

1 ***Campylobacter jejuni* modulates reactive oxygen species production and NADPH**
2 **oxidase 1 expression in human intestinal epithelial cells**

3 Geunhye Hong¹, Cadi Davies¹, Zahra Omole¹, Janie Liaw¹, Anna D. Grabowska², Barbara
4 Canonico³, Nicolae Corcionivoschi⁴, Brendan Wren¹, Nick Dorrell^{1#}, Abdi Elmi^{1#†} and Ozan
5 Gundogdu^{1#†}

6 ¹ Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine,
7 Keppel Street, London, WC1E 7HT, UK.

8 ² Department of Biophysics and Human Physiology, Medical University of Warsaw, Warsaw,
9 02-091, Poland.

10 ³ Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, 61029, Italy.

11 ⁴ Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute
12 (AFBI), Belfast, BT9 5PX, UK.

13 † **Joint senior authorship**

14 # **Correspondence**

15 Nick Dorrell
16 Faculty of Infectious and Tropical Diseases,
17 London School of Hygiene & Tropical Medicine,
18 Keppel Street, London, WC1E 7HT, UK.
19 Email: nick.dorrell@lshtm.ac.uk

20
21 Abdi Elmi
22 Faculty of Infectious and Tropical Diseases,
23 London School of Hygiene & Tropical Medicine,
24 Keppel Street, London, WC1E 7HT, UK.
25 Email: abdi.elmi@lshtm.ac.uk

26
27 Ozan Gundogdu
28 Faculty of Infectious and Tropical Diseases,
29 London School of Hygiene & Tropical Medicine,
30 Keppel Street, London, WC1E 7HT, UK.
31 Email: ozan.gundogdu@lshtm.ac.uk

32

33

34 **Abstract**

35 *Campylobacter jejuni* is the major bacterial cause of foodborne gastroenteritis worldwide.
36 Mechanistically, how this pathogen interacts with intrinsic defence machinery of human intestinal
37 epithelial cells (IECs) remains elusive. To address this, we investigated how *C. jejuni* counteracts the
38 intracellular and extracellular reactive oxygen species (ROS) in IECs. Our work shows that *C. jejuni*
39 differentially regulates intracellular and extracellular ROS production in human T84 and Caco-2 cells.
40 *C. jejuni* downregulates the transcription and translation of Nicotinamide adenine dinucleotide
41 phosphate (NAPDH) oxidase (Nox1), a key ROS-generating enzyme in IECs and antioxidant defence
42 genes *cat* and *sod1*. Furthermore, inhibition of Nox1 by diphenylene iodonium (DPI) and siRNA
43 reduced *C. jejuni* ability to interact, invade and intracellularly survive within T84 and Caco-2 cells.
44 Collectively, these findings provide mechanistic insight into how *C. jejuni* modulates the IEC defence
45 machinery.

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62 **KEYWORDS**

63 *Campylobacter jejuni*, Intestinal epithelial cells, NADPH oxidase 1, Reactive oxygen species

64 Introduction

65 Microbial pathogens have evolved to possess subversion strategies to alter the functionality
66 of host cells upon infection (Escoll, Mondino, Rolando, & Buchrieser, 2016). These include
67 modulation of host cell functions that involve vesicle trafficking, apoptosis, and immune
68 activation (Asrat, de Jesús, Hempstead, Ramabhadran, & Isberg, 2014; Pedron et al., 2007;
69 Rudel, Kepp, & Kozjak-Pavlovic, 2010). Crucially, these host cell functions are essential for
70 elimination of foreign pathogens. The evolving battle between pathogen and host adds to the
71 complexity of the pathogenesis of infection (Escoll et al., 2016).

72 *Campylobacter jejuni* is the leading foodborne bacterial cause of human gastroenteritis
73 worldwide (Silva et al., 2011). *C. jejuni* causes watery or bloody diarrhoea, abdominal pain,
74 and fever. *C. jejuni* infection can also lead to Guillain-Barré Syndrome (GBS), a rare but severe
75 post-infectious autoimmune complication of the peripheral nervous system (Kaakoush,
76 Castaño-Rodríguez, Mitchell, & Man, 2015; Silva et al., 2011; Willison, Jacobs, & van Doorn,
77 2016). Importantly, Campylobacteriosis in low-income countries is associated with child
78 growth impairment and can be fatal in children (Amour et al., 2016). Although *C. jejuni* is a
79 microaerophilic bacterium, its omnipresence in the environment and various hosts is mitigated
80 by regulatory mechanisms against oxidative stress (Gundogdu et al., 2016). Upon adhering
81 and invading human intestinal epithelial cells (IECs), *C. jejuni* manipulates host cytoskeleton
82 regulation to maximise its invasion (Negretti et al., 2021). Following invasion, *C. jejuni* resides
83 in cytoplasmic vacuoles named Campylobacter containing vacuoles (CCVs) which can escape
84 the canonical endocytic pathway and avoid fusion with lysosomes (M. E. Konkel, Hayes,
85 Joens, & Cieplak Jr, 1992; Watson & Galán, 2008). These findings demonstrate that
86 modulation and invasion of host IECs are a prerequisite for human intestinal disease caused
87 by *C. jejuni*.

88 A vital mechanism used by host cells in response to pathogens is the production of reactive
89 oxygen species (ROS) which are highly reactive molecules, such as oxygen radicals and non-
90 radicals, produced by the partial reduction of oxygen (Aviello & Knaus, 2017). When
91 phagocytes such as macrophages detect and engulf pathogens using the respiratory burst,
92 ROS are rapidly generated to eradicate the engulfed pathogens through oxidative damage
93 (Paiva & Bozza, 2014). Interestingly, the level of ROS produced by human IECs is lower in
94 comparison to resident macrophages and blood leukocytes (neutrophils and monocytes),
95 however, ROS in IECs can also exhibit antimicrobial activity by inducing inflammation
96 (Burgueño et al., 2019; Holmström & Finkel, 2014; Paiva & Bozza, 2014). The precarious
97 nature of ROS production by IECs is demonstrated by exhibiting both deleterious and
98 beneficial host effects, thus homeostasis of ROS is essential. To counter the damaging effects

99 of ROS, host IECs possess antioxidant components that neutralise ROS, such as catalase,
100 superoxide dismutase and glutathione peroxidase. Nicotinamide adenine dinucleotide
101 phosphate oxidase (NADPH oxidase; Nox) and mitochondria have central roles as
102 predominant sources of ROS in human IECs (Aviello & Knaus, 2017).

103 Nox is an essential multicomponent enzyme which catalyses production of superoxide (O_2^-)
104 (Brandes, Weissmann, & Schröder, 2014; Sumimoto, Miyano, & Takeya, 2005). In IECs, the
105 most abundant types of Nox are Nox1 and Nox4. Intriguingly, Nox4 is constitutively active,
106 whereas Nox1 is not. The Nox1 complex is composed of Nox1, p22phox, Nox organiser 1
107 (NoxO1), Nox activator (NoxA1) and small GTPase Rac1. Nox1 is the catalytic subunit of the
108 complex on the plasma membrane and its activation is dependent on supplementary cytosolic
109 subunits. Following this, p22phox is transported to the plasma membrane promoted by Nox1
110 expression (Brandes et al., 2014). Upon activation, NoxO1 binds to both NoxA1 and p22phox
111 targeting NoxA1 to the plasma membrane. In turn NoxA1 binds to GTP (guanosine
112 triphosphate)-bound Rac1 and promotes electron flow through flavocytochrome in Nox1 in a
113 GTP-dependent manner. Studies have shown that GTP-bound Rac1 is essential for activity of
114 Nox1 (Nisimoto et al., 2008; Ueyama, Geiszt, & Leto, 2006). Electrons travel from NADPH
115 initially to flavin adenine dinucleotide (FAD), then through the Nox heme groups and finally to
116 oxygen, forming O_2^- (Nisimoto et al., 2008). Notably, Nox1-mediated ROS play important roles
117 in IECs including regulation of growth and proliferation, epithelial wound healing, intestinal
118 host defence, and maintenance of bacterial homeostasis in the GI tract (Juhász et al., 2017;
119 Lipinski et al., 2019; Matziouridou et al., 2018).

