°C, relative to infection at 37°C with or without elevated temperature pre-incubation
199 (Figure 7A to C). Transcriptomic analysis revealed significant differences in the baseline
200 expression of genes associated with the cellular response to heat pathway (GO:0034605)
201 between respiratory tissue and undifferentiated HBE (p=0.0142) or VeroE6 (p<0.0001) cells
202 (Figure 7D, E), but not a reference set of genes expressed across a wide range of cell types
203 and tissues (Figure 7F, G, Supplemental File S8; (33-35)). We posit that such differences in
204 the constitutive expression of heat stress response genes is likely to influence the relative
205 restriction of SCV2 upon temperature elevation in a cell type, and potentially species-
206 specific, dependent manner. These data support our tissue analysis (Figure 4 to 6), which
207 demonstrates that the temperature-dependent restriction of SCV2 to occur independently of
208 the induction of IFN-mediated antiviral immune defences.

209 We next examined the stage at which SCV2 infection became restricted during
210 infection of respiratory tissue at elevated temperature. RNA-Seq analysis of SCV2 infected
211 tissues at 72 h post-infection demonstrated no significant difference in the total number of
212 mapped reads (human + SCV2) between 37 and 40°C (Figure 8A, p=0.3429). However,
213 analysis of viral reads identified a decrease in the relative abundance of viral transcripts
214 mapping to multiple ORFs upon temperature elevation (Figure 8B to D, p<0.0010). Indirect
215 immunofluorescence staining of tissue sections for SCV2 nucleocapsid (N) expression
216 demonstrated significantly fewer clusters of SCV2 positive cells within infected tissues at
217 40°C relative to 37°C (Figure 8E, F, p=0.0022). We conclude that infection of respiratory
218 tissue at elevated temperature restricts SCV2 replication through a mechanism that inhibits

219 viral transcription independently of the induction of IFN-mediated antiviral immune defences
220 that have been reported to restrict SCV2 propagation and spread (31, 38).

221

222 **Discussion**

223 A defining symptom of COVID-19 is the onset of fever with a febrile temperature range of
224 38 to 41°C (4-8). However, the effect of elevated temperature on SCV2 tissue tropism and
225 replication has remained to be determined. Here we identify a temperature-sensitive
226 phenotype in SCV2 replication in respiratory epithelial tissue that occurs independently of
227 the induction of IFN-mediated innate immune defences known to restrict SCV2 replication
228 (31, 38). The differentiation of pseudostratified respiratory epithelium has proven to be a
229 valuable research tool to investigate the cellular tropism, replication kinetics, and immune
230 regulation of SCV2 infection, as these tissue models mimic many aspects of infection
231 observed in animal models and COVID-19 patients (16-24, 29, 32, 39-42). While the use of
232 such 3D models represents an important advancement over traditional 2D cell culture
233 systems, the absence of circulating immune cells (e.g. macrophages, natural killer cells,
234 dendritic cells, and neutrophils) which modulate fever and pro-inflammatory immune
235 responses to microbial infection is an important limiting factor (9, 10, 43). Thus, we limit our
236 conclusions to the effect of elevated temperature on SCV2 infection and replication within
237 the respiratory epithelium. Additional animal studies are warranted to determine the overall
238 net effect of fever and pro-inflammatory immune responses on body temperature
239 thermoregulation and its corresponding influence on SCV2 tissue tropism and replication *in*
240 *vivo*.

241 Consistent with previous reports (16, 18-21, 30-32), SCV2 infection of respiratory
242 epithelium induced a pro-inflammatory immune response (Figure 2; IFN β , IFN λ 1-3, and IL-
243 6). While SCV2 has been reported to induce a relatively weak IFN immune signature in

244 comparison to other respiratory viruses (e.g. IAV) (20, 44), we regularly observed localized
245 areas of epithelial infection (Figure 1A, 2E, 8E) that were coincident with elevated levels of
246 ISG expression (Figure 2E, Mx1). Our findings are consistent with reports that have shown
247 similar patterns of localized infection in the respiratory airway of SCV2 infected ferrets and
248 surface epithelium of organoid cultures (29, 30, 32, 45). These data indicate that SCV2
249 induces a localized immune response during the opening phase of productive infection that is
250 likely to restrict the progress of infection through the epithelium (Figure 1A) (30, 31, 38).
251 Thus, differences in the proportion of epithelial infection and/or rate of intraepithelial spread
252 may account for the observed differences in immune signatures between different respiratory
253 pathogens (20, 46) and variance in apical yields of infectious virus at any given time point of
254 analysis (Figure 1B and Figure 4B, D). As we observed localized areas of ISG induction
255 (Figure 2E), our data support a model of epithelial infection where receptor-binding
256 consumption kinetics of secreted IFN may limit cytokine diffusion and the intraepithelial
257 induction of innate immune defences prior to the recruitment of immune cells and IFN γ
258 secretion (47, 48).

259 We demonstrate that respiratory epithelial cultures induce a significant heat stress
260 response upon temperature elevation without significant loss of tissue integrity, disruption to
261 cellular transcription, diminished levels of ACE2 expression, or DAMP (damage associated
262 molecular pattern) activation of immune defences at temperatures up to 40°C (Figure 3).
263 Importantly, the elevation of temperature alone was not sufficient to inhibit SCV2 entry into
264 respiratory tissue (Figure 4E), but restricted SCV2 replication leading to reduced levels of
265 SCV2 apical shedding (Figure 4A to D). Thus, we identify tissue temperature to play an
266 important role in the epithelial restriction of SCV2 replication. To our knowledge, these data
267 represent the first account of a growth defect in SCV2 replication at febrile temperatures
268 >37°C. Transcriptomic analysis identified substantial differences in the relative degree of

269 immune activation in respiratory tissue infected at 40°C (Figure 5 and 6), despite these
270 tissues having abundant levels of intracellular vRNA (Figure 4E). We posit that the lack of
271 immune induction observed at 40°C is likely a consequence of diminished levels of SCV2
272 replication, as vRNA replication has previously been shown to play an important role in the
273 production of PAMPs, including dsRNA intermediates, required for PRR detection and the
274 activation of innate immune defences during respiratory virus infection (49-52). We also
275 show the thermal restriction of SCV2 replication to occur in VeroE6 cells, a cell line
276 permissive to SCV2 infection but defective in type-I IFN-mediated antiviral immune
277 defences (26, 36, 37). Thus, we conclude that elevated temperature restricts SCV2 replication
278 independently of the induction of ISG expression. Importantly, however, PRR detection of
279 PAMPs which stimulate the production and secretion of pro-inflammatory cytokines
280 (including IL-6) is a pre-requisite requirement for a fever response to microbial challenge *in*
281 *vivo* (9, 10). Thus, additional animal studies are warranted to determine if the temperature
282 restriction of SCV2 observed in respiratory cultures can occur independently of IFN-
283 mediated immune defences *in vivo*. While speculative, we posit that low to moderate grade
284 fever may confer protection to respiratory tissue within SCV2 infected individuals as a
285 component of a homeostatically controlled non-hyperinflammatory immune response to
286 infection.

287 While we identify temperature to play an important role in the regulation of SCV2
288 replication in respiratory epithelia, the precise mechanism(s) of restriction remains to be
289 determined and warrants further investigation. The induction of a heat stress response alone
290 within respiratory tissue may be sufficient to limit SCV2 replication (Figure 2, Figure S3), as
291 the activation and/or induction of stress response proteins has been shown to play both a
292 positive (proviral) and negative (antiviral) role in the replication of many viruses (53-55).
293 Our transcriptomic analysis identified epithelial host responses to be differentially regulated

294 in response to both temperature elevation and SCV2 infection (Figure 5 and 6), with unique
295 profiles of induced gene expression between mock and SCV2 infected tissues at elevated
296 temperature (Figure 5, Figure S2). Thus, SCV2 infection of respiratory epithelia at elevated
297 temperature elicits a distinct host response that may directly contribute to the restriction of
298 SCV2 propagation, for example the induction of mucins (Figure S2, *MUC4* and *MUC5AC*)
299 which have been proposed to limit coronavirus disease progression and IAV replication (56,
300 57). We also identified a significant difference in the differential expression of lncRNAs and
301 miRNAs upon temperature elevation and SCV2 infection that warrants additional
302 investigation (Figure S4), as changes in the relative expression of non-coding RNAs are
303 known to influence the outcome of virus infection independently of IFN-mediated immune
304 defences (58). Thus, multiple gene products and/or pathways may contribute to the sequential
305 or accumulative restriction of SCV2 replication within respiratory tissue at elevated
306 temperature.

307 Importantly, we identify elevated temperature to lead to lower levels of SCV2
308 transcription and vRNA accumulation (Figure 4E, Figure 8D), suggesting that the
309 temperature-dependent block in SCV2 propagation occurs prior to and/or during vRNA
310 replication. We hypothesize that this block in transcription/replication may relate to the
311 inhibition of SCV2 RNA-dependent RNA-polymerase (RdRp) activity or RdRp RNA
312 binding affinity at elevated temperature, as the genomic replication activity of the IAV RdRp
313 polymerase is known to be restricted at elevated temperature (41°C) (59). Notably, IAV cold-
314 adaptation of RdRp activity has been shown to be a host determinant for avian IAV zoonosis,
315 which naturally replicate at higher temperatures (41°C) within the intestinal tracts of birds
316 (60). As circulating clinical strains of SCV2 are susceptible to non-synonymous mutations
317 within RdRp coding sequences (<http://cov-glue.cvr.gla.ac.uk/#/home>), detailed molecular
318 studies are warranted to determine if such amino acid substitutions influence the thermal

319 restriction of SCV2 replication. To our knowledge, no temperature sensitive growth defect
320 for coronaviruses has been reported to date, although a temperature-sensitive (ts) coronavirus
321 mutant has been identified for murine hepatitis virus (MHV, tsNC11) (61). The replication
322 defect of tsNC11 was attributed to coding substitutions within the macrodomain and papain-
323 like protease 2 domain of the non-structural protein 3, which displayed a severe growth
324 defect at 40°C in DBT (Delayed Brain Tumor) cells, whereas wild-type MHV replicated to
325 equivalent titres at 40°C to those observed at 37°C (61). Thus, we present the first evidence
326 demonstrating that a circulating clinical strain of coronavirus is sensitive to temperature
327 thermoregulation. As such, heat-adaptation gain of function experiments through serial
328 passage of SCV2 in respiratory cultures incubated at $\geq 39^\circ\text{C}$ may shed light on whether the
329 temperature restriction of SCV2 observed is related to viral or cellular host factors, although
330 appropriate levels of biosafety (ethical and genetic modification) should be considered prior
331 to such experimentation.

