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1 **Modulation of innate immune signalling by a *Coxiella burnetii* eukaryotic-like effector**
2 **protein**

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25 **Abstract**

26 The Q fever agent *Coxiella burnetii* uses a defect in organelle trafficking/intracellular
27 multiplication (Dot/Icm) Type 4b Secretion System (T4SS) to silence the host innate immune
28 response during infection. By investigating *C. burnetii* effector proteins containing eukaryotic-
29 like domains, here we identify NopA (for Nucleolar protein A), which displays 4 Regulator of
30 Chromosome Condensation (RCC) repeats, homologous to those found in the eukaryotic Ras-
31 related nuclear protein (Ran) guanine nucleotide exchange factor (GEF) RCC1. Accordingly,
32 NopA is found associated with the chromatin nuclear fraction of cells and uses the RCC-like
33 domain to interact with Ran. Interestingly, NopA triggers an accumulation of Ran-GTP, which
34 accumulates at nucleoli of transfected or infected cells, thus perturbing the nuclear import of
35 transcription factors of the innate immune signalling pathway. Accordingly, qRT-PCR analysis
36 on a panel of cytokines shows that cells exposed to the *C. burnetii nopA::Tn* or a Dot/Icm-
37 defective *dotA::Tn* mutant strains present a functional innate immune response, as opposed to
38 cells exposed to wt *C. burnetii* or the corresponding *nopA* complemented strain. Thus, NopA is
39 an important regulator of the innate immune response allowing *Coxiella* to behave as a stealth
40 pathogen.

41

42 **Significance statement**

43 *Coxiella burnetii* is a stealth pathogen that evades innate immune recognition by inhibiting the
44 NF- κ B signalling pathway. This process is mediated by the bacterial Dot/Icm secretion system;
45 however, the bacterial effector/s, as well as the molecular mechanism involved in this process
46 remained to date unknown. Here, by investigating *C. burnetii* proteins with eukaryotic-like
47 features (EUGENs), we discovered a new effector protein, NopA (for Nucleolar protein A),
48 which localizes at nucleoli of infected cells and perturbs nucleocytoplasmic transport by
49 manipulating the intracellular gradients of the GTPase Ran. In doing so, NopA reduces the
50 nuclear levels of transcription factors involved in the innate immune sensing of pathogens and
51 single-handedly downmodulates the expression of a panel of cytokines.

52

53 **Introduction**

54 The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) family of
55 transcription factors regulates the expression of genes associated with diverse cellular functions
56 and plays a central role in regulating the innate and acquired host immune response to bacterial
57 infections (1, 2). Under physiological conditions, the transcription factors of the NF- κ B family
58 are sequestered in the cytoplasm by specific interactions with nuclear factor kappa-light
59 polypeptide gene enhancer in B cells inhibitor alpha ($I\kappa B\alpha$), which mask the nuclear localisation
60 signal (NLS) on transcription factors. Exogenous signals, including recognition of the tumour
61 necrosis factor (TNF) by TNF receptor or the bacterial lipopolysaccharide (LPS) by Toll-like
62 Receptor 4 (TLR4), activate the NF- κ B signalling pathway by triggering the phosphorylation and
63 proteasomal degradation of $I\kappa B\alpha$, thus unmasking the NLS on transcription factors. The signal is
64 then recognised by importin- α and members of the importin- β family, which mediate the
65 translocation of transcription factors to the nucleus through nuclear pore complexes (1). Energy
66 for nuclear transport of NLS-containing proteins is provided by intracellular gradients of the
67 small GTPase Ras-related nuclear protein (Ran), which interacts with the importin complexes
68 upon nuclear import. GDP-bound Ran is largely cytoplasmic and nuclear translocation triggers
69 the conversion to the GTP-bound form by means of the Ran guanine nucleotide exchange factor
70 (GEF) RCC-1 (regulator of chromosome condensation-1). In its GTP-bound form, Ran triggers
71 the dissociation of importins from the cargo and importin complexes recycle back to the
72 cytoplasm. There, Ran GTPase activating protein (RanGAP) generates Ran-GDP, which
73 dissociates from importin complexes (3).