120 How *C. jejuni* interacts with the inherent defence machinery of human IECs remains unclear.
121 To explore this further, we examined the mechanisms *C. jejuni* uses to counteract the
122 intracellular and extracellular ROS in IECs. Previous findings demonstrated the upregulation
123 of Nox1 in IECs by enteric pathogens such as *Escherichia coli* (Elatrech et al., 2015),
124 *Salmonella* Enteritidis (Kawahara et al., 2016), and *Helicobacter pylori* (den Hartog et al.,
125 2016; Kawahara et al., 2005). However, given the invasion and survival properties of *C. jejuni*,
126 we hypothesised that *C. jejuni* may have distinct host cell modulation mechanisms in play. In
127 this study, we show that diverse *C. jejuni* strains downregulate both intracellular and
128 extracellular ROS production in human IECs by modulating the expression of Nox1. We
129 demonstrate inhibition of Nox1 by diphenylene iodonium (DPI) and siRNA reduced the ability
130 of *C. jejuni* to interact, invade and intracellularly survive within T84 and Caco-2 cells. Our
131 results highlight a unique strategy of *C. jejuni* survival and emphasise the importance of Nox1
132 in *C. jejuni*-IEC interactions. This represents a distinctive mechanism that *C. jejuni* uses to
133 modulate IEC defence machinery.

134

135 **Experimental procedures**

136 Bacterial strains and growth conditions

137 *C. jejuni* wild-type strains used in this study are listed in Table S1. For general growth, all *C.*
138 *jejuni* strains were grown on Columbia Blood Agar (CBA) plates (Oxoid, U.K) supplemented
139 with 7% (v/v) horse blood (TCS Microbiology, UK) and Campylobacter selective supplement
140 Skirrow (Oxoid) at 37°C under microaerobic conditions (10% CO₂, 5% O₂ and 85% N₂) (Don
141 Whitley Scientific, U.K).

142

143 Human intestinal epithelial cell culture

144 T84 cells (ECACC 88021101) and Caco-2 cells (ECACC 86010202) were obtained from
145 European Collection of Authenticated Cell Cultures (ECACC). T84 and Caco-2 cells were
146 cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium
147 (DMEM/F-12; Thermo Fisher Scientific, U.S.A) with 10% Fetal Bovine Serum (FBS; Labtech,
148 U.K), 1% non-essential amino acid (Sigma-Aldrich, U.S.A) and 1% penicillin-streptomycin
149 (Sigma-Aldrich). Both cell lines were cultured at 37°C in a 5% CO₂ humidified environment.
150 DMEM/F-12 without penicillin-streptomycin was used for the co-culture assays. DMEM/F-12
151 without phenol red was used for the ROS detection assays.

152

153 T84 and Caco-2 cells infection assays

154 Human IECs were counted using hemocytometer (Thermo Fisher Scientific, U.S.A) and for
155 general infection assays, approximately 10⁵ cells were seeded in 24-well tissue culture plates
156 7 days prior to initiation of the *C. jejuni* infection. The plates were incubated at 37°C in a 5%
157 CO₂ atmosphere. For Western blotting, approximately 2 x 10⁵ cells were seeded in 6-well
158 tissue culture plates. Prior to the infection, IECs were washed with phosphate-buffered saline
159 (PBS; Thermo Fisher Scientific) three times and the medium was replaced with DMEM/F-12
160 without penicillin-streptomycin. *C. jejuni* strains grown on CBA plates for 24 hours were
161 resuspended in PBS and bacterial suspension with appropriate OD₆₀₀ were then incubated
162 with IECs for various time periods giving a multiplicity of infection (MOI) of 200:1. In some
163 experiments, T84 and Caco-2 cells were pre-treated with 10 µM DPI for 1 hour, washed three
164 times with PBS and then infected with *C. jejuni*.

165

166 DCFDA measurement of intracellular reactive oxygen species (ROS)

167 To analyse the levels of intracellular ROS production in human IECs under experiments
168 conditions, DCFDA Cellular ROS Detection Assay Kit (Abcam U.K) was used according to the
169 manufacturer's instructions. Briefly, IECs grown in 96-well cell culture plates were washed
170 three times with PBS and incubated with *C. jejuni* for 3 or 24 hours (MOI 200:1). For positive
171 controls, IECs were treated with 500 μ M H₂O₂ for 45 minutes. 45 minutes prior to completion
172 of the infection, 100 μ M 2',7'-dichlorofluorescein diacetate (DCFDA) was added into each well
173 giving a final concentration of 50 μ M. After *C. jejuni* infection, the fluorescence was detected
174 using SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, U.S.A) with 485 nm
175 excitation and 535 nm emission.

176

177 Measurement of extracellular H₂O₂

178 Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, U.S.A) was used to
179 measure extracellular H₂O₂ in culture media after incubation with *C. jejuni*. Briefly, Amplex[®]
180 Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) with horseradish peroxidase (HRP) reacts
181 with H₂O₂ in a 1:1 stoichiometry producing a fluorescent product called resorufin. After
182 incubation with *C. jejuni* for 3 or 24 hours, 100 μ l of culture media was transferred to a 96-well
183 plate and 100 μ l of reaction mixture containing 50 μ M Amplex[®] Red reagent was added
184 followed by incubation for 10 minutes at 37°C under microaerobic condition. Using
185 SpectraMax M3 Multi-Mode Microplate Reader, fluorescence was measured at 530 nm
186 excitation and 590 nm emission.

187

188 Real time-quantitative polymerase chain reaction (qRT-PCR) analysis

189 For qRT-PCR, RNA was isolated from infected and uninfected IECs using PureLink[™] RNA
190 Mini Kit (Thermo Fisher Scientific) and contaminating DNA was removed using TURBO DNA-
191 free kit (Ambion, U.S.A) according to manufacturer's instructions. Concentration and purity of
192 RNA samples were determined in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher
193 Scientific). 400 ng of RNA per sample were first denatured at 65°C for 5 minutes and snap
194 cooled on ice. Complementary DNA (cDNA) was generated with random hexamers and
195 SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Each reaction had 10 μ l of
196 SYBR Green PCR Master Mix (Applied Biosystems, U.S.A), 1 μ l of primer (20 pmol), 1 μ l of
197 cDNA and 10 μ l of HyClone[™] water (Thermo Fisher Scientific). The sequence of each primer
198 is described in Table S2. All reactions were run in triplicate on an ABI-PRISM 7500 instrument
199 (Applied Biosystems) and expression levels of all target genes were normalised to *gapdh*
200 expression determined in the same sample. Relative expression changes were calculated

201 using the comparative threshold cycle (C_T) method (Pfaffl, 2001). A minimum of three
202 biological replicates were always analysed, each in technical triplicate.

203

204 Semi-quantitative reverse transcription (RT-PCR) analysis

205 Each PCR reaction had 50 μ l of FasTaq PCR master mix (Qiagen, Netherlands), 2 μ l of primer
206 (0.4 nmol) described in Table S2 and 1.5 μ l of cDNA. For PCR reactions Tetrad-2 Peltier
207 thermal cycler (Bio-Rad, U.K) was used. One cycle of PCR programme performs 95°C for 15
208 seconds after 2 minutes in the first cycle, annealing at 50°C for 20 seconds, and extension at
209 72°C for 30 seconds. Total 36 cycles were repeated. The PCR products were loaded on the
210 1% agarose gel and the gel was running for 1 hour at 120V. The gel was imaged using G:BOX
211 Chime XRQ (Syngene, U.S.A). Quantification of relative mRNA level was performed using
212 ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