332 In summary, we identify an important role for tissue temperature in the restriction of
333 SCV2 replication in respiratory epithelia that occurs independently of the induction of IFN-
334 mediated antiviral immune defences. We demonstrate tissue temperature to significantly
335 influence the differential regulation of epithelial host responses induced in response to SCV2
336 infection. Future investigation is warranted to determine the precise mechanism(s) of
337 restriction, as this may uncover novel avenues for therapeutic intervention in the treatment of
338 COVID-19.

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344 **Materials and Methods**

345 **Virus**

346 Severe Acute Respiratory Syndrome Virus-2 (SARS-CoV-2, SCV2)

347 BetaCoV/England/02/2020/EPI_ISL_407073 was isolated from a COVID-19 patient (a gift
348 from Public Health England). The virus was passaged three times in VeroE6 cells and
349 genotype sequence confirmed by Illumina sequencing. All experiments were performed in a
350 Biosafety level 3-laboratory at the MRC-University of Glasgow Centre for Virus Research
351 (SAPO/223/2017/1a).

352 **SCV2 replication kinetics in VeroE6 cells**

353 VeroE6 cells (a gift from Michelle Bouloy, Institute Pasteur, France) were propagated in
354 Dulbecco's Modified Eagle Medium (DMEM GlutaMAX; ThermoFisher, 31966-021)
355 supplemented with 10% fetal calf serum (FCS; ThermoFisher, 10499044 [Lot Number
356 08G8293K]) at 37°C with 5% CO₂. Cells were seeded the day before infection at a density of
357 2x10⁵ cells per well in a 12-well plate and infected with 10⁴ PFU of SCV2 diluted in serum-
358 free DMEM for 120 minutes (mins) with occasional rocking. The inoculum was removed and
359 replaced by DMEM + 10% FCS. Supernatant was collected every 24 hours (h) and snap-
360 frozen prior to analysis. For temperature inhibition experiments, cells were either pre-
361 incubated at 37, 39, or 40°C for 24 h prior to infection with continued incubation at their
362 respective temperatures or incubated at the indicated temperatures after the addition of the
363 medium.

364 **Differentiation of respiratory epithelium**

365 Primary human bronchiolar epithelial (HBE) cells from a healthy 63-year-old white
366 Caucasian male (non-smoker) were sourced from Epithelix Sarl (Geneva, Switzerland). Cells
367 were propagated at 37°C with 5% CO₂ in hAEC culture medium (Epithelix, EP09AM). All
368 differentiation experiments were performed on cells that had been passaged a total of four

369 times. Cells were seeded onto transwells (Falcon, 734-0036) at a cell density of 3×10^4 cells
370 per transwell and grown to confluency. Cells were differentiated under air-liquid interface
371 (ALI) in PneumaCult-ALI medium containing hydrocortisone and heparin supplements
372 (STEMCELL Technologies, 05001) as specified by the manufacturer's guidelines. Cells were
373 differentiated for a minimum of 35 days, with media changed every 48 h and twice weekly
374 apical washing in serum-free DMEM after day 20 at ALI. ALI cultures were apically washed
375 twice before infection, 24 h prior to infection and immediately preceding infection. Cultures
376 were inoculated apically with 100 μ l of serum-free DMEM containing 10^4 PFU of SCV2
377 (based on VeroE6 titres) for 120 mins. The inoculum was removed, and the apical surface
378 washed once in serum-free DMEM (0 h time point). Unless stated otherwise, ALI cultures
379 were incubated at 37°C with 5% CO₂. For temperature inhibition experiments, ALI cultures
380 were pre-incubated at 37, 39, or 40°C for 24 h prior to infection with continued incubation at
381 their respective temperatures. Apical washing for 30 mins in 200 μ l of serum-free DMEM
382 was used to collect infectious virus. Supernatants were divided into two 100 μ l aliquots; one
383 for virus quantitation (TCID₅₀) and the other for vRNA extraction in TRIzol (ThermoFisher,
384 15596026; 1 in 3 dilution). Samples were stored at -80°C until required. Tissues were fixed at
385 the indicated time points in 8% formaldehyde (Fisher Scientific, F/1501/PB17) for 16 to 24 h
386 prior to paraffin embedding and processing.

387 **TCID₅₀**

388 VeroE6 cells (subclone 6F5, MESO) were seeded the day before infection into 96-well-plates
389 at a density of 1.1×10^5 cells/well in DMEM + 10% FCS at 37°C with 5% CO₂. Virus
390 supernatants were serially diluted 1:10 in DMEM + 2% FCS in a total volume of 100 μ l.
391 Cells were infected in triplicate and incubated for three days at 37°C with 5% CO₂. The
392 inoculum was removed and fresh media overlaid. Plates were incubated for three days at
393 37°C in 5% CO₂. Cells were fixed in 8% formaldehyde (Fisher Scientific, F/1501/PB17) for

394 ≥ 2.5 h. Cytopathic effect was scored by staining with 0.1% Coomassie Brilliant Blue
395 (BioRad, 1610406) in 45% methanol and 10% glacial acetic acid. TCID₅₀ was calculated
396 according to Reed–Muench and Spearman–Kärber as described (62).

397 **Plaque assay**

398 VeroE6 cells were seeded at a density of 2.5×10^5 cells/well in 12-well plates the day before
399 infection. Ten-fold serial dilutions of virus were prepared in DMEM + 2% FCS in a total
400 volume of 500 μ l. Cells were infected for 60 mins with occasional rocking at 37°C in 5%
401 CO₂. The inoculum was removed and 1 ml of overlay containing 0.6% Avicel (Avicel
402 microcrystalline cellulose, RC-591) made up in DMEM containing 2% FCS was added to
403 each well. Cells were incubated for three days at 37°C in 5% CO₂ prior to fixation in 8%
404 formaldehyde and Coomassie brilliant blue staining (as described above). Plaques were
405 counted manually and the plaque forming unit (PFU) per ml calculated.

406 **Reverse transcription quantitative PCR (RTq-PCR)**

407 RNA was extracted from TRIzol treated apical wash samples using a QIAGEN RNeasy Mini
408 kit (Qiagen, 74106) following the manufacture's protocol. SCV2 RNA was quantified using a
409 NEB Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, E3006) and
410 2019-nCoV CDC N1 primers and probes (IDT, 10006713). Genome copy numbers were
411 quantified using a standard curve generated from serial dilutions of an RNA standard used
412 throughout the study. The RNA standard was calibrated using a plasmid (2019-nCoV_N;
413 IDT, 10006625) that was quantified using droplet digital PCR. Values were normalized to
414 copies/ml for apical washes or copies/tissue for cell-associated RNA.

415 ***In situ*-hybridization and immunostaining of respiratory tissue sections**

416 Paraffin-embedded tissues were sectioned using a microtome (~2-3 μ m thick) and mounted
417 on glass slides. Tissue sections were stained with Haematoxylin and Eosin (H&E). For
418 immunofluorescence staining, tissue sections underwent antigen retrieval by citrate (pH 6) or

419 EDTA (pH 8) pressure cooking (as indicated). RNAscope was used for the detection of
420 SCV2 RNA using a Spike-specific probe set (Advanced Cell Diagnostics, 848561 and
421 322372) following the manufacturer's protocol, which included a pre-treatment with boiling
422 in target solution and proteinase K treatment. ACE2 was detected using a rabbit polyclonal
423 anti-hACE2 antibody (Cell Signalling, 4355S; citrate antigen-retrieval) and EnVision+ anti-
424 rabbit HRP (Agilent, K4003). Slides were scanned with a bright field slide scanner (Leica,
425 Aperio Versa 8). Mx1 was detected using a mouse monoclonal anti-Mx1 antibody ((63);
426 EDTA antigen-retrieval). SCV2 nucleocapsid protein was detected using a sheep polyclonal
427 anti-SCV2 N protein antibody (University of Dundee, DA114; EDTA antigen-retrieval).
428 Secondary antibodies for detection were rabbit anti-sheep AlexaFluor 555 (abcam, ab150182)
429 and rabbit anti-mouse AlexaFluor 488 (Sigma-Aldrich, SAB4600056). Nuclei were stained
430 with ProLong Gold (Life Technologies, P36941). Images were collected using a Zeiss LSM
431 710 confocal microscope using 40x Plan-Apochromat oil objective lens (numerical aperture
432 1.4) using 405, 488, and 543 nm laser lines. Zen black software (Zeiss) was used for image
433 capture and exporting images, with minimal adjustment (image rotation) in Adobe Photoshop
434 for presentation.

435 **Tissue section image analysis**

436 Scanned Haematoxylin and Eosin (H&E) stained sections were analyzed using Aperio
437 ImageScope analysis software (Leica). Epithelial thickness was measured by manually
438 outlining the bottom and top surfaces of each epithelium (excluding cilia) and measuring the
439 vertical point-to-point distance between each surface across the epithelium. One hundred
440 evenly distributed distance measurements were acquired per epithelium per experimental
441 condition in triplicate.