74 Given its pivotal role in the antimicrobial response, it is not surprising to observe that a
75 considerable number of bacterial pathogens deploy effector proteins that modulate the NF- κ B

76 signalling pathway (1, 2). These are mostly involved in phosphorylation, ubiquitination and
77 proteasomal degradation of components of the NF- κ B complex, whereas other modulate NF- κ B-
78 mediated transcription (1, 2). Interestingly, it has been recently reported that *Salmonella* and
79 *Orientia tsutsugamushi* effector proteins can interfere with nucleocytoplasmic transport, thereby
80 inhibiting nuclear translocation of the p65/RelA transcription factor (4, 5).

81 The Q fever pathogen *Coxiella burnetii* is an obligate intracellular bacterium that relies on
82 the translocation of effector proteins by a defect in organelle trafficking/intracellular
83 multiplication (Dot/Icm) Type 4b Secretion System (T4SS) to replicate within large
84 autolysosomal-like compartments inside infected cells (6, 7). Bioinformatics analysis identified
85 over 140 *C. burnetii* genes encoding candidate effector proteins (7); however, the majority of
86 these remain under-investigated due to the technical constraints associated with the genetic
87 manipulation of this organism. A subset of effector proteins is involved in the biogenesis of
88 *Coxiella*-containing vacuoles (CCVs), by rerouting membrane traffic to the bacterial replicative
89 niche, while other effectors manipulate the apoptotic and inflammatory pathways to ensure
90 intracellular persistence (6). Importantly, *C. burnetii* behaves as a stealth pathogen, evading the
91 host innate immune response by down-modulating the NF- κ B and the inflammasome signalling
92 pathways (8, 9). The *C. burnetii* effector protein IcaA (Inhibition of caspase activation A) inhibits
93 NOD-like receptor family pyrin domain containing 3 (NLRP3)-mediated inflammasome
94 activation induced by caspase-11 (8), whereas the NF- κ B signalling pathway is down-modulated
95 in a Dot/Icm-dependent manner, by perturbing the nuclear translocation of the p65/RelA subunit,
96 without affecting the overall cellular levels of p65 (9). However, the bacterial effector/s involved
97 in this process remain uncharacterised (9). We have previously reported the large-scale
98 phenotypic characterisation of *C. burnetii* transposon mutants library, which allowed to gain
99 important insights into the function of the Dot/Icm secretion system, and highlight an important

100 set of virulence determinants (10–12). Importantly, several genes involved in intracellular
101 replication of *C. burnetii* encode proteins with predicted eukaryotic-like domains, which
102 prompted us to investigate eukaryotic-like genes (EUGENs) on a genome-wide scale. Here, we
103 identify and validate the Dot/Icm-mediated translocation of 7 *C. burnetii* EUGENs. Among these,
104 NopA (for Nucleolar protein A) displays 4 Regulation of Chromosome Condensation (RCC)
105 repeats, which are partially homologous to the 7 repeats found in the bladed β -propeller structure
106 of the Ran GEF RCC1 (13–15). Similar to RCC1, NopA also localises at the nucleus of infected
107 or transfected cells, it is found associated with the chromatin nuclear fraction, and uses the RCC-
108 like domain to interact with Ran. Differently from RCC1 however, NopA accumulates at nucleoli
109 and sequesters Ran, thus perturbing nucleocytoplasmic transport. Indeed, NopA perturbs nuclear
110 translocation of p65 upon cell treatment with TNF- α or challenge with *C. burnetii*. Conversely,
111 transposon insertions in the *nopA* gene restore nuclear translocation of p65 during infections, to
112 levels that are similar to those observed with the Dot/Icm-deficient *C. burnetii dotA* mutant.
113 Accordingly, myeloid cells challenged with the *C. burnetii nopA* or *dotA* mutant strains present a
114 functional innate immune response, as opposed to myeloid cells exposed to wt *C. burnetii* or the
115 *nopA* complemented strain.