213

214 SDS-PAGE and Western blot analysis

215 After infection, IECs were washed three times with PBS and lysed with cold RIPA lysis and
216 extraction buffer (Thermo Fisher Scientific) with cOmplete™ Mini EDTA-free Protease
217 Inhibitor Cocktail (Roche, Switzerland) and cleared by centrifugation (4 °C, 13,000 \times g, 20
218 min). Protein concentration was determined using Pierce™ Bicinchoninic acid (BCA) Protein
219 Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Afterward,
220 samples were diluted to a desired concentration in HyClone™ water and 4X Laemmli sample
221 buffer (Sigma-Aldrich) and incubated for 5 minutes at 95°C. Equal amounts of protein samples
222 were separated using 4-12% NuPAGE™ Bis-Tris gel in 1 \times NuPAGE™ MES buffer or MOPS
223 buffer (Thermo Fisher Scientific). Proteins were transferred from the gel using the iBlot® 2
224 transfer stacks (Life Technologies, U.S.A) using the iBlot® Gel Transfer Device (Invitrogen).
225 These stacks were integrated with nitrocellulose transfer membrane. After the transfer,
226 membranes were blocked with 1X PBS containing 2% (w/v) milk. Membranes were then
227 probed with primary antibodies overnight as described previously (Elmi et al., 2016). The
228 following primary antibodies were used; GAPDH (ab181602; Abcam); Nox1 (ab101027;
229 Abcam) or Nox1 (NBP-31546; Novus Biologicals). Blots were developed using LI-COR
230 infrared secondary antibody (IRDye 800CW Donkey anti-rabbit IgG) and imaged on a LI-COR
231 Odyssey Classic (LI-COR Biosciences, U.S.A). Quantification of relative protein levels were
232 performed using ImageJ software (Schneider et al., 2012).

233

234 Detection of GTP-bound Active Rac1

235 The levels of active GTP-bound Rac1 were measured by using Rac1 G-LISA kit (Cytoskeleton
236 Inc., U.S.A) according to the manufacturer's instructions. Briefly, before the infection, IECs
237 were incubated with reduced serum (0.1% FBS) for 24 hours. Infected or uninfected human
238 IECs were washed with 1X PBS and lysed using the supplied 1X Lysis Buffer. Cell lysates
239 were centrifuged for 1 minute at 10,000 x g at 4°C and adjusted to 1 mg/ml for the further
240 process of the assay. As a positive control, constitutively active Rac1 (RCCA) was provided
241 in the kit. Three biological replicates were conducted in all experiments, along with two
242 technical replicates for each assay.

243

244 Inhibition of Nox1 with diphenyleneiodonium chloride (DPI)

245 A stock solution of 3.25 mM DPI (Sigma-Aldrich) in dimethyl sulfoxide (DMSO; Sigma Aldrich)
246 was prepared and stored at -20°C. For treatment, the DPI stock solution was diluted to 10 µM
247 DPI in culture media without antibiotics, then incubated with IECs for 1 hour at 37°C in a 5%
248 CO₂ atmosphere. After treatment, IECs were washed with PBS for three times before co-
249 incubation with *C. jejuni* for various time points.

250

251 Small interfering (si) RNA transfection

252 On the day of reverse transfection, 500 µl of Caco-2 cells (10⁵ cells/ml) were seeded in 24-
253 well plates and treated for 24 hours with 30 pmol siRNA from either Nox1 siRNA (sc-43939;
254 Santa Cruz Biotechnology, Inc, U.S.A) or Ambion® Silencer Negative Control #1 siRNA
255 (Invitrogen) for the negative control. For preparation of siRNA transfection reagent complex,
256 3 µl of 10 µM stock siRNA was diluted with 100 µl of Opti-MEM® Reduced-Serum Medium
257 (Thermo Fisher Scientific) and mixed with 1.5 µl of Lipofectamine® RNAiMAX Transfection
258 Reagent (Thermo Fisher Scientific). After 24 hours transfection, media was replaced with
259 DMEM/F-12 containing 10% FBS. After additional 48 or 72 hours, RNA and protein were
260 extracted to check efficacy of transfection.

261

262 Adhesion, invasion and intracellular survival assay

263 Adherence, invasion and intracellular assays were performed as described previously with
264 minor modifications (Gundogdu et al., 2011). T84 and Caco-2 cells seeded in a 24-well plate
265 were washed three times with PBS and treated with 10 µM DPI for 1 hour or transfected with

266 Nox1 siRNA as described in above. Then IECs were inoculated with *C. jejuni* with OD₆₀₀ 0.2
267 at a MOI of 200:1 and incubated for 3 hours at 37°C in 5% CO₂. For the interaction (adhesion
268 and invasion) assay, monolayers were washed three times with PBS to remove unbound
269 extracellular bacteria and then lysed with PBS containing 0.1% (v/v) Triton X-100 (Sigma-
270 Aldrich) for 20 min at room temperature. The cell lysates were diluted and plated on blood
271 agar plates to determine the number of interacting bacteria (CFU/ml).

272 Invasion assays were performed by additional step of treatment of gentamicin (150 µg/ml) for
273 2 hours to kill extracellular bacteria, washed three times with PBS, lysed and plated as
274 described above. For intracellular survival assays, after infection with *C. jejuni* for 3 hours, T84
275 and Caco-2 cells were treated with gentamicin (150 µg/ml) for 2 hours to kill extracellular
276 bacteria followed by further 18 hours incubation with gentamicin (10 µg/ml). Cell lysis and
277 inoculation were performed as described above.

278

279 Cytotoxicity assay with trypan blue exclusion methods

280 After treatment with DPI and gentamicin or transfection with siRNA as previously described,
281 IECs were washed three times with PBS and were detached using trypsin-EDTA (Thermo
282 Fisher Scientific) and resuspended with culture media. 50 µl of cell suspension were added
283 into 50 µl of 0.4% Trypan Blue solution (Thermo Fisher Scientific) and the numbers of viable
284 and dead cells were counted using hemocytometer under microscope.

285

286 *Campylobacter jejuni* viability test with DPI treatment

287 T84 cells were treated with 10 µM DPI for 1 hour and the cells were washed three times with
288 PBS. After DPI treatment for 1 hour, *C. jejuni* strains (OD₆₀₀ 0.2) were co-incubated for 1 hour
289 with PBS from the last wash. After incubation, serial dilution was performed and each dilution
290 was spotted on to blood agar plates. The plates were incubated under microaerobic condition
291 at 37°C for 48 hours. CFU of each spot was recorded.

292

293 Statistical analysis and graphing

294 At least three biological replicates were performed in all experiments. Each biological replicate
295 was performed in three technical replicates. For statistical analysis and graphing, GraphPad
296 Prism 8 for Windows (GraphPad Software, U.S.A) was used. One sample *t*-test or unpaired *t*-

297 test were used to compare two data sets for significance with * indicating $p < 0.05$, ** indicating
298 $p < 0.01$, *** indicating $p < 0.001$, and **** indicating $p < 0.0001$.

299

300 **Results**

301 ***Campylobacter jejuni* modulates intracellular and extracellular ROS in T84 and Caco-2**
302 **cells in a time- and strain-dependent manner.**

303 As *C. jejuni* possesses distinct physiological characteristics compared to more studied enteric
304 pathogens, we assessed the ability of three distinct *C. jejuni* strains to modulate intracellular
305 and extracellular ROS in T84 and Caco-2 cells (Burnham & Hendrixson, 2018). We observed
306 strain-specific ROS modulation at 3- and 24-hours post-infection (Figure 1). All three *C. jejuni*
307 strains reduced the levels of intracellular ROS in T84 and Caco-2 cells compared to the
308 uninfected control (Figure 1A, 1B, 1E, 1F). A similar pattern was observed for extracellular
309 ROS where all *C. jejuni* strains reduced the levels of extracellular ROS in T84 and Caco-2
310 cells compared to the uninfected control (Figure 1C, 1D, 1G, 1H). A distinct pattern was
311 observed when assessing levels of extracellular ROS for *C. jejuni* 81-176 strain at 3 hours
312 post-infection (Figure 1C and 1G). At this early time point, extracellular ROS is increased in
313 T84 and Caco-2 cells infected with *C. jejuni* 81-176, although we observed similar reduced
314 levels of ROS at 24 hours. These results indicate a strain and time-specific pattern linking the
315 ability of different *C. jejuni* strains to modulate intracellular and extracellular ROS levels in T84
316 and Caco-2 cells.