442 **RNA sequencing (RNA-Seq)**

443 Transwell tissue or undifferentiated cells seeded into 12-well dishes at a cell density of 2×10^5
444 cells per well were harvested by scraping into TRIzol Reagent and transferred into tubes
445 containing 2.8 mm ceramic beads (Stretton Scientific, P000916-LYSK0-A.0). Samples were
446 homogenized (two 20 sec pulses with a 30 sec interval at room temperature, RT) using a
447 Percellys Cryolys Evolution Super Homogeniser (Bertin Instruments, P000671-CLYS2-A) at
448 5500 rounds per minute. The homogenised suspension was loaded into QIAshredder tubes
449 (Qiagen, 79654) and centrifuged ($12,000 \times g$ for 2 mins at RT). 0.25 ml of chloroform (VWR
450 Life Sciences, 0757) was added to the eluate and incubated at RT for 7 mins prior to
451 centrifugation ($12,000 \times g$, 15 minutes, 4°C). The aqueous phase was transferred into a fresh
452 tube, mixed with 250 μl of 100% ethanol, and RNA isolated using RNeasy columns
453 (Qiagen, 74104) following the manufacturer's protocol, that included a 15 min DNase
454 treatment (Qiagen, 79254) treatment at RT. Eluted RNA was quantified using a NanoDrop
455 2000 Spectrophotometer (ThermoFisher Scientific, ND-2000) and quality controlled on a
456 TapeStation (Agilent Technologies, G2991AA). All samples had a RIN score of ≥ 9 . One
457 microgram of total RNA was used to prepare libraries for sequencing using an Illumina
458 TruSeq Stranded mRNA HT kit (Illumina, 20020594) and SuperScript2 Reverse
459 Transcriptase (Invitrogen, 18064014)
460 according to the manufacturer's instructions. Libraries were pooled in equimolar
461 concentrations and sequenced using an Illumina NextSeq 500 sequencer (Illumina, FC-404-
462 2005). At least 95% of the reads generated presented a Q score of ≥ 30 . RNA-Seq reads were
463 quality assessed (FastQC; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and
464 sequence adaptors removed (TrimGalore;
465 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). RNA-Seq reads were
466 aligned to the *Homo sapiens* genome (GRCh38) or *Chlorocebus Sabaeus* genome
467 (ChlSab1.1), downloaded via Ensembl using HISAT2. HISAT2 is a fast and sensitive splice

468 aware mapper, which aligns RNA sequencing reads to mammalian-sized genomes using FM
469 index strategy (64). RNA-Seq reads were also mapped to SARS-CoV-2 (GISAID accession
470 ID:EPI_ISL_407073) using Bowtie2 (65). FeatureCount (66) was used to count reads
471 mapping to gene annotation files. Reads counts were normalized to counts per million (CPM)
472 unless otherwise stated. The edgeR package was used to calculate the gene expression level
473 and to analyze differentially expressed genes between sample groups (67). Sequences have
474 been deposited in the European Nucleotide Archive
475 (<https://www.ebi.ac.uk/ena/browser/home>), accession number PRJEB41332. Only high
476 confidence ($p < 0.05$) differentially expressed genes (DEGs; ≥ 1.5 or ≤ -1.5 log₂ fold change,
477 log₂ FC) were used for pathway analysis in Reactome (<https://reactome.org>) (68, 69) or
478 differential pathway enrichment in Metascape
479 (<https://metascape.org/gp/index.html#/main/step1>) (70). In Reactome, the gene mapping tool
480 was used as a filter to identify pathways enriched (over-represented) for mapped DEG
481 entities. FDR (False Discovery Rate) values < 0.05 were considered significant for pathway
482 enrichment. In Metascape, all DEGs were used for differential pathway analysis. Pathway p-
483 values < 0.05 were considered significant. Differentially expressed ($p < 0.05$, ≥ 1.5 or ≤ -1.5 log₂
484 FC) lncRNAs and miRNAs were identified using the Ensembl BioMart tool
485 (<http://www.ensembl.org/biomart/martview/05285d5f063a05a82b8ba71fe18a0f18>). Heat
486 maps were plotted in GraphPad Prism (version 9). Mean counts per million (CPM) values of
487 zero were normalized to 0.01 for log₂ presentation. Venn diagrams were plotted using
488 <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

489 **Statistical analysis**

490 GraphPad Prism (version 9) was used for statistical analysis. For unpaired non-parametric
491 data, a Kruskal-Wallis one-way ANOVA or Mann-Whitney *U*-test was applied. For paired
492 non-parametric data, a one-way ANOVA Friedman test or Wilcoxon matched-pairs sign rank

493 test was applied. Statistical p-values are shown throughout. Statistically significant
494 differences were accepted at $p \leq 0.05$.

495

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512

513 **Availability of data**

514 The datasets generated and analyzed during the current study are available in the University
515 of Glasgow data repository (a doi will be generated upon manuscript acceptance). RNA-Seq

516 data sets are available from the European Nucleotide Archive, accession number

517 PRJEB41332 (available upon manuscript acceptance).

518

519 **Author contributions**

520 VH, KD (joint first authors): Conceptualization, Data curation, Formal Analysis,

521 Investigation, Methodology, Project administration, Validation, Visualization, Writing –

522 original draft, Writing – review & editing.

523 JKW, RJ, CR, DG, IE, AS, SMF, QG: Data curation, Formal Analysis, Investigation,

524 Methodology, Validation

525 MS, AMS, RMP.: Resources, Methodology

526 AMG: Data curation, Formal Analysis

527 SG, PRM: Conceptualization, Funding acquisition, Methodology, Supervision, Writing –

528 review & editing.

529 CB: Conceptualization, Methodology, Data curation, Formal Analysis, Investigation,

530 Funding acquisition, Project administration, Supervision, Validation, Writing – Original

531 draft, Writing – review & editing.

532

533 **Conflicts of interest**

534 The authors declare no conflict of interest.

535

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- 724

726 **Figure legends**

727 **Figure 1. Differentiation of primary bronchial epithelial airway cultures supports**

728 **SARS-CoV-2 replication.** Primary bronchiole epithelial cells were seeded onto 6.5mm
729 transwells and grown to confluency prior to differentiation under air liquid interface (ALI)
730 conditions for ≥ 35 days. Ciliated respiratory cultures were mock (media only) or SARS-CoV-
731 2 (SCV2; 10^4 PFU/well) infected at 37°C for the indicated times (hours, h). (A)
732 Representative images of H&E (left-hand panels), ACE2 (brown, middle panels), or
733 detection of SCV2 RNA by *in situ* hybridization (red, right-hand panels) stained sections.
734 Haematoxylin was used as a counter stain. Scale bars = $20\ \mu\text{m}$. (B/C) Genome copies per ml
735 of SCV2 in apical washes harvested over time as determined RT-qPCR. $N \geq 7$ derived from a
736 minimum of three independent biological experiments per sample condition. (B) Black line,
737 median; all data points shown. (C) Means and SD shown. (D) Specificity of RT-qPCR to
738 detect vRNA in apical washes taken from mock (blue circles) or SCV2 infected (red circles)
739 tissues at 72 h (as in B). $N \geq 5$ derived from a minimum of three independent biological
740 experiments per sample condition. Black line, median; all data points shown; $p=0.0003$,
741 Mann-Whitney *U*-test. (E) TCID₅₀ assay measuring infectious viral load in apical washes
742 harvested from mock (blue circles) or SCV2 infected (red circles) tissues over time. Means
743 and SD shown. $N \geq 5$ derived from a minimum of three independent biological experiments
744 per sample condition.

745

746 **Figure 2. SARS-CoV-2 infection of respiratory airway cultures induces a type-I and**

747 **type-III IFN response.** Ciliated respiratory cultures were mock (media only) or SARS-CoV-
748 2 (SCV2; 10^4 PFU/well) infected at 37°C for 72 h prior to RNA extraction and RNA-Seq.
749 (A) Scatter plots showing high confidence ($p < 0.05$) differentially expressed gene (DEG)
750 transcripts identified between mock and SCV2 infected cultures (grey circles); DEGs ≥ 1.5

751 log₂ FC, red circles; DEGs ≤ -1.5 log₂ FC, blue circles. (B) Reactome pathway analysis of
752 mapped DEGs ($p < 0.05$, ≥ 1.5 log₂ FC). Pathways enriched for DEGs with an FDR (corrected
753 over-representation P value) < 0.05 (plotted as $-\log_{10}$ FDR) shown (red bars). Dotted line,
754 threshold of significance ($-\log_{10}$ FDR of 0.05). (C) Expression profile (log₂ CPM) of
755 Immune system (R-HSA168256) related DEGs (identified in B). Black line, median; dotted
756 lines; 5th and 95th percentile range; $p < 0.0001$, Mann-Whitney U -test. (D) Expression levels
757 (log₂ CPM) of individual Immune system DEGs (identified in B); $p < 0.0001$, Wilcoxon
758 matched-pairs sign rank test. (A to D) RNA-Seq data derived from RNA isolated from three
759 technical replicates per sample condition. Data analysis is presented in Supplemental File S1.
760 (E) Indirect immunofluorescence staining of tissue sections showing Mx1 (green) and SCV2
761 nucleocapsid (N, red) epithelial localization. Nuclei were stained with DAPI. Scale bars = 20
762 μm .

763

764 **Figure 3. Respiratory airway cultures induce a heat stress response upon incubation at**
765 **elevated temperature.** Ciliated respiratory cultures were incubated at 37, 39, or 40°C for 72
766 h prior to fixation or RNA extraction and RNA-Seq. (A) Representative images of H&E
767 stained sections. Scale bars = 20 μm . (B) Quantitation of respiratory tissue thickness (μm) at
768 72 h. $N=300$ measurements derived from three technical replicates per sample condition.
769 Black line, median; dotted lines; 5th and 95th percentile range; p -values shown, one-way
770 ANOVA Kruskal-Wallis test. (C) ACE2 expression levels (CPM) in respiratory cultures
771 incubated at 37 or 40°C; means and SD shown; $p=0.4001$, Mann-Whitney U -test. (D)
772 Expression values (log₂ CPM) of a reference gene set (24 genes; (33-35)) in respiratory
773 cultures incubated at 37 or 40°C; $p=0.9106$, Mann-Whitney U -test. (E) Scatter plots showing
774 high confidence ($p < 0.05$) differentially expressed gene (DEG) transcripts identified between
775 respiratory cultures incubated at 37 or 40°C (grey circles); DEGs ≥ 1.5 log₂ FC, red circles;

776 DEGs $\leq -1.5 \log_2$ FC, blue circles. (F) Reactome pathway analysis of mapped DEGs ($p < 0.05$,
777 $\geq 1.5 \log_2$ FC). Pathways enriched for DEGs with an FDR < 0.05 (plotted as $-\log_{10}$ FDR)
778 shown (red bars; every 4th bar labelled). Dotted line, threshold of significance ($-\log_{10}$ FDR of
779 0.05). (G) Expression values (\log_2 CPM) of DEGs identified with cellular response to heat
780 stress (R-HSA-3371556; arrow in B) and cellular response to external stimuli (R-HSA-
781 8953897) pathways; $p = 0.001$, Wilcoxon matched-pairs sign ranked test. (H) Expression
782 levels (\log_2 CPM) for all genes associated with cellular response to heat stress pathway (R-
783 HSA-3371556); $p = 0.0013$, Wilcoxon matched-pairs sign ranked test. (C to H) RNA-Seq data
784 derived from RNA isolated from three technical replicates per sample condition. Data
785 analysis is presented in Supplemental File S2.