116

117 **Results**

118 **Identification of *C. burnetii* EUGENS**

119 The Searching Algorithm for Type IV Effector proteins (S4TE) 2.0 (16) was used to
120 identify *C. burnetii* eukaryotic-like genes (EUGENS) encoding candidate effector proteins. This
121 allowed the identification of 56 genes, which were validated using the PFAM, SMART, CDD
122 and ELM databases (Table S1). Of these, 20 candidate EUGENS were retained for further
123 analysis (Table S2), based on the S4TE score (16), the eukaryotic-like domain encoded and the
124 presence of corresponding transposon mutants in our library (10). *cbu0072* (*ankA*), *cbu0201*
125 (*ankC*), *cbu0447* (*ankF*), *cbu0781* (*ankG*) and *cbu1213* (*ankI*) encode Ankyrin repeats (17, 18);
126 *cbu0295*, *cbu0547* and *cbu1457* (*cig43*) encode tetratricopeptide repeats; *cbu0175* and *cbu1379a*
127 encode predicted Ser/Thr kinases; *cbu0801* (*rimI*), *cbu0505* (*cig14*) and *cbu1799* encode
128 acetyltransferases; *cbu0096* encode a predicted phospholipase D; *cbu0519* (*dedA*) encodes a
129 SNARE-like domain-containing protein; *cbu1206* encodes a predicted sterol reductase; *cbu1217*
130 encodes a protein with 4 Regulation of Chromosome Condensation (RCC) repeats; *cbu1724*
131 encodes a predicted F-box protein; *cbu1366* (*cig40*) encodes a coiled-coil domain-containing
132 protein and *cbu0542* (*ligA*) encodes a predicted DNA-ligase (Table S2). Of note, Dot/Icm-
133 dependent translocation of proteins encoded by 8 of these genes has been previously validated
134 using *L. pneumophila* as a surrogate system (17, 19, 20) (Table S2). Selected genes were cloned
135 into pXDC61K-*blaM* vector, thus generating N-terminal fusions with β -lactamase, and
136 transformed into *C. burnetii* NMII RSA439. The expression of 16 out of 20 chimeric proteins
137 was validated by Western blot using an anti- β -lactamase antibody (Fig. S1A). Candidate effector
138 protein translocation was assessed at 6, 12, 24, 48, and 72 hours post-infection using the β -
139 lactamase assay. *C. burnetii* expressing β -lactamase alone or β -lactamase-tagged CvpB
140 (CBU0021) (12) were used as negative and positive controls, respectively. CvpB, CBU0295, and

141 CBU1217 were efficiently translocated from 12 hours post-infection, whereas AnkA, F and G
142 were translocated from 24 hours post-infection (Fig. 1A, B). Finally, AnkC, CBU0175 and
143 CBU1724 were also translocated, albeit less efficiently, at later time points of infection (Fig. 1A,
144 B). Plasmids encoding translocated effectors were then transformed into the *C. burnetii dotA::Tn*
145 strain to validate their Dot/Icm-dependent secretion at 72 hours post-infection. The expression of
146 6 chimeric proteins was validated by Western blot using an anti- β -lactamase antibody (Fig. S1B).
147 None of the effector proteins were secreted by the Dot/Icm-defective mutant as expected (Fig.
148 1A). Next, *cbu0072 (ankA)*, *cbu0295*, *cbu0447 (ankF)*, *cbu0781 (ankG)* and *cbu1217* were
149 cloned into a pLVX-mCherry vector to tag effector proteins at their N-terminal domain and
150 investigate their localisation in non-infected and *C. burnetii*-infected U2OS cells (Fig. 1C). AnkA
151 and CBU0295 were mostly diffuse in the cytoplasm and did not localise at CCVs in infected
152 cells. AnkF displayed a punctate pattern in the cytoplasm, which partially colocalised with the
153 lysosomal marker LAMP1 in non-infected and infected cells alike (Fig. 1C). Differently from
154 previous reports, indicating a translocation of AnkG from mitochondria to the nucleus of
155 transfected cells following staurosporine treatment (21), in our hands, this effector protein
156 displayed nuclear localisation even in the absence of staurosporine, in both infected and non-
157 infected cells (Fig. 1C). Of note, CBU1217 was exclusively localised at sub-nuclear structures in
158 over 90% of either infected or non-infected cells (Fig. 1C).

159

160 **The effector protein CBU1217 localises at nucleoli in infected and transfected cells**

161 The localisation of CBU1217 was further investigated by cloning the gene into a pJA-
162 LacO-4HA plasmid, to express the effector protein carrying an N-terminal 4xHA tag in *C.*
163 *burnetii*, under the control of an IPTG promoter, and monitor its localisation during infection.
164 GFP-expressing wt *C. burnetii* or the *dot/icm* mutant *dotA::Tn* (10) were transformed either with