317

318 ***Campylobacter jejuni* modulates intracellular and extracellular ROS in T84 and Caco-2**
319 **cells via the downregulation of Nox1 complex.**

320 Given the observed modulation of intracellular and extracellular ROS in T84 and Caco-2 cells,
321 we next explored the mechanism by which *C. jejuni* strains orchestrate ROS modulation. We
322 analysed the transcription and translation of Nox1 which is the main source of ROS production
323 in IECs (Brandes et al., 2014; Sumimoto et al., 2005). As shown in Figure 2, Nox1 transcription
324 and translation levels were significantly reduced in both T84 (Figure 2A) and Caco-2 cells
325 (Figure 2B) infected with *C. jejuni* when compared to uninfected cells. Notably, at 24 hours
326 post-infection, mRNA levels of Nox1 in T84 cells are significantly reduced compared with *C.*
327 *jejuni*-infected Caco-2 cells. We measured the relative levels of mRNA between T84 and
328 Caco-2 cells and identified T84 cells expressed a higher basal level of Nox1 mRNA compared
329 to Caco-2 cells (Figure 2C). As a result of this higher basal level of Nox1 mRNA in T84 cells
330 we validated our qRT-PCR data using RT-PCR where less expression of Nox1 in *C. jejuni*-
331 infected T84 cells was observed (Figure 2D and 2E). Reduction in the translational level of
332 Nox1 in *C. jejuni*-infected T84 cells was confirmed independently by Western blotting (Figure
333 2F and 2G).

334

335 ***Campylobacter jejuni* modulates activity of small GTPase Rac1 in T84 and Caco-2 cells**
336 **in a time-dependent manner.**

337 To gain further insight into the mechanism that leads to *C. jejuni* modulation of ROS in T84
338 and Caco-2 cells, we examined the ability of *C. jejuni* to activate Rac1, a member of the Rho
339 family of small GTPases. Although Rac1 is implicated in Nox1 activation in several eukaryotic
340 cell lines (Nisimoto et al., 2008; Ueyama et al., 2006), the contribution of Rac1 in *C. jejuni*-
341 mediated Nox1 modulation is unknown. As shown in Figure 3, Rac1 is an integral part of the
342 Nox1 complex. Given that *C. jejuni* activates Rac1 in human INT 407 cells via *Campylobacter*
343 invasion antigen D (CiaD) (Krause-Gruszczynska et al., 2007; Negretti et al., 2021), and that
344 Rac1 supports Nox1 activity only in its GTP-bound active form, we examined if downregulation
345 of Nox1 is linked to GTPase Rac1 by *C. jejuni*. Interestingly, *C. jejuni* 11168H strain induced
346 Rac1 1- and 3-hours after infection in T84 cells (Figure 4A). After 24 hours infection, Rac1
347 activity was reduced (though not statistically significant; $p = 0.0714$) (Figure 4A). Similarly, *C.*
348 *jejuni* 11168H induced Rac1 activity after 1 hour infection in Caco-2 cells (Figure 4B). However,
349 this activity was reduced after 3- and 24-hours infection (Figure 4B). These results suggest
350 that the downregulation of Nox1 by *C. jejuni* is inversely correlated with an increase in Rac1
351 GTPase activity.

352

353 ***Campylobacter jejuni* modulates transcription of antioxidant-related genes in T84 and**
354 **Caco-2 cells**

355 To gain further insight into the ability of *C. jejuni* to modulate intracellular and extracellular
356 ROS in T84 and Caco-2 cells, we sought to understand if *C. jejuni* modulates the expression
357 of two important antioxidant genes, superoxide dismutase 1 (*sod1*) and catalase (*cat*). Sod1
358 decomposes O_2^- to H_2O_2 , and Cat breaks down H_2O_2 to H_2O and O_2 (Aviello & Knaus, 2017).
359 Intriguingly, as shown in Figure 5A and 5B, there is a significant downregulation of the mRNA
360 levels of *cat* and *sod1* at 24 hours post-infection in T84 cells. A similar pattern was observed
361 when compared with Caco-2 cells where the expression of *cat* and *sod1* at 24 hours post-
362 infection is significantly downregulated (Figure 5C and 5D). In contrast, the expression of *cat*
363 and *sod1* at 3 hours post-infection is unaffected. These results may indicate *C. jejuni*-mediated
364 reduction in intracellular and extracellular ROS is independent of modulation of *cat* and *sod1*.

365

366 **Chemical inhibition of Nox1 activity by DPI impairs *Campylobacter jejuni* interaction,**
367 **invasion and intracellular survival of T84 and Caco-2 cells *in vitro*.**

368 Having established that *C. jejuni* significantly reduced the transcription and translation of Nox1
369 in T84 and Caco-2 cells in a time-dependent manner, and that Rac1 is not only known as a
370 key component of the Nox1 complex, but also implicated in cell dynamic morphology (Nisimoto
371 et al., 2008; Ueyama et al., 2006), we hypothesised Rac1-mediated Nox1 might modulate
372 membrane ruffling and cytoskeleton rearrangement which might in turn affect *C. jejuni*
373 interaction with IECs. Therefore, we investigated the role of Nox1 in *C. jejuni* interaction,
374 invasion and intracellular survival in IECs by transiently pre-treating T84 and Caco-2 cells with
375 DPI (10 μ M) which is known to inhibit activity of flavoenzymes including Nox complex (Riganti
376 et al., 2004). First, we demonstrated that DPI reduced extracellular ROS in T84 and Caco-2
377 cells (Figure S1). As shown in Figure 6A, 6C, 6E, pre-treatment of T84 cells by DPI significantly
378 reduced the ability of *C. jejuni* to interact, invade, and survive intracellularly in T84 cells.
379 Similarly, as shown in Figure 6B, 6D and 6F, *C. jejuni* infected with DPI-treated Caco-2 cells
380 showed significant reduction in interaction, invasion, and intracellular survival compared to
381 untreated Caco-2 cells. Since our data revealed *C. jejuni* reduced interaction, invasion and
382 intracellular survival between the control and DPI-treated T84 and Caco-2 cells, we next
383 evaluated the viability of *C. jejuni*, T84 and Caco-2 cells co-incubated with DPI. Treatment with
384 DPI did not affect viability of IECs (Figure S2) or *C. jejuni* (Figure S3). Thus, our observations
385 suggest further inhibition of Nox1 with DPI is detrimental to *C. jejuni* interaction, invasion and
386 intracellular survival in IECs.

387

388 **Nox1 silencing by siRNA impairs *Campylobacter jejuni* interaction, invasion and** 389 **intracellular survival in Caco-2 cells *in vitro*.**

390 As DPI is a pan-Nox inhibitor, we silenced Nox1 expression in Caco-2 cells by delivering
391 specific small interfering RNA (siRNA) into cultured Caco-2 cells. We used siRNA sequence
392 which target regions of Nox1 for silencing. As a negative control, we used a non-targeting
393 scrambled RNA sequence which is not complementary to the Nox1 mRNA. As shown in Figure
394 7A and 7B, transcriptional and translational levels of Nox1 were significantly decreased in cells
395 treated with Nox1 siRNA, relative to that in mock-treated Caco-2 controls. We further
396 confirmed reduced activity of Nox1 by demonstrating significant reduction in extracellular ROS
397 (Figure 7C). We showed that Nox1 siRNA transfection did not affect viability of Caco-2 cells
398 (Figure S4). Based on these results, we further investigated interaction, invasion and
399 intracellular survival of *C. jejuni* within Caco-2 cells (Figure 7D, 7E and 7F). Our result showed
400 significant decrease in *C. jejuni* interaction, invasion and intracellular survival when compared
401 to non-transfected controls. This result highlights a correlation between reduced Nox1

402 expression with a reduction in *C. jejuni* infection. Taken together, our results demonstrate that
403 Nox1 is a critical host factor for *C. jejuni* interaction, invasion, and intracellular survival.