786

787 **Figure 4. Elevated temperature restricts SARS-CoV-2 replication in respiratory airway**

788 **cultures.** Ciliated respiratory cultures were incubated at 37, 39, or 40°C for 24 h prior to
789 mock (media only) or SARS-CoV-2 (SCV2; 10^4 PFU/well) infection and incubation at the
790 indicated temperatures. Apical washes were collected over time (as indicated) and tissues
791 harvested at 72 h for RNA extraction and RNA-Seq. (A/B) Genome copies per ml of SCV2
792 in apical washes harvested over time as determined RT-qPCR. $N \geq 11$ derived from a
793 minimum of three independent biological experiments per sample condition. (A) Means and
794 SD shown. (B) Black line, median; whisker, 95% confidence interval; all data points shown;
795 p-values shown, one-way ANOVA Kruskal-Wallis test. (C/D) TCID₅₀ assay measuring
796 infectious viral load in apical washes harvested from SCV2 infected respiratory cultures over
797 time. $N \geq 11$ derived from a minimum of three independent biological experiments. (C) Means
798 and SD shown. (D) Black line, median; whisker, 95% confidence interval; all data points
799 shown; p-values shown, one-way ANOVA Kruskal-Wallis test. (E) Genome copies per tissue
800 of SCV2 as determined RT-qPCR. $N \geq 11$ derived from a minimum of three independent

801 biological experiments per sample condition. Black line, median; whisker, 95% confidence
802 interval; all data points shown; p-values shown, one-way ANOVA Kruskal-Wallis test.
803
804 **Figure 5. Respiratory airway cultures induce distinct transcriptional host responses to**
805 **SARS-CoV-2 infection at elevated temperature.** Ciliated respiratory cultures were
806 incubated at 37 or 40°C for 24 h prior to mock (media only) or SARS-CoV-2 (SCV2; 10⁴
807 PFU/well) infection. Tissue were incubated at their respective temperatures for 72 h prior to
808 RNA extraction and RNA-Seq. DEGs ($p < 0.05$, $\geq 1.5 \log_2 \text{FC}$ [top panels] or $\leq -1.5 \log_2 \text{FC}$
809 [bottom panels]) were identified for each paired condition; blue ellipses, SCV2 37°C/Mock
810 37°C (SCV37/Mock37); green ellipses, SCV2 40°C/Mock 40°C (SCV40/Mock40); red
811 ellipses, SCV2 40°C/Mock 37°C (SCV40/Mock37); yellow ellipses, Mock 40°C/Mock 37°C
812 (Mock40/Mock37). (A) Venn diagram showing the number of unique or shared DEGs
813 between each paired condition analyzed. (B) Circos plot showing the proportion of unique
814 (light orange inner circle) or shared (dark orange inner circle + purple lines) DEGs between
815 each paired condition analyzed. Blue lines, DEGs which share pathway gene ontology terms.
816 (C) Metascape pathway analysis showing DEG enrichment p-value ($-\log_{10}$) for each paired
817 condition analyzed. Grey boxes, $p > 0.05$. (A to C) RNA-Seq data derived from RNA isolated
818 from three technical replicates per sample condition. Data analysis is presented in
819 Supplemental Files S1 to S5.

820

821 **Figure 6. Elevated temperature restricts SARS-CoV-2 replication in respiratory airway**
822 **cultures independently of the induction of IFN-mediated innate immune defences.**

823 Ciliated respiratory cultures were incubated at 37 or 40°C for 24h prior to mock (media) or
824 SARS-CoV-2 (SCV2; 10⁴ PFU/well) infection. Tissue were incubated at their respective
825 temperatures for 72 h prior to RNA extraction and RNA-Seq. (A) Scatter plots showing high

826 confidence ($p < 0.05$) differentially expressed gene (DEG) transcripts identified between 37
827 and 40°C SCV2 infected respiratory cultures (grey circles); DEGs $\geq 1.5 \log_2$ FC, red circles;
828 DEGs $\leq -1.5 \log_2$ FC, blue circles. (B) Reactome pathway analysis of mapped DEGs
829 ($p < 0.05$, $\leq -1.5 \log_2$ FC). Pathways enriched for DEGs with an FDR < 0.05 (plotted as $-\log_{10}$
830 FDR) shown (blue bars). Dotted line, threshold of significance ($-\log_{10}$ FDR of 0.05). (C)
831 Expression profile (\log_2 CPM) of Immune system (R-HSA168256) DEGs (identified in B)
832 relative to expression levels in Mock tissue incubated at 37 and 40°C. Black line, median;
833 dotted lines, 5th and 95th percentile range; p-values shown, Wilcoxon matched-pairs signed
834 rank test (top), one-way ANOVA Friedman test (bottom). (D) Expression levels (\log_2 CPM)
835 for individual Immune system (R-HAS-168256) DEGs (identified in B) relative to mock at
836 37 and 40°C. (A to D) RNA-Seq data derived from RNA isolated from three technical
837 replicates per sample condition. Data analysis is presented in Supplemental File S7.

838

839 **Figure 7. Elevated temperature restricts SARS-CoV-2 replication in VeroE6 cells.**

840 Permissive VeroE6 cells were infected with SARS-CoV-2 (SCV2; 10^4 PFU/well) at 37°C
841 prior to temperature elevation and incubation at 37, 39, or 40°C (A/B) or pre-incubated at 37,
842 39, or 40°C for 24 h prior to infection and continued incubation at their respective
843 temperatures (C). (A) TCID₅₀ growth curve of SCV2 infected VeroE6 cells incubated at 37°C
844 over time (h). Means and SD shown. (B/C) TCID₅₀ viral titres at 24 and 48 h post-infection.
845 Left-hand panel; means and SD. Right-hand panel; black line, median; whisker, 95%
846 confidence interval; all data points shown; p-values shown, one-way ANOVA Kruskal-
847 Wallis test. (A-C) N=9 derived from a minimum of three independent biological experiments
848 per sample condition. (D) Expression profile (\log_2 CPM) for genes associated with cellular
849 response to heat (GO:0034605) in respiratory cultures (Resp. tissue), undifferentiated
850 primary HBE, and VeroE6 cells incubated at 37°C; p-values shown, One-way ANOVA

851 Friedman test. (E) Expression levels (log₂ CPM) of individual genes associated with cellular
852 response to heat (as in D). (F) Expression profile (log₂ CPM) of a reference gene set (18
853 genes; (33-35)); p-values shown, One-way ANOVA Friedman test. (G) Expression values
854 (log₂ CPM) of a reference gene set (as in F). (D to G) RNA-Seq data derived from RNA
855 isolated from three technical replicates per sample condition. Data analysis is presented in
856 Supplemental File S8.

857

858 **Figure 8. Elevated temperature restricts SARS-CoV-2 transcription in respiratory**

859 **airway cultures.** Ciliated respiratory cultures were incubated at 37 or 40°C for 24 h prior to
860 mock (media only) or SARS-CoV-2 (SCV2; 10⁴ PFU/well) infection. Tissue were incubated
861 at their respective temperatures for 72 h prior to RNA extraction and RNA-Seq. (A) Total
862 mapped reads (MR; human + SCV2) from infected tissues; means and SD shown; p=0.3429,
863 one-way ANOVA Kruskal-Wallis test. (B) SCV2 mapped reads from infected tissues; means
864 and SD shown. (C) % SCV2 mapped reads of total mapped read count (human + SCV2);
865 means and SD shown. (D) Expression values (log₂ MR) of SCV2 gene transcripts; p<0.0010,
866 Wilcoxon matched-pairs sign rank test. (A-D) RNA-Seq data derived from RNA isolated
867 from three technical replicates per sample condition. (E) Indirect immunofluorescence
868 staining of tissue sections showing SCV2 nucleocapsid (N, red) epithelial localization. Nuclei
869 were stained with DAPI. Scale bars = 20 μm. (F) Quantitation of SCV2 N infectious foci in
870 respiratory tissue sections. N=6 independently stained tissue sections per sample condition.
871 Black line, median; whisker, 95% confidence interval; all data points shown; p=0.0022,
872 Mann-Whitney *U*-test.

873

874 **Figure S1. Respiratory airway cultures induce a heat stress response.** Ciliated respiratory

875 cultures were incubated at 37 or 40°C for 72 h prior to RNA extraction and RNA-Seq. Heat

876 map showing expression values (log₂ CPM) of genes associated with the cellular response to
877 heat pathway (GO: 0034605); p=0.0018, Wilcoxon matched-pairs sign rank test; every
878 second row labelled. RNA-Seq data derived from RNA isolated from three technical
879 replicates per sample condition. Data analysis is presented in Supplemental File S2.

880

881 **Figure S2. Identification of DEGs in mock and SARS-CoV-2 infected respiratory**

882 **cultures incubated at 40°C.** Ciliated respiratory cultures were incubated at 40°C for 24 h

883 prior to mock (media only) or SARS-CoV-2 (SCV2; 10⁴ PFU/well) infection and continued

884 incubation at 40°C. Tissues were harvested at 72 h for RNA extraction and RNA-Seq. (A)

885 Scatter plots showing high confidence (p<0.05) differentially expressed gene (DEG)

886 transcripts identified between mock and SCV2 infected respiratory cultures at 40°C (grey

887 circles); DEGs ≥ 1.5 log₂ FC, red circles; DEGs ≤ -1.5 log₂ FC, blue circles. (B) Reactome

888 pathway analysis of mapped DEGs (p<0.05, ≥ 1.5 log₂ FC [red bars] or ≤ -1.5 log₂ FC [blue

889 bars]). Top 10 enriched pathways shown. Dotted line, threshold of significance (-log₁₀ FDR

890 of 0.05). (C) Expression values (log₂ CPM) of Reactome mapped DEGs; p-values shown,

891 Wilcoxon matched-pairs sign rank test. (A to C) RNA-Seq data derived from RNA isolated

892 from three technical replicates per sample condition. Data analysis is presented in

893 Supplemental File S3.

894

895

896 **Figure S3. Identification of DEGs in mock and SARS-CoV-2 infected respiratory**

897 **cultures incubated at 37 and 40°C, respectively.** Ciliated respiratory cultures were

898 incubated at 37 or 40°C for 24 h prior to mock (media only) or SARS-CoV-2 (SCV2; 10⁴

899 PFU/well) infection and continued incubation at their respective temperatures. Tissues were

900 harvested at 72 h for RNA extraction and RNA-Seq. (A) Scatter plots showing high