165 pJA-LacO-4HA or with pJA-LacO-4HA-*cbu1217*. Expression of 4HA-CBU1217 was validated
166 by Western blot using an anti-HA antibody (Fig. S1C). U2OS cells were challenged with the
167 transformed *C. burnetii* strains and NopA localisation was assessed, in the presence or absence of
168 IPTG, using anti-HA and anti-fibrillarin antibodies and Hoechst dye. Infections by *C. burnetii*
169 transformed with pJA-LacO-4HA-*cbu1217* in the absence of IPTG did not show specific HA
170 labelling (Fig. 2A). Addition of 1mM IPTG triggered 4HA-CBU1217 expression, which co-
171 localised with fibrillarin in over 90% of HA-positive cells (Fig. 2B). The intracellular localisation
172 of 4HA-CBU1217 was lost when cells were infected with the *dotA::Tn* mutant transformed with
173 pJA-LacO-4HA-*cbu1217* in the presence of IPTG (Fig. 2D), confirming that CBU1217 is a
174 Dot/Icm substrate. Induction of the expression of the HA tag alone did not show specific
175 localisation (Fig. 2C). We thus named the new *C. burnetii* EUGEN NopA, for Nucleolar protein
176 A.

177 As mentioned above, NopA encodes 4 RCC repeats in its C-terminal domain (Fig. 2E). In
178 the eukaryotic protein RCC1, 7 repeats are arranged in a 7-bladed propeller, which associates
179 with nuclear chromatin and acts as a GEF for Ran, thus regulating nucleocytoplasmic protein
180 transport (3). To determine the role of the RCC-like domain in NopA localisation and function,
181 the effector protein was cloned into a pRK5-HA plasmid to generate HA-tagged NopA. U2OS
182 cells transfected with pRK5-HA-NopA were processed for immunofluorescence using Hoechst
183 dye, anti-HA and anti-fibrillarin antibodies. In parallel, HA-NopA localisation was investigated
184 by Western blot using U2OS cells transfected as above, lysed and separated into cytoplasmic,
185 nuclear and chromatin fractions. Full length NopA (NopA_{FL}) localised at nucleoli in over 90% of
186 transfected cells, confirming our observations in the context of *C. burnetii* infections (Fig. 2E).
187 Western blot analysis confirmed that NopA is excluded from the cytoplasmic fraction and
188 localised at the soluble and chromatin nuclear fractions (Fig. 2E). Next, we generated HA-tagged

189 NopA deletions to exclude (NopA_{N-ter}; aa 1-195, Fig. 2F) or include (NopA_{C-ter}; aa 196-497, Fig.
190 2G) the RCC repeats. Ectopically expressed HA-NopA_{N-ter} was excluded from nuclei and
191 remained diffuse in the cytoplasm (Fig. 2F) whereas HA-NopA_{C-ter} retained the nucleolar
192 localisation (Fig. 2G). Cell fractionation confirmed the cytoplasmic localisation of HA-NopA_{N-ter}
193 and the nuclear localisation of HA-NopA_{C-ter}, as well as the association with the chromatin
194 fraction (Fig. 2F, G). Thus, despite the lack of typical nuclear or nucleolar localisation signals,
195 the C-terminal domain of NopA encoding the RCC-like domain, is necessary and sufficient for
196 the nucleolar targeting of the effector protein. The role of the RCC repeats in the intracellular
197 localisation of NopA was further dissected by generating increasing deletions of single RCC
198 repeats (numbered from 1 to 4 from the N-terminal) from either the N-terminal or C-terminal
199 ends of HA-NopA_{C-ter} (Fig. S2A). The intracellular localisation of each construct was tested by
200 immunofluorescence and cell fractionation following ectopic expression in U2OS cells.
201 Interestingly, this revealed that the first RCC repeat is critical for targeting NopA to the nucleus
202 as removal of this repeat from NopA_{C-ter} displaces the protein to the cytoplasm (Fig. S2B, C). The
203 first 2 RCC repeats (RCC12; aa 196-310) alone localise within the nucleus but are excluded from
204 nucleoli (Fig. S2E) and instead localise at promyelocytic leukaemia (PML) bodies (Fig. S2G).
205 This localisation remains unchanged with the addition of the 3rd RCC repeat (Fig. S2D, F), and it
206 is only with the addition of the complete NopA_{C-ter} that the protein localises at nucleoli (Fig. 2G),
207 suggesting the presence of a nucleolar-targeting motif in the 4th RCC repeat. Unfortunately, we
208 were unable to express detectable amounts of single RCC repeats (RCC1 and RCC4, Fig. S2A).