404

405 **Discussion**

406 Upon infection, host cells induce a range of cellular responses to remove offending pathogens.
407 However, bacterial pathogens often target host organelle(s), signalling pathway(s) or immune
408 responses to evade host defence mechanisms (Escoll et al., 2016). Disruption of ROS
409 production in host cells by bacterial pathogens has been previously reported (Gallois, Klein,
410 Allen, Jones, & Nauseef, 2001; Vareechon, Zmina, Karmakar, Pearlman, & Rietsch, 2017). *S.*
411 Typhimurium pathogenicity island-2 encoding Type III Secretion System (T3SS) inhibits ROS
412 production in human macrophages by preventing Nox2 assembly (Antoniou et al., 2018;
413 Gallois et al., 2001). In addition, *Pseudomonas aeruginosa* T3SS effector, ExoS disrupts ROS
414 production in human neutrophils by ADP-ribosylating Ras and inhibiting its activity which is
415 essential for Nox2 assembly (Vareechon et al., 2017).

416

417 We have characterised the ability of distinct *C. jejuni* strains to modulate intracellular and
418 extracellular ROS from human IECs *in vitro*. ROS production by human IECs is a major
419 defence mechanism, yet how *C. jejuni* evades ROS remains unclear. Our work establishes
420 that in contrast to other enteric pathogens, *C. jejuni* uses a different mechanism involving
421 downregulation of Nox1 expression to modulate ROS in human IECs (den Hartog et al., 2016;
422 Elatrech et al., 2015; Kawahara et al., 2005; Kawahara et al., 2016). We examined three
423 different *C. jejuni* strains using two different human IECs and showed that *C. jejuni* strains
424 modulate intracellular and extracellular ROS from human IECs via the differential regulation
425 of the transcription and translation of Nox1 which is a major ROS source in IECs (Aviello &
426 Knaus, 2017). Interestingly, a previous study demonstrated that *C. jejuni* 81-176 induces
427 extracellular ROS production through Nox1 activation in human ileocecal adenocarcinoma
428 derived HCT-8 cells (Corcionivoschi et al., 2012). To further understand the implications of *C.*
429 *jejuni* transcriptional and translational downregulation of Nox1 in T84 and Caco-2 cells, we
430 revealed similarities with some other enteropathogens, and also differences amongst others
431 including the *C. jejuni* strain 81-176 (den Hartog et al., 2016; Elatrech et al., 2015; Kawahara
432 et al., 2005; Kawahara et al., 2016). Enteropathogens such as *E. coli*, *Salmonella* spp., and
433 *H. pylori* upregulate expression of Nox1 and ROS production in infected IECs (den Hartog et
434 al., 2016; Elatrech et al., 2015; Kawahara et al., 2005; Kawahara et al., 2016). Our findings
435 confirmed downregulation of ROS production by *C. jejuni* is strain dependent. In contrast to
436 *C. jejuni* 11168H and 488 strains, *C. jejuni* 81-176 induced extracellular ROS in T84 and Caco-
437 2 cells at 3 hours post-infection. Induction of extracellular ROS by *C. jejuni* 81-176 at this

438 earlier infection time point was also observed previously (Corcionivoschi et al., 2012). We
439 hypothesise *C. jejuni* 81-176 might have additional bacterial determinants which may induce
440 host extracellular ROS independent of Nox1 modulation (e.g. the pVir and pTet plasmids
441 which encode putative Type IV Secretion Systems (T4SS)) (Bacon et al., 2002; Batchelor,
442 Pearson, Friis, Guerry, & Wells, 2004). We also noted a difference between the ability of *C.*
443 *jejuni* strains to regulate expression of Nox1 in T84 and Caco-2 cells. This difference could be
444 due to variations between the two cell lines. Caco-2 cells possess characteristic enterocytes
445 whereas T84 cells possess characteristic colonocytes throughout differentiation (Devriese et
446 al., 2017). In addition, previous studies have shown that reduced Nox1 mRNA was present in
447 the ileum than in the colon of healthy patients suggesting there is a gradient in Nox1
448 expression from small intestine to large intestine (Schwerd et al., 2018). In our study, the lower
449 expression of Nox1 mRNA detected in Caco-2 cells compared to T84 cells was also observed.

450

451 As ROS homeostasis in the GI tract is regulated by multiple antioxidant enzymes (Aviello &
452 Knaus, 2017), *C. jejuni*-mediated modulation of Cat and Sod1 at the transcriptional level was
453 investigated. Our data demonstrated *C. jejuni* strains did not affect transcriptional levels of *cat*
454 and *sod1* in T84 and Caco-2 cells after 3 hours infection, but they significantly downregulated
455 expression of both genes after 24 hours. To our knowledge, this is the first data on *C. jejuni*
456 modulation of antioxidant-related genes in human IECs *in vitro*. Our observations imply *C.*
457 *jejuni* might modulate intracellular or extracellular ROS after 3 hours infection without
458 modulating expression of *cat* and *sod1*. These results also suggest that there could be
459 additional mechanisms of *C. jejuni*-mediated reduction of ROS because *C. jejuni* was able to
460 reduce ROS after 24 hours infection even though transcription levels of antioxidant-related
461 genes *cat* and *sod1* were downregulated. However, we cannot disregard the possibilities that
462 *C. jejuni* might secrete its own antioxidant-related proteins that may mitigate host cellular ROS
463 and/or *C. jejuni* might induce expression of other host antioxidant genes such as mitochondrial
464 superoxide dismutase (Sod2), extracellular superoxide dismutase (Sod3) and glutathione
465 peroxidase (Aviello & Knaus, 2017).

466

467 Upon adhering to host cells, *C. jejuni* modulates small GTPase Rac1 resulting in actin filament
468 reorganisation to promote invasion. Activation of Rac1 in human embryonic INT 407 cells was
469 observed between 45 minutes and 4 hours after *C. jejuni* infection (Krause-Gruszczynska et
470 al., 2007; Negretti et al., 2021). In accordance with previous studies, we demonstrated *C.*
471 *jejuni* activates Rac1 at early infection time points. In contrast, a decrease of active Rac1 was
472 detected at the later infection time point. Given the association of the active GTP-bound Rac1
473 and Nox1 activity, the early activation of Rac1 in IECs suggest that *C. jejuni* uses an intriguing
474 system which we hypothesise could have temporally nonoverlapping mechanisms. The GTP-

475 bound Rac1 observed in early time points may be linked to the requirement for *C. jejuni* to
476 establish adhesion/invasion utilising a distinct mechanism in its infection cycle. Although the
477 inactive GDP-bound Rac1 observed at the later time point of 24 hours, suggests *C. jejuni*
478 clearly possesses yet to be discovered mechanisms that enable differential regulation of Nox1
479 relative to modulation of Rac1. We also observe the pattern of active GTP-bound Rac1 in
480 Caco-2 cells that is different to T84 cells. Such a difference may be due to the signalling cues
481 between the cells as well as *C. jejuni* preference to efficiently interact with individual cells by
482 binding, invading, and intracellularly surviving from distinct states during its infection.

483

484 The impact of differential regulation of Nox1 on *C. jejuni* interaction, invasion and intracellular
485 survival in human IECs remains unclear. Surprisingly, chemical inhibition of Nox1 significantly
486 reduced the ability of *C. jejuni* to interact, invade, and survive intracellularly in T84 and Caco-
487 2 cells. It is possible that DPI may inadvertently affect local cellular receptors that *C. jejuni*
488 uses to bind human IECs. Since DPI is not a specific inhibitor of Nox1 (Riganti et al., 2004),
489 we repeated these experiments using siRNA silencing of Nox1 which demonstrated similar
490 findings, suggesting that Nox1 is indirectly necessary for *C. jejuni* interaction, invasion, and
491 intracellular survival. Previous studies have demonstrated that DPI treatment reduced
492 fibronectin expression in rat renal tubular epithelial cells (Rhyu et al., 2005), and a pan-Nox
493 inhibitor APX-115 reduced fibronectin production in mesangial cells (Cha et al., 2017). As
494 fibronectin has been demonstrated as a key host receptor that *C. jejuni* uses to bind and
495 invade human IECs (Michael E. Konkel, Talukdar, Negretti, & Klappenbach, 2020), we
496 hypothesise that silencing Nox1 might also affect expression of a key receptor fibronectin as
497 is the case following DPI treatment, and this might be responsible for the reduced interaction
498 and invasion of *C. jejuni* strains. However, the broader non-specificity of DPI and siRNA
499 silencing experiments mean that there could be alternative mechanisms in play.