901 confidence ($p < 0.05$) differentially expressed gene (DEG) transcripts identified between mock
902 (37°C) and SCV2 infected (40°C) respiratory cultures (grey circles); DEGs $\geq 1.5 \log_2 \text{FC}$,
903 red circles; DEGs $\leq -1.5 \log_2 \text{FC}$, blue circles. (B) Reactome pathway analysis of mapped
904 DEGs ($p < 0.05$, $\geq 1.5 \log_2 \text{FC}$ [red bars, every sixth row labelled] or $\leq -1.5 \log_2 \text{FC}$ [blue
905 bars]). Dotted line, threshold of significance ($-\log_{10} \text{FDR}$ of 0.05). Black arrow,
906 identification of cellular response to heat stress pathway. (C) Expression values ($\log_2 \text{CPM}$)
907 of Reactome mapped DEGs; p-values shown, Wilcoxon matched-pairs sign rank test. Every
908 third column labelled. (A to C) RNA-Seq data derived from RNA isolated from three
909 technical replicates per sample condition. Data analysis is presented in Supplemental File S4.
910

911 **Figure S4. Respiratory airway cultures induce distinct lncRNA and miRNA**
912 **transcriptional host responses to SARS-CoV-2 infection at elevated temperature.**
913 Ciliated respiratory cultures were incubated at 37 or 40°C for 24 h prior to mock (media
914 only) or SARS-CoV-2 (SCV2; 10^4PFU/well) infection. Tissue were incubated at their
915 respective temperatures for 72 h prior to RNA extraction and RNA-Seq. DEGs ($p < 0.05$, ≥ 1.5
916 $\log_2 \text{FC}$ [top panels] or $\leq -1.5 \log_2 \text{FC}$ [bottom panels]) were identified for each paired
917 condition; blue circles/ellipses, SCV2 $37^{\circ}\text{C}/\text{Mock } 37^{\circ}\text{C}$ (SCV37/Mock37); green
918 circles/ellipses, SCV2 $40^{\circ}\text{C}/\text{Mock } 40^{\circ}\text{C}$ (SCV40/Mock40); red circles/ellipses, SCV2
919 $40^{\circ}\text{C}/\text{Mock } 37^{\circ}\text{C}$ (SCV40/Mock37); yellow circles/ellipses, Mock $40^{\circ}\text{C}/\text{Mock } 37^{\circ}\text{C}$
920 (Mock40/Mock37). (A) Proportion of lncRNA + miRNA (grey numbers and lines [relative
921 %]) identified per DEG population (coloured numbers and lines [relative %]). (B) Venn
922 diagram showing the number of unique or shared lncRNA + miRNAs identified between
923 each paired condition analyzed. (C) Circos plot showing the proportion of unique (light
924 orange inner circle) or shared (dark orange inner circle + purple lines) lncRNA + miRNAs
925 between each paired condition analyzed. (D) Expression values ($\log_2 \text{CPM}$) of lncRNA +

926 miRNA identified per sample condition analyzed; p-values shown, one-way ANOVA
927 Friedman test (top), Wilcoxon matched-pairs sign rank test (bottom). (A to D) RNA-Seq data
928 derived from RNA isolated from three technical replicates per sample condition. Data
929 analysis is presented in Supplemental File S6.

930

931 **Supplementary Files**

932 **Supplemental File S1.** RNA-Seq data analysis for mock (Mck)-treated or SARS-CoV-2
933 (SCV2) infected respiratory tissues at 37°C.

934 **Supplemental File S2.** RNA-Seq data analysis for mock (Mck)-treated respiratory tissues
935 incubated at 37 and 40°C.

936 **Supplemental File S3.** RNA-Seq data analysis for mock (Mck)-treated or SARS-CoV-2
937 (SCV2) infected respiratory tissues at 40°C.

938 **Supplemental File S4.** RNA-Seq data analysis for mock (Mck)-treated or SARS-CoV-2
939 (SCV2) infected respiratory tissues at 37°C and 40°C, respectively.

940 **Supplemental File S5.** Comparative DEG analysis of RNA-Seq data derived from mock
941 (Mck)-treated or SARS-CoV-2 (SCV2) infected respiratory tissues at 37 and 40°C.

942 Supplemental Files S1-S4.

943 **Supplemental File S6.** Comparative lncRNA and miRNA DEG analysis of RNA-Seq data
944 derived from mock (Mck)-treated or SARS-CoV-2 (SCV2) infected respiratory tissues at 37
945 and 40°C. Supplemental Files S1-S4.

946 **Supplemental File S7.** RNA-Seq data analysis for SARS-CoV-2 (SCV2) infected respiratory
947 tissues at 37 and 40°C.

948 **Supplemental File S8.** DEG analysis of the Cellular response to heat (GO:0034605) from
949 RNA-Seq data derived from mock (Mck)-treated respiratory tissues (Resp. tissue) and
950 undifferentiated HBEC and VeroE6 cells incubated at 37°C.