209

210 **NopA is not involved in *C. burnetii* intracellular replication**

211 Given the early translocation of NopA observed using the β -lactamase assay, we
212 determined the time course of NopA production during infection. To this aim, we have

213 complemented the *nopA* mutation, using a mini Tn7 transposon to integrate a wild type copy of
214 HA-tagged *nopA*, under the regulation of its predicted endogenous promoter, in the chromosome
215 of the *C. burnetii* Tn227 strain, which carries the transposon insertion closest to the *nopA* start
216 codon (10). Protein expression was then monitored by Western blot, using an anti-HA antibody,
217 from cells challenged with the complemented *nopA::Tn* strain for 12, 24, 48, and 72 hours. By
218 this approach, detectable amounts of NopA were observed from 12 hours post-infection (Fig.
219 S1D).

220 We previously reported that transposon insertions in *nopA* do not affect bacterial
221 replication in Vero cells (10). To further investigate the role of NopA in *C. burnetii* infections,
222 bacterial replication and virulence of the wild type, *dotA::Tn*, *nopA::Tn*, and the *nopA::Tn*
223 complemented strain (*nopA::Tn* Comp.) described above, were tested using either bone marrow-
224 derived macrophages (BMDM) or a SCID mouse model of infection. Confirming our initial
225 observations, transposon insertions in *nopA* do not affect *C. burnetii* replication (Fig. 2H, I) or
226 virulence (as determined here by splenomegaly measurements, Fig. 2J).

227

228 **NopA interacts with the small GTPase Ran**

229 Given that NopA localises at nucleoli and presents 4 out of the 7 RCC repeats present in
230 the eukaryotic Ran-GEF RCC1, we investigated whether NopA can interact with Ran. To this
231 aim, U2OS cells incubated either with the *nopA::Tn* mutant or the complemented strain
232 expressing 4HA-tagged NopA under the control of the predicted endogenous promoter (*nopA::Tn*
233 Comp.). Twenty-four hours post-infection, cells were lysed, separated into cytoplasmic, nuclear
234 and chromatin fractions, and NopA was immuno-captured from cell fractions using an anti-HA
235 antibody. As expected, NopA was not detected in cells infected with the *nopA::Tn* mutant strain,
236 whereas it was efficiently isolated from the nuclear and chromatin fractions of cells challenged

237 with the complemented strain (Fig. 3A). Of note, whole cell lysates of cells incubated with the
238 complemented strain also presented an accumulation of Ran in the chromatin fraction, which was
239 not observed in cells challenged with the *nopA*::Tn mutant strain (Fig. 3A). Importantly, Ran was
240 efficiently detected, together with NopA, in immunoprecipitates from the nuclear and chromatin
241 fractions of cells challenged with the *nopA*::Tn complemented strain, indicating indeed an
242 interaction with the *C. burnetii* effector protein (Fig. 3A). The NopA/Ran interaction was further
243 investigated in U2OS cells, following the ectopic expression of either HA-tagged NopA_{N-ter},
244 NopA_{C-ter} or the *C. burnetii* effector protein CvpF as negative control (22). Unfortunately, under
245 these conditions, we were unable to immuno-precipitate full-length NopA from transfected cells.
246 Similarly to infected cells, 24 hours post-transfection, cells were lysed, separated into
247 cytoplasmic, nuclear and chromatin fractions, and NopA truncations and CvpF were immuno-
248 captured from cell fractions using an anti-HA antibody. As expected, NopA_{N-ter} and CvpF were
249 efficiently isolated from the cytoplasmic fractions, whereas NopA_{C-ter} was isolated from the
250 nuclear and cytoplasmic fractions (Fig. 3B). In agreement with what we observed in infected
251 cells, the ectopic expression of NopA_{C-ter} triggered an accumulation of Ran to the chromatin
252 fractions (Fig. 3B). Moreover, Ran was specifically detected in the nuclear and chromatin
253 fractions upon immune-capturing of NopA_{C-ter}, confirming an interaction between the two
254 proteins (Fig. 3B). Of note, no interaction was detected between Ran and NopA_{N-ter}, despite their
255 shared cytoplasmic localisation (Fig. 3B). Furthermore, NopA_{C-ter} did not interact with other
256 small GTPases such as DRP1 or RAB26, nor with the nucleolar marker fibrillarin (Fig. 3B).
257 Conversely, the *C. burnetii* effector protein CvpF (22), was readily immuno-captured from the
258 cytoplasm of transfected cells and interacted with RAB26 as reported (22) (Fig. 3B).