500

501 We have demonstrated that *C. jejuni* modulates intracellular and extracellular ROS in human
502 T84 and Caco-2 cells. Our observations link *C. jejuni* ROS modulation to the transcriptional
503 and translational downregulation of Nox1. These findings also point to a further role of Rac1
504 in Nox1 modulation and downstream interaction. Based on chemical inhibition and silencing
505 of Nox1 expression and translation, our findings suggest an indirect role of Nox1 for adhesion,
506 invasion and intracellular survival of *C. jejuni*. In this context, further understanding *C. jejuni*
507 determinants that lead to ROS and/or Nox1 modulation in IECs will provide greater insights
508 into how *C. jejuni* manipulate host defence mechanisms and cause diarrhoeal disease.

509 References

- 510 Amour, C., Gratz, J., Mduma, E., Svensen, E., Rogawski, E. T., McGrath, M., . . . Platts-Mills, J. A.
511 (2016). Epidemiology and impact of *Campylobacter* infection in children in 8 low-resource
512 settings: Results from the MAL-ED study. *Clinical infectious diseases*, *63*(9), 1171-1179.
513 doi:10.1093/cid/ciw542
- 514 Antoniou, A. N., Lenart, I., Kriston-Vizi, J., Iwawaki, T., Turmaine, M., McHugh, K., . . . Powis, S. J.
515 (2018). *Salmonella* exploits HLA-B27 and host unfolded protein responses to promote
516 intracellular replication. *Annals of the Rheumatic Diseases*, *78*(1), 74-82.
517 doi:10.1136/annrheumdis-2018-213532
- 518 Asrat, S., de Jesús, D. A., Hempstead, A. D., Ramabhadran, V., & Isberg, R. R. (2014). Bacterial
519 pathogen manipulation of host membrane trafficking. *Annual review of cell and*
520 *developmental biology*, *30*(1), 79-109. doi:10.1146/annurev-cellbio-100913-013439
- 521 Aviello, G., & Knaus, U. (2017). ROS in gastrointestinal inflammation: rescue or sabotage? In (Vol.
522 174, pp. 1704-1718).
- 523 Bacon, D. J., Alm, R. A., Burr, D. H., Hu, L., Kopecko, D. J., Ewing, C. P., . . . Guerry, P. (2002). DNA
524 sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176.
525 *Infection and Immunity*, *70*(11), 6242-6250. doi:10.1128/IAI.70.11.6242-6250.2002
- 526 Batchelor, R. A., Pearson, B. M., Friis, L. M., Guerry, P., & Wells, J. M. (2004). Nucleotide Sequences
527 and Comparison of Two Large Conjugative Plasmids from Different *Campylobacter* species.
528 *Microbiology*, *150*, 3507-3517. doi:10.1099/mic.0.27112-0
- 529 Brandes, R. P., Weissmann, N., & Schröder, K. (2014). Nox family NADPH oxidases: Molecular
530 mechanisms of activation. *Free Radical Biology and Medicine*, *76*, 208-226.
531 doi:10.1016/j.freeradbiomed.2014.07.046
- 532 Burgueño, J. F., Fritsch, J., Santander, A. M., Brito, N., Fernández, I., Pignac-Kobinger, J., . . . Abreu,
533 M. T. (2019). Intestinal Epithelial Cells Respond to Chronic Inflammation and Dysbiosis by
534 Synthesizing H2O2. *Frontiers in physiology*, *10*, 1484-1484. doi:10.3389/fphys.2019.01484
- 535 Burnham, P. M., & Hendrixson, D. R. (2018). *Campylobacter jejuni*: collective components promoting
536 a successful enteric lifestyle. *Nature reviews. Microbiology*, *16*(9), 551-565.
537 doi:10.1038/s41579-018-0037-9
- 538 Cha, J. J., Min, H. S., Kim, K. T., Kim, J. E., Ghee, J. Y., Kim, H. W., . . . Cha, D. R. (2017). APX-115, a
539 first-in-class pan-NADPH oxidase (Nox) inhibitor, protects db/db mice from renal injury.
540 *Laboratory investigation; a journal of technical methods and pathology*, *97*(4), 419-431.
- 541 Corcionivoschi, N., Alvarez, Luis A. J., Sharp, Thomas H., Strengert, M., Alemka, A., Mantell, J., . . .
542 Bourke, B. (2012). Mucosal reactive oxygen species decrease virulence by disrupting
543 *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host & Microbe*, *12*(1), 47-59.
544 doi:10.1016/j.chom.2012.05.018
- 545 den Hartog, G., Chattopadhyay, R., Ablack, A., Hall, E. H., Butcher, L. D., Bhattacharyya, A., . . .
546 Blanke, S. R. (2016). Regulation of Rac1 and reactive oxygen species production in response
547 to infection of gastrointestinal epithelia. *PLOS Pathogens*, *12*(1), 1-20.
548 doi:10.1371/journal.ppat.1005382
- 549 Devriese, S., Van den Bossche, L., Van Welden, S., Holvoet, T., Pinheiro, I., Hindryckx, P., . . . Laukens,
550 D. (2017). T84 monolayers are superior to Caco-2 as a model system of colonocytes.
551 *Histochemistry and Cell Biology*, *148*(1), 85-93. doi:10.1007/s00418-017-1539-7
- 552 Elatrech, I., Marzaioli, V., Boukemara, H., Bournier, O., Neut, C., Darfeuille-Michaud, A., . . . Marie, J.-
553 C. (2015). *Escherichia coli* LF82 differentially regulates ROS production and mucin expression
554 in intestinal epithelial T84 cells: implication of Nox1. *Inflammatory Bowel Disease*, *21*, 1018-
555 1026. doi: 10.1097/MIB.0000000000000365
- 556 Elmi, A., Nasher, F., Jagatia, H., Gundogdu, O., Bajaj-Elliott, M., Wren, B., & Dorrell, N. (2016).
557 *Campylobacter jejuni* outer membrane vesicle-associated proteolytic activity promotes