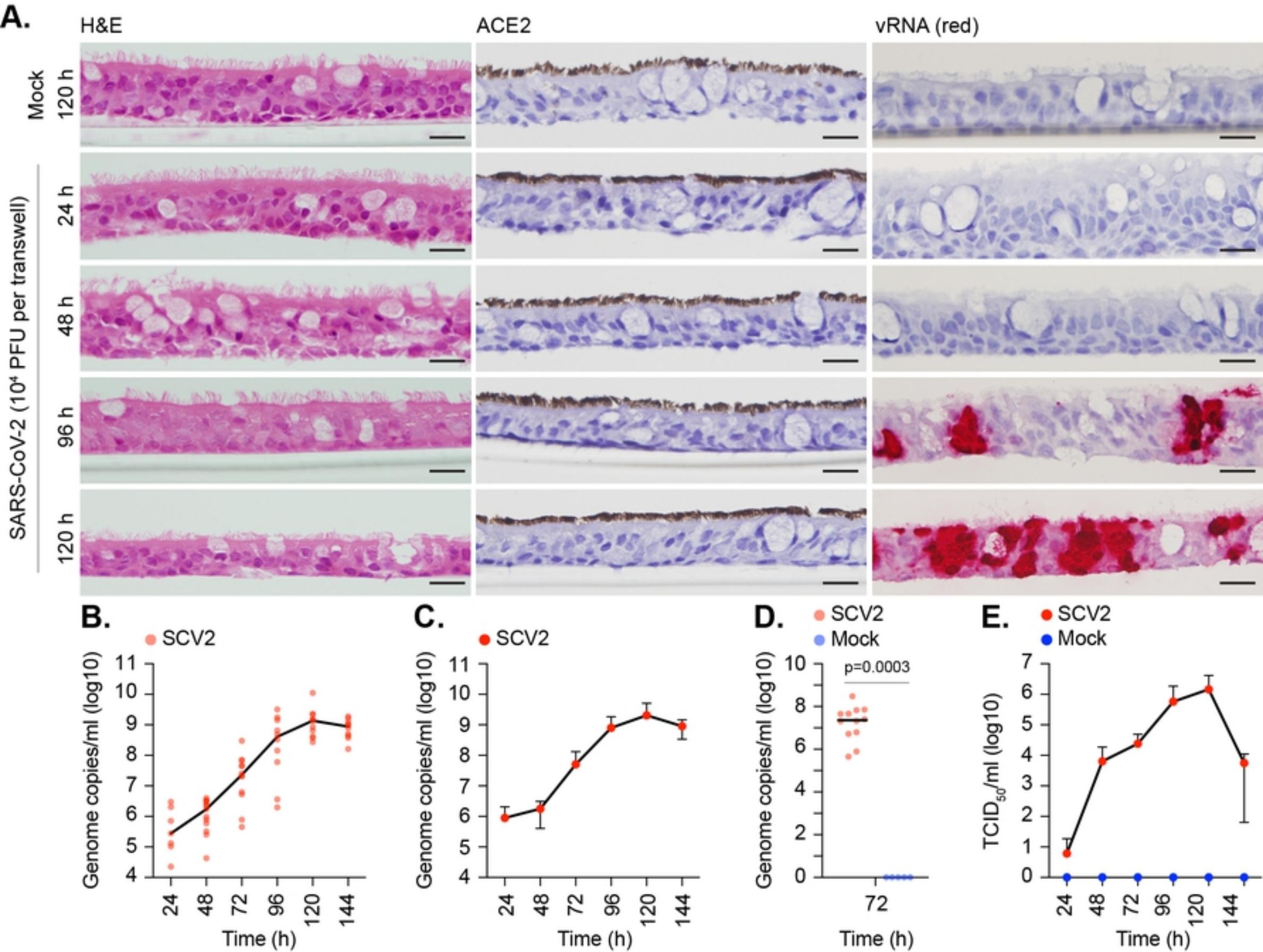


Fig 1

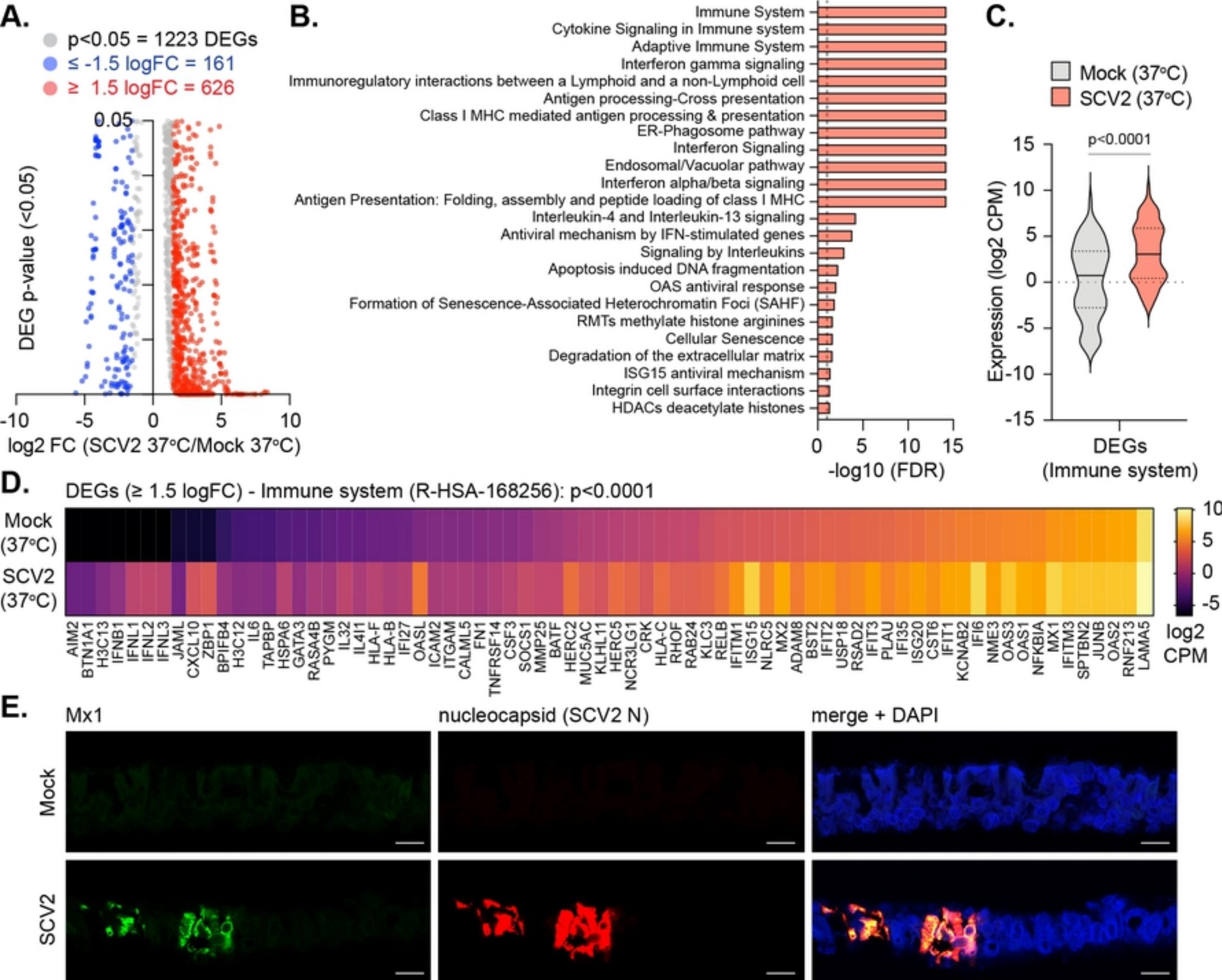


Fig 2

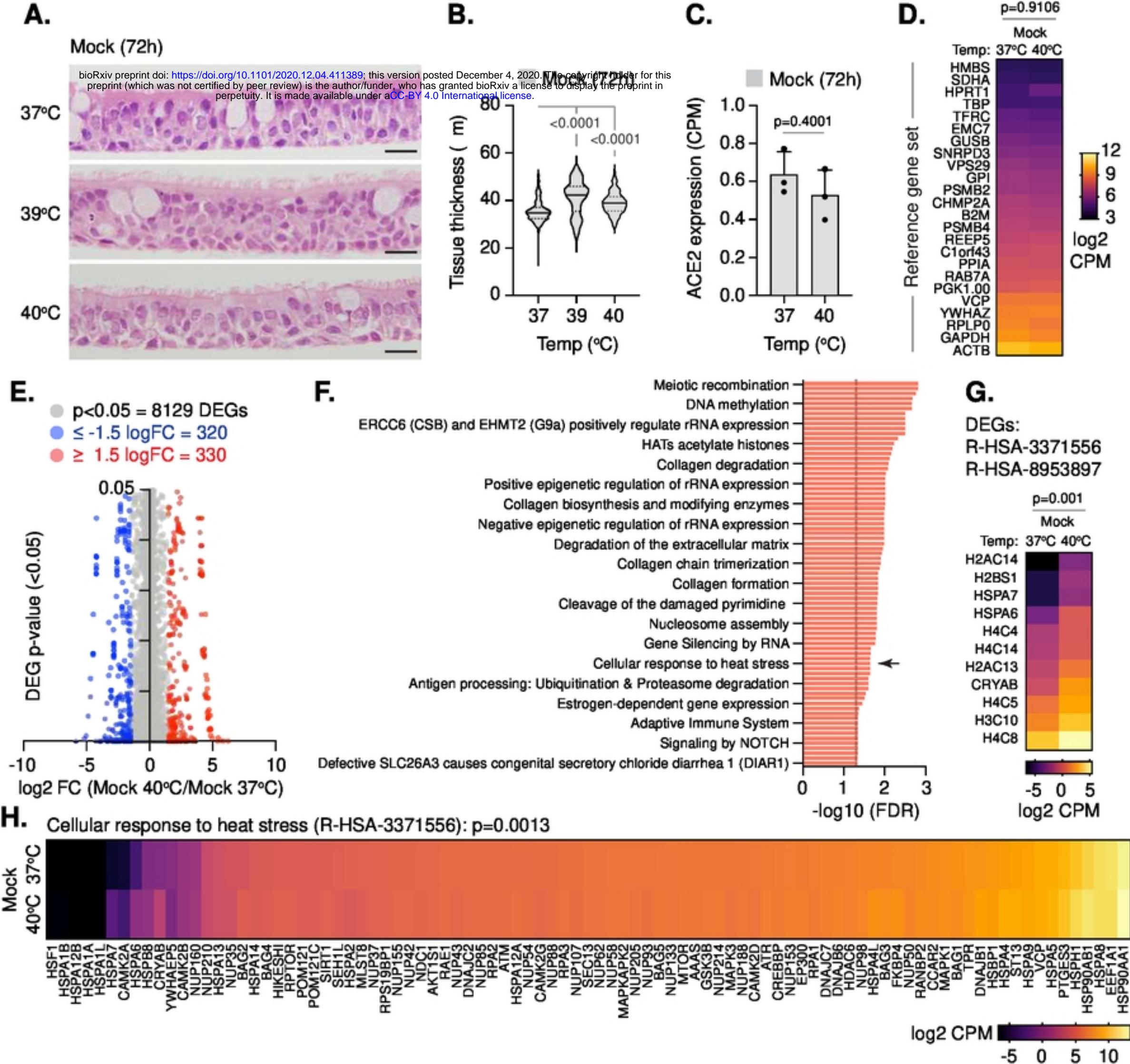
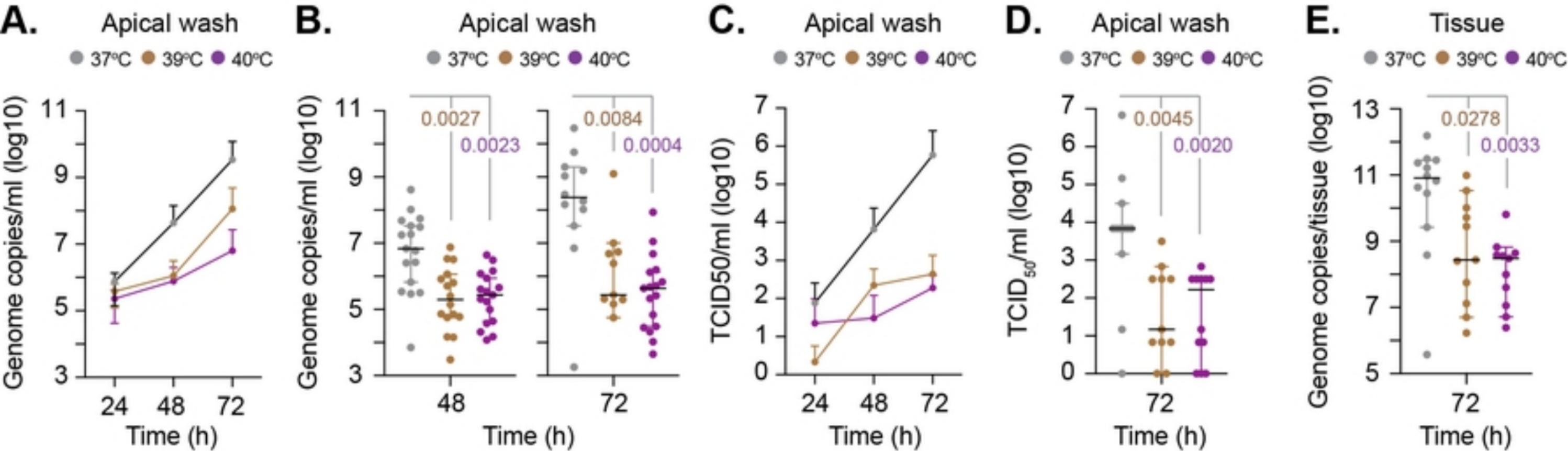


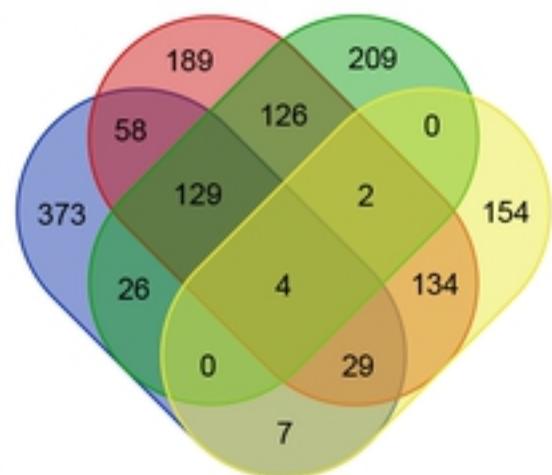
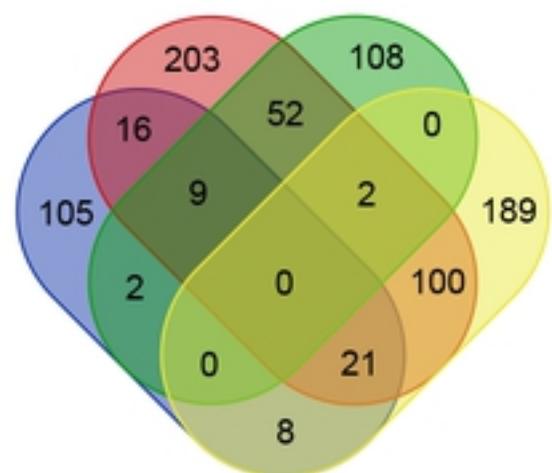
Fig 3



Manuscript

A.