259 Finally, the direct interaction between NopA and Ran was further investigated using the
260 tripartite split-GFP interaction sensor (23). Briefly, the assay is based on a tripartite association

261 between two GFP β -strands (GFP10 and GFP11), fused to proteins of interest, and the
262 complementary GFP1-9 detector. If proteins interact, GFP10 and GFP11 self-associate with
263 GFP1-9 to reconstitute a functional GFP. pCDNA3-zipper-GFP10 and pCDNA3-zipper-GFP11
264 were used as negative control, whereas a plasmid encoding GFP10 and GFP11 linked by a zipper
265 motif (GFP10-zip-GFP11) was used as positive control (23). *ran* cDNA was cloned into the
266 pCDNA3-GFP10-zipper plasmid to generate the GFP10-Ran, whereas *nopA*, *rcc1* and *fb1* (the
267 gene encoding fibrillarlin) were cloned into the pCDNA3-zipper-GFP11 plasmid to generate the
268 corresponding GFP11 fusion proteins. Combinations of the above-mentioned constructs with a
269 pCMV plasmid encoding GFP1-9 were used for triple transfections in U2OS cells. After fixation,
270 an anti-GFP antibody was used to identify cells expressing GFP1-9 (which is not fluorescent) and
271 protein interactions were analysed by monitoring GFP reconstitution. As expected, co-expression
272 of GFP1-9 with GFP10 and GFP11 did not result in the reconstitution of GFP (Fig. 3C, top row,
273 and 3D). The co-expression of GFP1-9 with GFP10-zip-GFP11 led to the reconstitution of GFP
274 fluorescence in over 93% of transfected cells, demonstrating the functionality of the assay (Fig.
275 3C, centre row, and 3D). Importantly, over 60% of cells expression GFP1-9 in combination with
276 GFP10-Ran and GFP11-NopA showed reconstitution of GFP, with a fluorescent signal detected
277 at nuclei, with a strong accumulation at nucleoli (Fig. 3C, bottom row, and 3D). On the contrary,
278 the expression GFP1-9 in combination with GFP10-Ran and GFP11-RCC1, which allowed
279 reconstitution of GFP fluorescence homogeneously detected in the nucleus in over 73% of
280 transfected cells (Fig. S3A, and 3D). Lack of GFP reconstitution upon expression of GFP1-9 in
281 combination with GFP10-Ran and GFP11-fibrillarlin indicated that the shared nucleolar
282 localisation was not sufficient for GFP reconstitution (Fig. S3A, and 3D).

283 Ectopic expression of either mCherry-NopA_{FL} or mCherry-NopA_{C-ter} in combination with
284 GFP-Ran in U2OS cells also confirmed the co-localisation of both proteins at nucleolar structures
285 labelled with the anti-fibrillarin antibody (Fig. S3B). Conversely, Ran-GFP accumulation at
286 nucleoli was lost when the small GTPase was ectopically expressed in U2OS cells in
287 combination either with mCherry alone or mCherry-NopA_{N-ter} (Fig. S3B). Collectively, these
288 observations indicate that NopA specifically interacts with Ran and may sequester it at nucleoli.

289

290 **NopA preferentially interacts with GDP-bound Ran and triggers an increase in Ran-GTP**

291 To determine whether NopA displays preferential binding to Ran in its GDP- versus GTP-
292 bound form, a GFP-trap assay was carried out on U2OS cells co-transfected with plasmids
293 encoding HA-NopA_{C-ter} in combination with either GFP alone, GFP-Ran, GFP-Ran_{T24N} (GDP-
294 locked), GFP-Ran_{Q69L} (GTP-locked) or GFP-Ran_{N122I} (nucleotide-free form). Similar to RCC1,
295 NopA displayed preferential binding to either GDP-locked Ran_{T24N} or the nucleotide-free form
296 Ran_{N122I} (Fig. 4A).