- 558 bacterial invasion by mediating cleavage of intestinal epithelial cell E-cadherin and occludin.
559 *Cellular Microbiology*, 18(4), 561-572. doi:10.1111/cmi.12534
- 560 Escoll, P., Mondino, S., Rolando, M., & Buchrieser, C. (2016). Targeting of host organelles by
561 pathogenic bacteria: a sophisticated subversion strategy. *Nature reviews. Microbiology*,
562 14(1), 5-19. doi:10.1038/nrmicro.2015.1
- 563 Gallois, A., Klein, J. R., Allen, L.-A. H., Jones, B. D., & Nauseef, W. M. (2001). *Salmonella* pathogenicity
564 island 2-Encoded type III secretion system mediates exclusion of NADPH oxidase assembly
565 from the phagosomal membrane. *The Journal of immunology (1950)*, 166(9), 5741.
- 566 Gundogdu, O., da Silva, D. T., Mohammad, B., Elmi, A., Wren, B. W., van Vliet, A. H. M., & Dorrell, N.
567 (2016). The *Campylobacter jejuni* oxidative stress regulator RrpB is associated with a
568 genomic hypervariable region and altered oxidative stress resistance. *Frontiers in*
569 *Microbiology*, 7, 2117-2117. doi:10.3389/fmicb.2016.02117
- 570 Gundogdu, O., Mills, D. C., Elmi, A., Martin, M. J., Wren, B. W., & Dorrell, N. (2011). The
571 *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and
572 aerobic stress response and is important for bacterial survival in vivo. *Journal of*
573 *Bacteriology*, 193(16), 4238-4249. doi:10.1128/JB.05189-11
- 574 Holmström, K. M., & Finkel, T. (2014). Cellular mechanisms and physiological consequences of redox-
575 dependent signalling. *Nature reviews. Molecular cell biology*, 15(6), 411-421.
576 doi:10.1038/nrm3801
- 577 Juhasz, A., Markel, S., Gaur, S., Liu, H., Lu, J., Jiang, G., . . . Doroshov, J. H. (2017). NADPH oxidase 1
578 supports proliferation of colon cancer cells by modulating reactive oxygen species-
579 dependent signal transduction. *The Journal of Biological Chemistry*, 292, 7866-7887.
580 doi:10.1074/jbc.M116.768283
- 581 Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., & Man, S. M. (2015). Global epidemiology of
582 *Campylobacter* infection. *Clinical Microbiology Reviews*, 28(3), 687-720.
583 doi:10.1128/CMR.00006-15
- 584 Kawahara, T., Kohijima, M., Kuwano, Y., Mino, H., Teshima-Kondo, S., Takeya, R., . . . Rokutan, K.
585 (2005). *Helicobacter pylori* lipopolysaccharide activates Rac1 and transcription of NADPH
586 oxidase Nox1 and its organizer NoxO1. *The American Journal of Physiology-Cell Physiology*,
587 288, C450-C457. doi:10.1152/ajpcell.00319.2004
- 588 Kawahara, T., Kuwano, Y., Teshima-Kondo, S., Takeya, R., Sumimoto, H., Kishi, K., . . . Rokutan, K.
589 (2016). Role of nicotinamide adenine dinucleotide phosphate oxidase 1 in oxidative burst
590 response to toll-like receptor 5 signaling in large intestinal epithelial cells. *The Journal of*
591 *Immunology*, 172, 3051-3058. doi:10.4049/jimmunol.172.5.3051
- 592 Konkel, M. E., Hayes, S. F., Joens, L. A., & Cieplak Jr, W. (1992). Characteristics of the internalization
593 and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microbial*
594 *Pathogenesis*, 13(5), 357-370. doi:10.1016/0882-4010(92)90079-4
- 595 Konkel, M. E., Talukdar, P. K., Negretti, N. M., & Klappenbach, C. M. (2020). Taking control:
596 *Campylobacter jejuni* binding to fibronectin sets the stage for cellular adherence and
597 invasion. *Frontiers in Microbiology*, 11, 564-564. doi:10.3389/fmicb.2020.00564
- 598 Krause-Gruszczynska, M., Rohde, M., Hartig, R., Genth, H., Schmidt, G., Keo, T., . . . Backert, S. (2007).
599 Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*.
600 *Cellular Microbiology*, 9(10), 2431-2444. doi:10.1111/j.1462-5822.2007.00971.x
- 601 Lipinski, S., Petersen, B.-S., Barann, M., Pieczyk, A., Tran, F., Mayr, G., . . . Rosenstiel, P. (2019).
602 Missense variants in NOX1 and p22phox in a case of very-early-onset inflammatory bowel
603 disease are functionally linked to NOD2. *Cold Spring Harbor Molecular Case Studies*, 5(1).
604 doi:10.1101/mcs.a002428
- 605 Matziouridou, C., Rocha, S. C., Haabeth, O., Rudi, K., Carlsen, H., & Kielland, A. (2018). iNOS- and
606 Nox1-dependent ROS production maintains bacterial homeostasis in the ileum of mice.
607 *Mucosal immunology*, 11, 774-784. doi:10.1038/mi.2017.106

- 608 Negretti, N. M., Gourley, C. R., Talukdar, P. K., Clair, G., Klappenbach, C. M., Lauritsen, C. J., . . .
609 Konkell, M. E. (2021). The *Campylobacter jejuni* CiaD effector co-opts the host cell protein
610 IQGAP1 to promote cell entry. *Nature Communications*, *12*(1), 1339-1339.
611 doi:10.1038/s41467-021-21579-5
- 612 Nisimoto, Y., Tsubouchi, R., Diebold, B. A., Qiao, S., Ogawa, H., Ohara, T., & Tamura, M. (2008).
613 Activation of NADPH oxidase 1 in tumour colon epithelial cells. *Biochemical Journal*, *415*, 57-
614 65. doi:10.1042/BJ20080300
- 615 Paiva, C. N., & Bozza, M. T. (2014). Are reactive oxygen species always detrimental to pathogens?
616 *Antioxidants & Redox Signaling*, *20*, 1000-1037. doi:10.1089/ars.2013.5447
- 617 Pedron, T., Parsot, C., Kim, D. W., Mateescu, B., Sansonetti, P. J., Arbibe, L., . . . Batsche, E. (2007). An
618 injected bacterial effector targets chromatin access for transcription factor NF- κ B to alter
619 transcription of host genes involved in immune responses. *Nature Immunology*, *8*(1), 47-56.
620 doi:10.1038/ni1423
- 621 Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR.
622 *Nucleic acids research*, *29*(9), 45e-45. doi:10.1093/nar/29.9.e45
- 623 Rhyu, D. Y., Yang, Y., Ha, H., Lee, G. T., Song, J. S., Uh, S. T., & Lee, H. B. (2005). Role of reactive
624 oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and
625 epithelial-mesenchymal transition in renal tubular epithelial cells. *Journal of the American
626 Society of Nephrology*, *16*(3), 667-675.
- 627 Riganti, C., Gazzano, E., Polimeni, M., Costamagna, C., Bosia, A., & Ghigo, D. (2004).
628 Diphenyleneiodonium inhibits the cell redox metabolism and induces oxidative stress. *The
629 Journal of Biological Chemistry*, *279*, 47726-47731. doi:10.1074/jbc.M406314200
- 630 Rudel, T., Kepp, O., & Kozjak-Pavlovic, V. (2010). Interactions between bacterial pathogens and
631 mitochondrial cell death pathways. *Nature reviews. Microbiology*, *8*(10), 693-705.
632 doi:10.1038/nrmicro2421
- 633 Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image
634 analysis. *Nature methods*, *9*(7), 671-675. doi:10.1038/nmeth.2089
- 635 Schwerd, T., Bryant, R. V., Pandey, S., Capitani, M., Meran, L., Cazier, J. B., . . . Uhlig, H. H. (2018).
636 NOX1 loss-of-function genetic variants in patients with inflammatory bowel disease.
637 *Mucosal immunology*, 562-574. doi:10.17863/CAM.18641
- 638 Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., & Teixeira, P. (2011). *Campylobacter* spp. as
639 a Foodborne Pathogen: A Review. *Frontiers in Microbiology*, *2*, 1-12.
640 doi:10.3389/fmicb.2011.00200
- 641 Sumimoto, H., Miyano, K., & Takeya, R. (2005). Molecular composition and regulation of the Nox
642 family NAD(P)H oxidases. *Biochemical and Biophysical Research Communications*, *338*(1),
643 677-686. doi:10.1016/j.bbrc.2005.08.210
- 644 Ueyama, T., Geiszt, M., & Leto, T. L. (2006). Involvement of Rac1 in activation of multicomponent
645 Nox1- and Nox3-based NADPH oxidases. *Molecular and Cellular Biology*, *26*(6), 2160-2174.
646 doi:10.1128/MCB.26.6.2160-2174.2006
- 647 Vareechon, C., Zmina, S. E., Karmakar, M., Pearlman, E., & Rietsch, A. (2017). *Pseudomonas
648 aeruginosa* effector ExoS inhibits ROS production in human neutrophils. *Cell Host & Microbe*,
649 *21*(5), 611-618.e615. doi:10.1016/j.chom.2017.04.001
- 650 Watson, R. O., & Galán, J. E. (2008). *Campylobacter jejuni* survives within epithelial cells by avoiding
651 delivery to lysosomes (*C. jejuni* intracellular survival). *PLoS Pathogens*, *4*(1), e14.
652 doi:10.1371/journal.ppat.0040014
- 653 Willison, H. J., Jacobs, B. C., & van Doorn, P. A. (2016). Guillain-Barré syndrome. *The Lancet*,
654 *388*(10045), 717-727. doi:10.1016/S0140-6736(16)00339-1