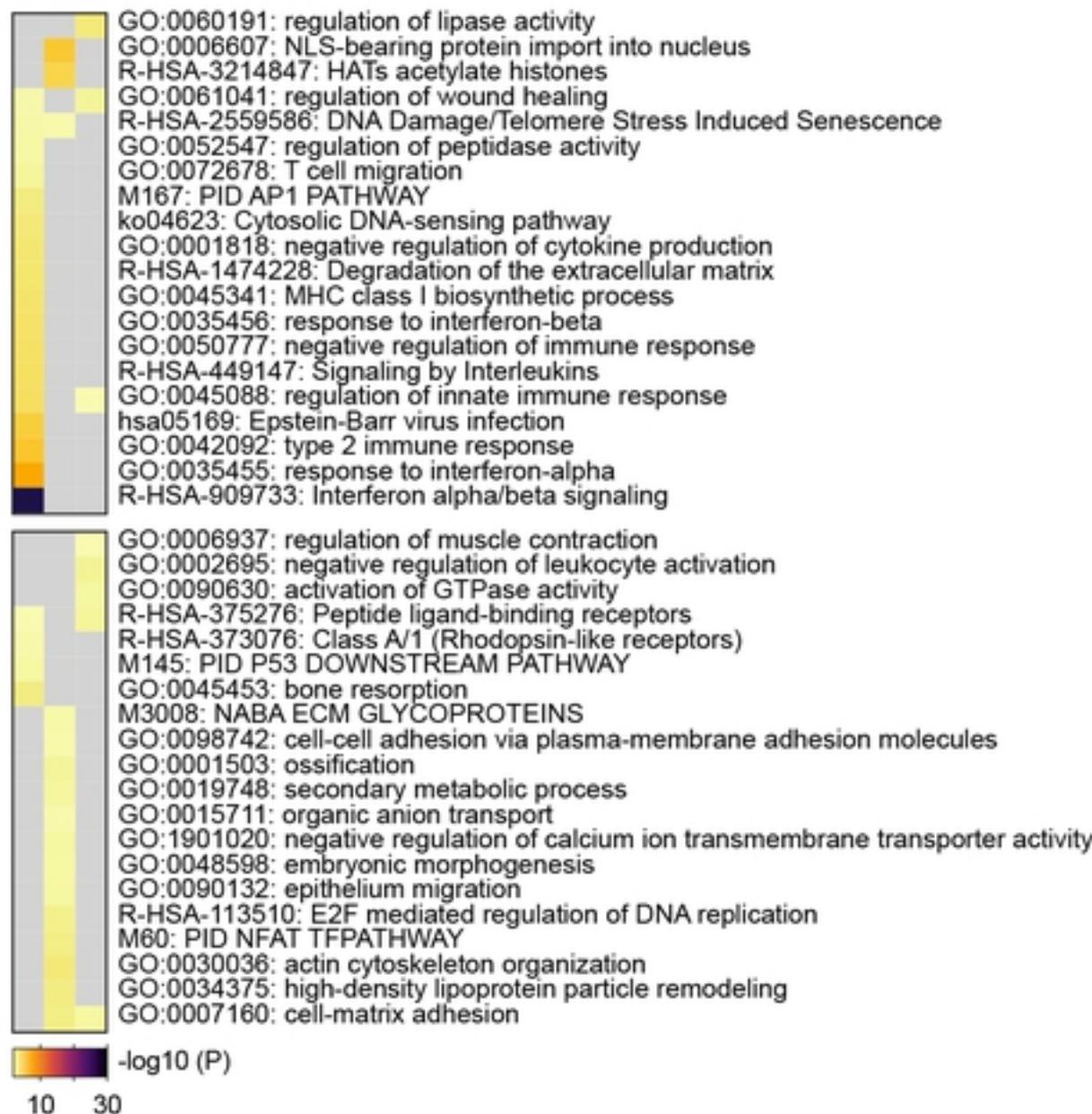
● SCV40/Mock40 ● SCV37/Mock37
● SCV40/Mock37 ● Mck40/Mock37

upregulated (FDR $\geq 1.5 \log_2$ FC)downregulated (FDR $\leq -1.5 \log_2$ FC)**B.**