297 Next, we investigated whether NopA binding to Ran can affect the Ran GDP/GTP ratio
298 that is required to fuel nucleocytoplasmic transport. U2OS cells were challenged either with wt
299 *C. burnetii*, the *dotA::Tn*, *nopA::Tn*, or the *nopA::Tn* complemented strains. Non-infected cells
300 were used as control. Twenty-four hours post-infection, cells were lysed and incubated with
301 agarose beads coated with the Ran effector Ran-Binding Protein 1 (RanBP1), to specifically pull-
302 down the GTP-bound form of Ran. Indeed, infection with wt *C. burnetii* triggered a 40-fold
303 increase in the intracellular levels of Ran-GTP, as compared to non-infected cells (Fig. 4B). This
304 phenotype was lost in cells challenged with the *dotA::Tn* mutant strain and only an 8.5-fold
305 increase was observed in cells challenged with the *nopA::Tn* mutant strain. Increased levels of
306 Ran-GTP were largely restored (35-fold increase) in cells exposed to the complemented strain

307 (*nopA::Tn* Comp., Fig. 4B). The effects of NopA on the intracellular levels of Ran-GTP were
308 further investigated in U2OS cells transfected with plasmids encoding either HA alone, HA-
309 NopA, HA-NopA_{N-ter}, HA-NopA_{C-ter}, or the *C. burnetii* effector protein CvpB (12) used here as
310 negative control. A three-fold increase in the intracellular levels of Ran-GTP was observed in
311 cells expressing either HA-NopA or HA-NopA_{C-ter}, as compared to cells transfected with HA
312 alone or HA-NopA_{N-ter} (Fig. 4C). As expected, ectopic expression of CvpB had negligible impact
313 on the intracellular levels of Ran-GTP (Fig. 4C). These observations suggest that NopA
314 sequestration of Ran at nucleoli leads to an increase in the intracellular levels of Ran-GTP, which
315 may negatively regulate nuclear import (24).

316

317 **NopA perturbs protein translocation to the nucleus**

318 Given the role of Ran in nucleocytoplasmic traffic, and the previously reported
319 observation that *C. burnetii* infections modulate nuclear translocation of p65 by a Dot/Icm-
320 dependent mechanism (9), we investigated whether NopA affects the nuclear localisation of p65,
321 which follows the activation of the NF- κ B signalling pathway. U2OS cells transfected with
322 plasmids encoding either HA- or mCherry-tagged versions of NopA were either left untreated or
323 challenged with 10 ng/ml TNF- α for 30 minutes, and the nuclear translocation of p65 was
324 monitored using an anti-p65 antibody either by fluorescence microscopy or Western blot
325 following cell fractionation. Cells expressing either HA- or mCherry-tagged CvpB or the tags
326 alone were used as controls. TNF- α treatment efficiently activated the NF- κ B pathway, as
327 indicated by the significant degradation of I κ B α (Fig. 5A). Accordingly, p65 was readily re-
328 localised to the nucleus of cells expressing either the HA or mCherry tags alone or tagged
329 versions of the *C. burnetii* effector CvpB (Fig. 5B, C and D). However, p65 translocation was

330 largely inhibited in cells expressing either HA-NopA or mCherry-NopA (Fig. 5B, C and D). In
331 all cases, the intracellular levels of p65 remained largely unaltered. To determine whether NopA
332 modulates the intracellular levels of p65 by perturbing its nuclear import or by accelerating its
333 nuclear export, U2OS cells expressing either mCherry-NopA, mCherry-CvpB or mCherry alone
334 as controls, were incubated for 4 hours with 5 nM leptomycin B (LMB), a fungal metabolite that
335 blocks nuclear export by covalently binding to exportin 1. As p65 shuttles continuously between
336 the nucleus and the cytoplasm, treatment with LMB in mCherry- or mCherry-CvpB expressing
337 cells led to an accumulation of the transcription factor in the nucleus (Fig. 5D, S4). Interestingly
338 however, ectopic expression of mCherry-NopA significantly prevented p65 nuclear accumulation
339 in response to LMB treatment (Fig. 5D, S4). A similar phenotype was observed in cells treated
340 with LMB for 4 hours, followed by 30 min incubation with TNF- α (Fig. 5D, S4). These data
341 indicate that indeed, NopA perturbs nuclear import.

342 Next, we tested whether the perturbation of nuclear import triggered by NopA was
343 specific to p65 and *C. burnetii* infections. The nuclear translocation of the transcription factor
344 IRF3 was monitored in U2OS cells co-transfected with 3FLAG-tagged IRF3 in combination with
345 either mCherry alone, mCherry-NopA or mCherry-CvpB, and infected with the Sendai Virus for
346 18 hours. Non-infected cells were used as control (Fig. S5A). Similar to what we reported for
347 p65, IRF3 was readily translocated to the nuclei of cells expressing either mCherry alone or
348 mCherry-CvpB but remained largely cytoplasmic in cells expressing mCherry-NopA (Fig. S5A,
349 B).