655

656

657 **Figure Legends**

658 **FIGURE 1.** Detection of intracellular and extracellular ROS in T84 and Caco-2 cells after
659 infection with *C. jejuni* 11168H, 81-176 or 488 strains. Intracellular ROS in T84 cells after
660 infection with *C. jejuni* for (A) 3 hours or (B) 24 hours and extracellular ROS from T84 cells
661 after infection with *C. jejuni* for (C) 3 hours or (D) 24 hours were measured. Intracellular ROS
662 in Caco-2 cells after infection of *C. jejuni* for (E) 3 hours or (F) 24 hours and extracellular ROS
663 from Caco-2 cells after infection for (G) 3 hours or (H) 24 hours were measured. For detection
664 of intracellular ROS, DCFDA was used. For detection of extracellular ROS, Amplex[®] Red
665 reagent with HRP were used. H₂O₂ was used as a positive control. Experiments were repeated
666 in three biological and three technical replicates. Asterisks denote a statistically significant
667 difference (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

668

669 **FIGURE 2.** *C. jejuni* modulates Nox1 expression in T84 and Caco-2 cells. qRT-PCR showing
670 expression of Nox1 in (A) T84 and (B) Caco-2 cells. (C) RT-PCR showing expression of Nox1
671 in uninfected T84 and Caco-2 cells. *gapdh* was used as an internal control. (D) RT-PCR
672 showing expression of Nox1 in T84 cells infected with *C. jejuni* for 24 hours and (E) relative
673 mRNA levels as a percentage from RT-PCR data. (F) Western blotting showing Nox1 in T84
674 cells infected with *C. jejuni* for 24 hours and (G) relative protein level as a percentage from
675 Western blotting. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p <$
676 0.01 ; *** = $p < 0.001$).

677

678 **FIGURE 3.** Proposed structure of the Nox1 complex consisting of Nox1, p22phox, GTP-bound
679 Rac1, NoxA1 and NoxO1. p22phox and other subcellular subunits are assembled to activate
680 catalytic subunit Nox1 which results in the generation of O₂⁻ by oxidising NADPH (Brandes et
681 al., 2014). Created with BioRender.com

682

683 **FIGURE 4.** *C. jejuni* modulates activity of small GTPase Rac1 in T84 and Caco-2 cells. (A)
684 T84 and (B) Caco-2 cells were infected with *C. jejuni* 11168H strain for 1, 3, and 24 hours and
685 the activation of small GTPase Rac1 in each time point was measured. Constitutively active
686 Rac1 (RCCA) was used as a positive control. Experiments were repeated in three biological
687 and three technical replicates. Asterisks denote a statistically significant difference (* = $p <$
688 0.05 , ** = $p < 0.001$).

689

690 **FIGURE 5.** qRT-PCR showing expression of human catalase (*cat*) and superoxide dismutase
691 1 (*sod1*) in T84 and Caco-2 cells. (A, B) T84 and (C, D) Caco-2 cells were infected with *C.*
692 *jejuni* for 3- or 24-hours and transcriptional levels of *cat* and *sod1* were measured. *gapdh* was
693 used as an internal control. Experiments were repeated in three biological and three technical
694 replicates. Asterisks denote a statistically significant difference (** = $p < 0.01$; *** = $p < 0.001$;
695 **** = $p < 0.0001$).

696

697 **FIGURE 6.** The effect of DPI on *C. jejuni* interaction, invasion and intracellular survival. T84
698 and Caco-2 cells were pre-treated with 10 μ M of DPI for 1 hour and infected with *C. jejuni* for
699 3 hours. (A) T84 and (B) Caco-2 cells were washed with PBS and lysed, and the numbers of
700 interacting bacteria were assessed. (C, D) For invasion assay, after infection with *C. jejuni*,
701 IECs were incubated with gentamicin (150 μ g/ml) for 2 hours to kill extracellular bacteria and
702 then lysed, and the numbers of intracellular bacteria were assessed. (E, F) For intracellular
703 survival assay, 2 hours gentamicin treatment was followed by further incubation with
704 gentamicin (10 μ g/ml) for 18 hours. Then cells were lysed, and the number of intracellular
705 bacteria were assessed. Experiments were repeated in three biological and three technical
706 replicates. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$; ****
707 = $p < 0.0001$).

708

709 **FIGURE 7.** The effect of Nox1 silencing on *C. jejuni* interaction, invasion and intracellular
710 survival. Caco-2 cells were transfected with Nox1 siRNA or scrambled siRNA (Scr siRNA). (A)
711 qRT-PCR showing expression of Nox1 after siRNA transfection. (B) Western blotting showing
712 expression of Nox1 after 72 hours siRNA transfection. (C) Detection of extracellular ROS from
713 Caco-2 cells after 72 hours siRNA transfection followed by co-incubation of *C. jejuni* for 3
714 hours. (D) After 72 hours siRNA transfection followed by *C. jejuni* infection for 3 hours, Caco-
715 2 cells were washed with PBS and lysed and the numbers of interacting bacteria were
716 assessed or (E) for invasion assay, the cells were incubated with gentamicin (150 μ g/ml) for
717 2 hours to kill extracellular bacteria and then lysed, and the numbers of intracellular bacteria
718 were assessed. (F) For intracellular survival assay, 2 hours gentamicin treatment was followed
719 by further incubation with gentamicin (10 μ g/ml) for 18 hours. Then the cells were lysed, and
720 the number of intracellular bacteria determined. Experiments were repeated in three biological
721 and three technical replicates. Asterisks denote a statistically significant difference (* = $p <$
722 0.05; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

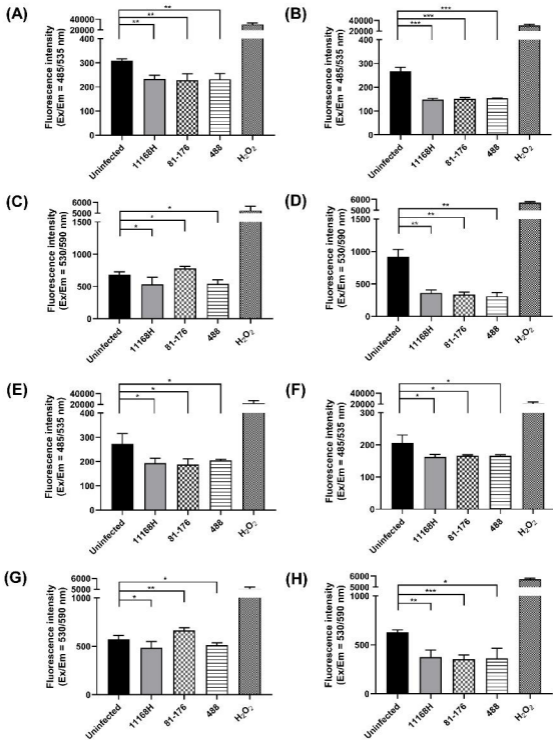
723

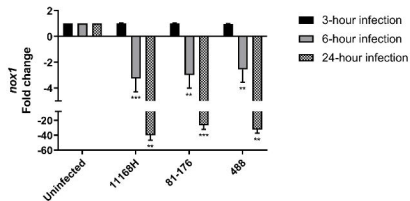
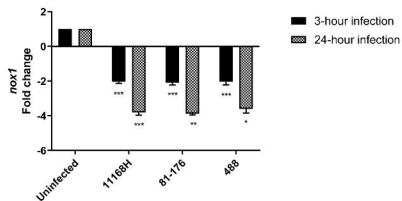
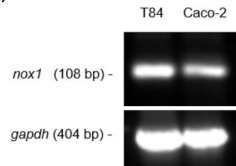
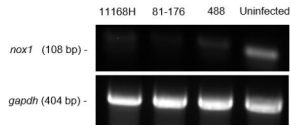
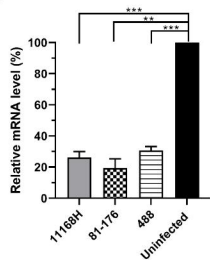
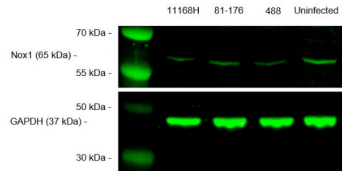
724

725 **ACKNOWLEDGEMENTS**

726 We would like to acknowledge Marta Mauri for kind advice on siRNA transfection.

727



(A)**(B)****(C)****(D)****(E)****(F)****(G)**