● Unique gene ID
● Shared gene ID
— Shared pathway ontology

**C.**

● SCV37/Mock37
● SCV40/Mock37
● SCV40/Mock40

**Fig 5**

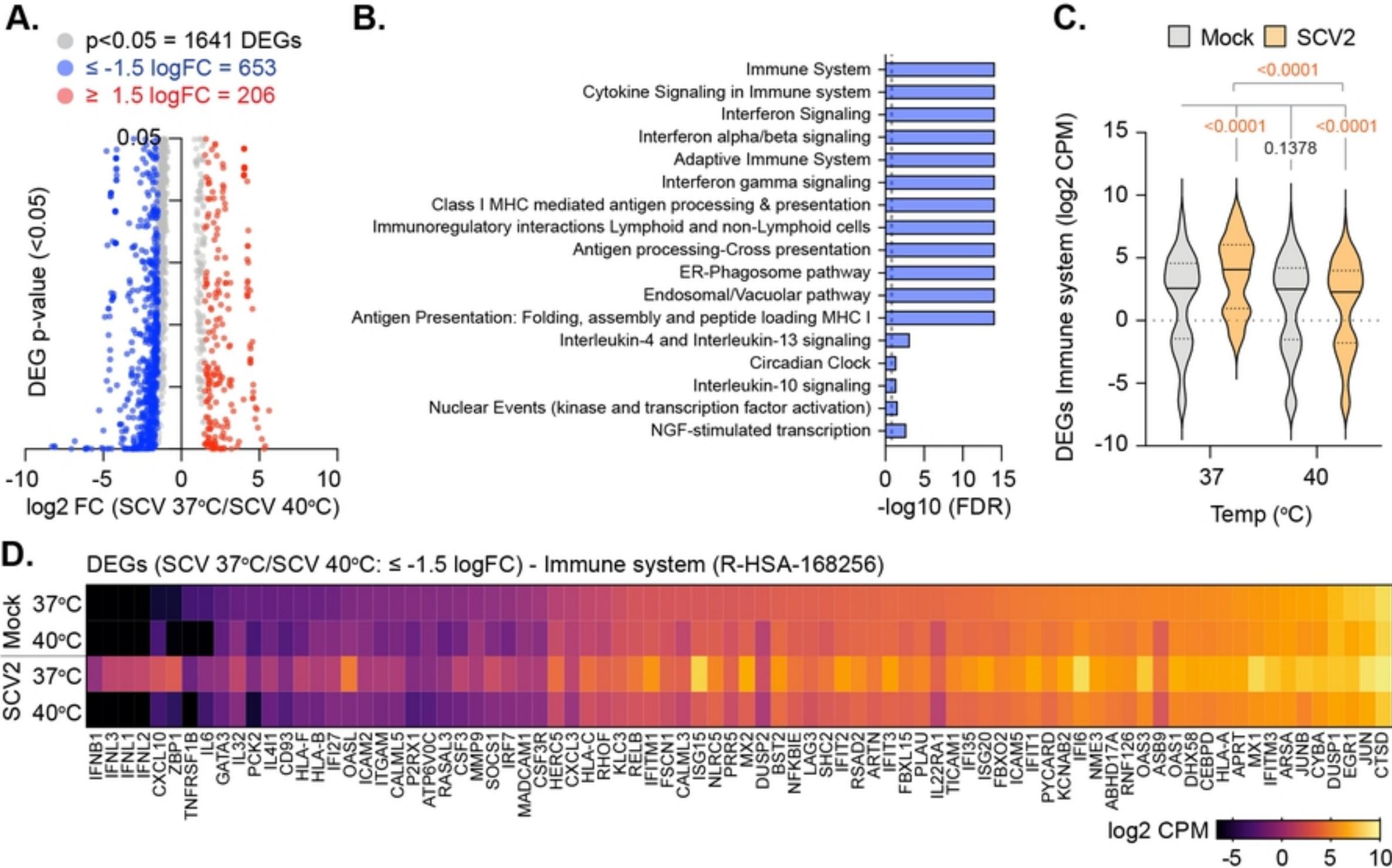


Fig 6

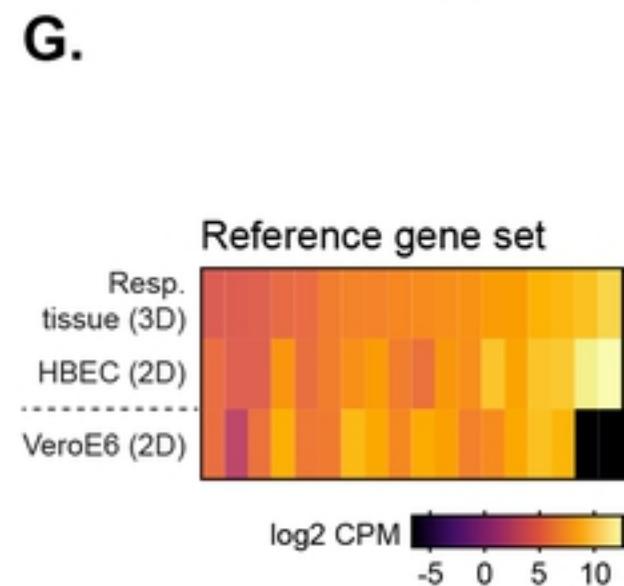
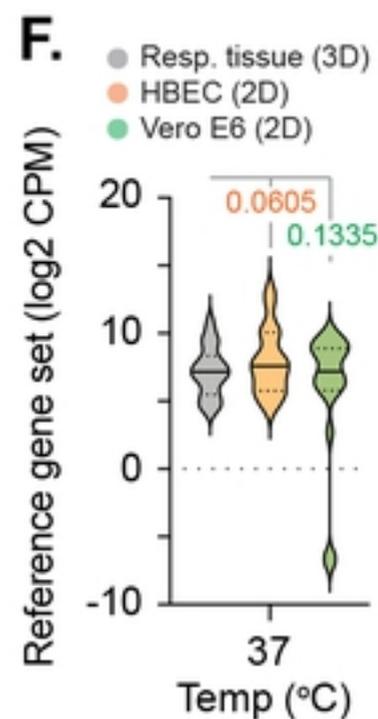
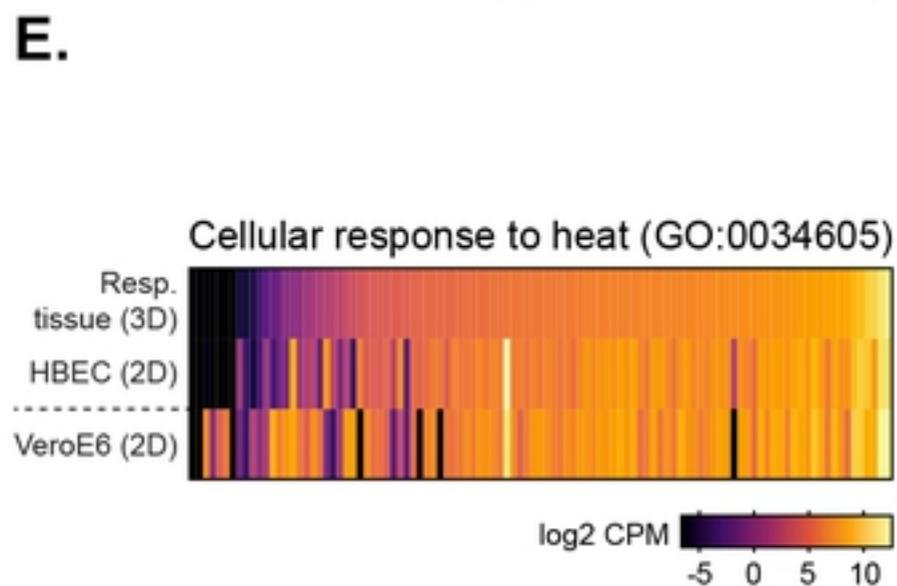
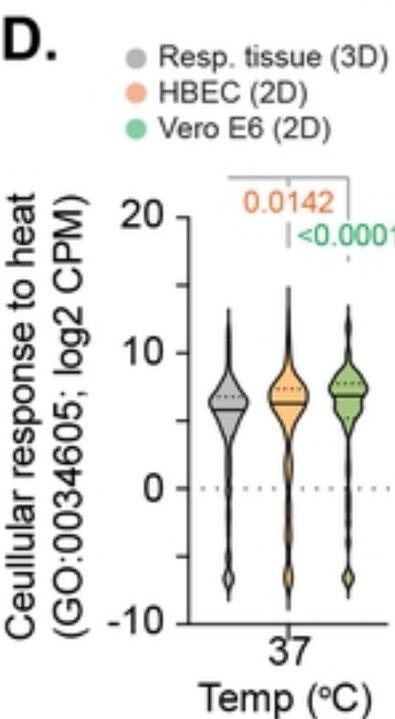
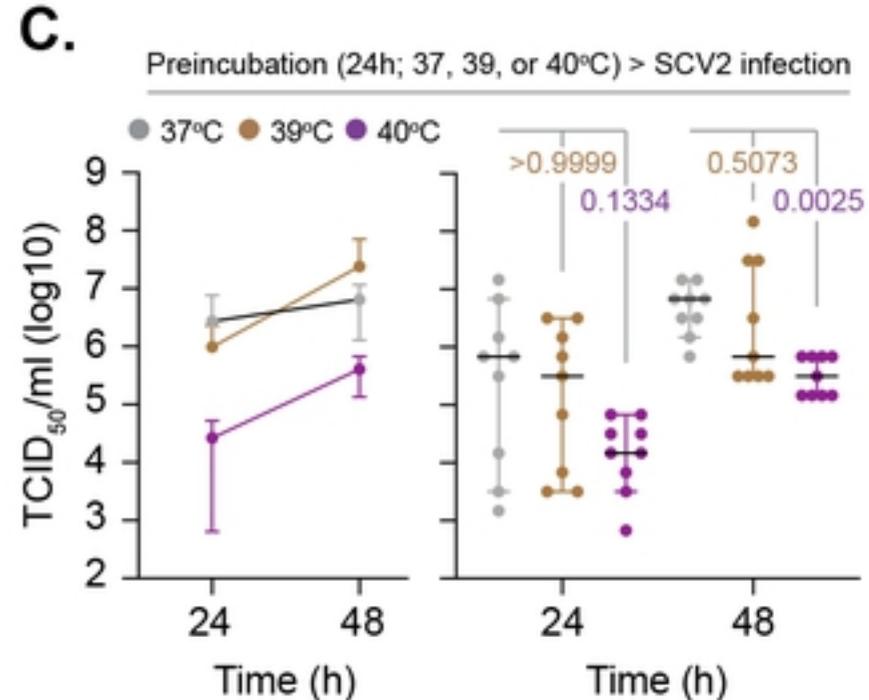
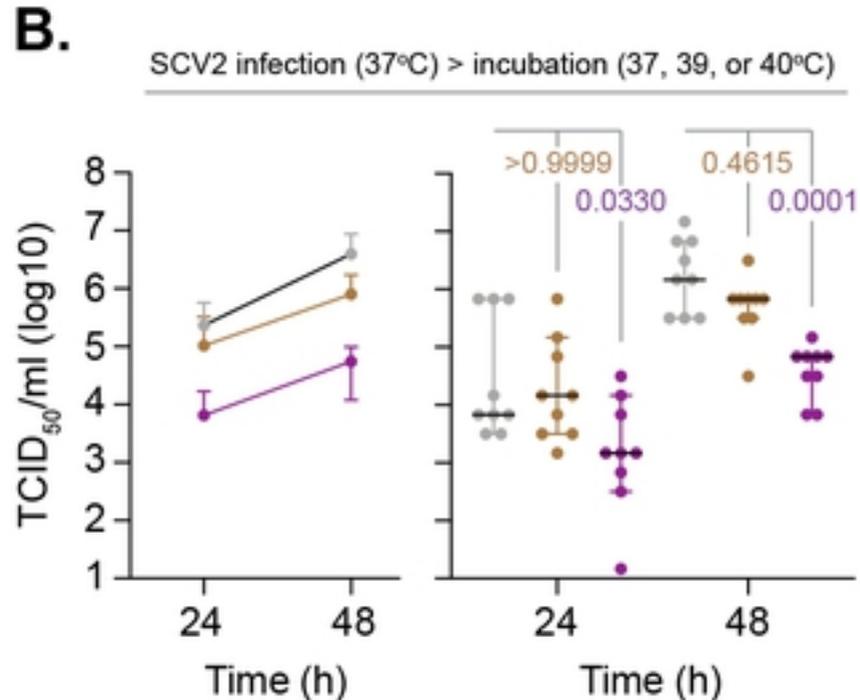
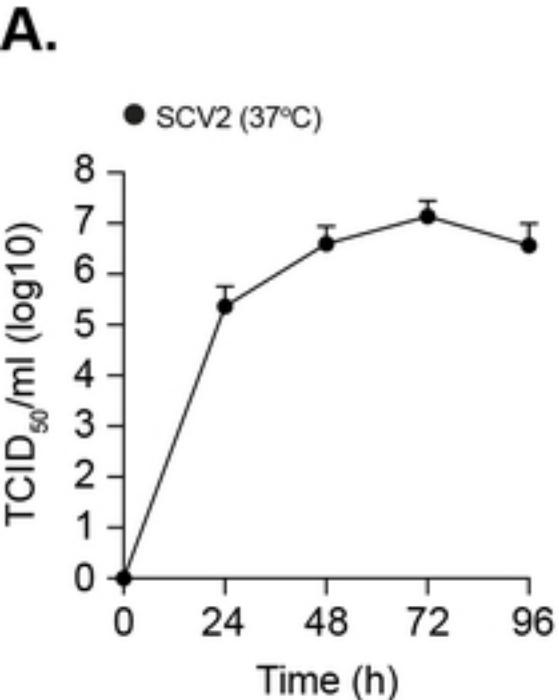


Fig 7

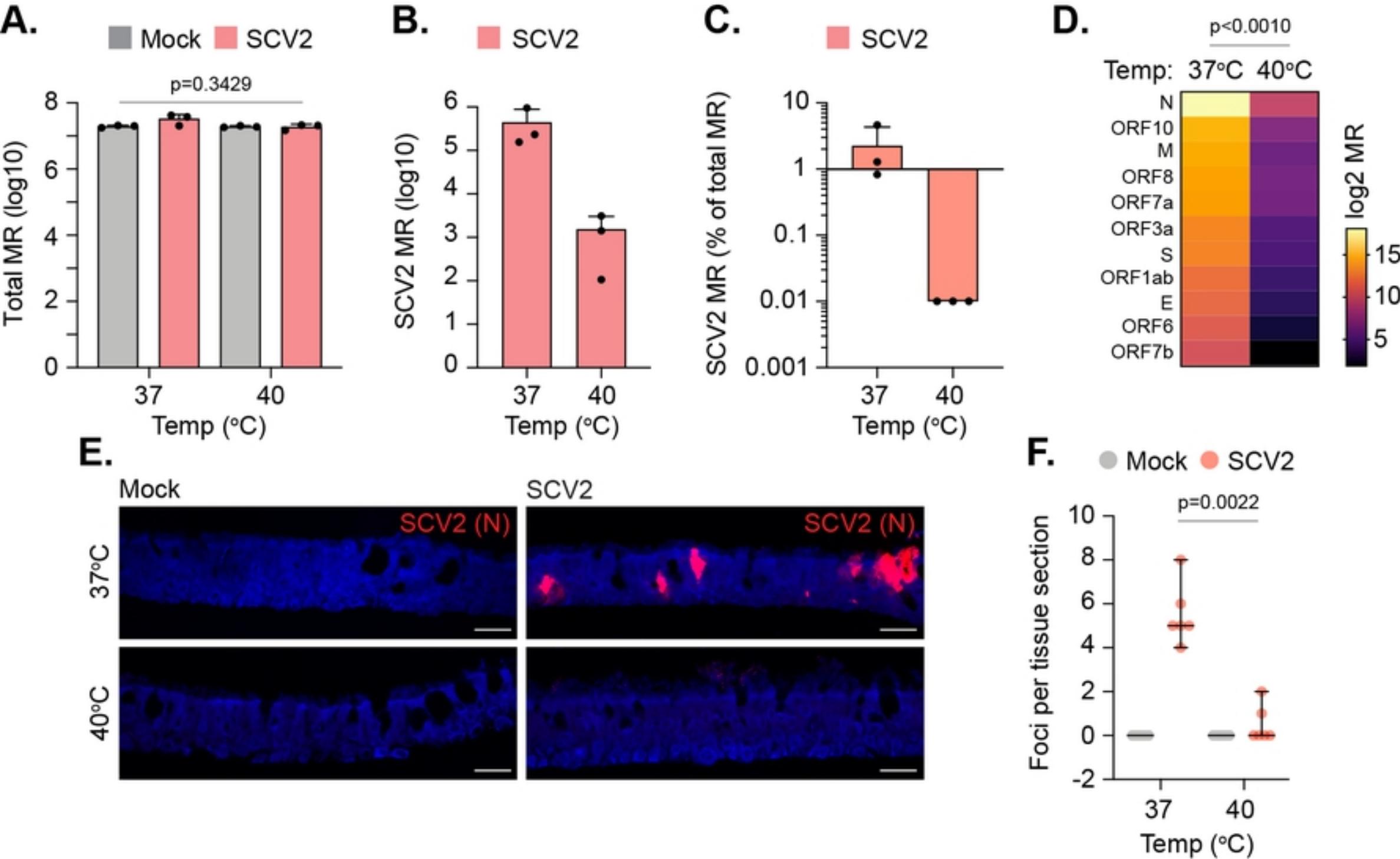


Fig 8