350
351 **NopA is involved in the silencing of the innate immune response during *C. burnetii***
352 **infections**

353 p65 nuclear translocation was further monitored in U2OS cells non-infected or challenged
354 either with 10 ng/ml TNF- α for 30 minutes, wt *C. burnetii*, the Dot/Icm-defective mutant
355 *dotA::Tn*, the *nopA::Tn* or the complemented strain (*nopA::Tn Comp.*), for 24, 48 and 72 hours by
356 fluorescence microscopy and, for the 72 hours time point, by Western blot following cell
357 fractionation. I κ B α was significantly degraded in all conditions as compared to non-infected
358 cells, indicating an efficient activation of the NF- κ B pathway (Fig. 6A). Translocation of p65 to
359 the nucleus was readily detected in cells treated with TNF- α , either by Western blotting (Fig. 6B)
360 or by immunofluorescence (Fig. 6C, D). Cells challenged with wt *C. burnetii* or the *nopA::Tn*
361 complemented strain showed a small but significant increase in nuclear p65 fluorescence as
362 compared to non-infected, untreated cells (Fig. 6B, C, D). However, incubation with either the
363 *dotA::Tn* or the *nopA::Tn* mutants triggered an accumulation of p65 to the nucleus which was
364 comparable to the TNF- α treatment (Fig. 6B, C, D). Measurement of p65 nuclear translocation
365 by immunofluorescence, which was specifically measured in infected cells, resulted in a stronger
366 phenotype as compared to Western blot analysis, which was carried out on the total cell
367 population.

368 To investigate the downstream effects of perturbing the nuclear translocation of
369 transcription factors involved in the immune response to *C. burnetii* infections, differentiated
370 THP-1 macrophages were exposed to either wt *C. burnetii*, the Dot/Icm-deficient *dotA::Tn*
371 mutant, the *nopA::Tn* mutant or the corresponding complemented strain (*nopA::Tn Comp.*) for
372 24, 48 and 72 hours. Total RNA was extracted from cell lysates and qRT-PCR analysis was used
373 to monitor the expression of a panel of cytokines (Fig. 7A, S6A). A slight increase in the mRNA
374 expression levels of all tested cytokines was observed in cells exposed to wt *C. burnetii* or the
375 *nopA::Tn* complemented strain, as compared to non-infected cells. Interestingly however, cells

376 exposed to either the *dotA*::Tn mutant or the *nopA*::Tn mutant displayed a comparable, significant
377 increase in the production of the majority of the cytokines tested, ranging from a two-fold
378 increase to a 100-fold increase for IL8 (Fig. 7A, S6A, S7). Downmodulation of the innate
379 immune response was further confirmed by monitoring TNF- α and IFN- α production in THP-1
380 macrophages infected as above for 72 and 96 hours. As *C. burnetii* effectors are known to perturb
381 the secretory pathway of infected cells (20, 25), THP-1 cells were treated with brefeldin A (BFA)
382 24 hours prior to fixation and the intracellular levels of TNF- α and IFN- α were assessed by flow
383 cytometry (Fig. 7B, C), following the application of a specific gating to isolate the population of
384 infected cells (Fig. S6B). A significant increase in the intracellular levels of both cytokines was
385 observed in cells infected either with the *nopA*::Tn or the *dotA*::Tn strains as compared to cells
386 infected with wt *C. burnetii* or the *nopA*::Tn complemented strain (Fig. 7B, C). Overall, our data
387 indicate that *C. burnetii* uses the Dot/Icm secretion system to down-modulate the NF- κ B
388 signalling pathway as previously reported (9), and that NopA is a key effector for this process.
389

390 **Discussion**

391 Intracellular bacterial pathogens and symbionts establish intimate interactions with their
392 eukaryotic hosts, which have evolved by co-evolution over time. Part of their adaptation to their
393 intracellular niches has been mediated by trans-kingdom acquisition and functional integration of
394 eukaryotic genes in bacterial genomes (26). Indeed, Eukaryotic-like GENes (or EUGENs),
395 represent a hallmark of intracellular bacteria, and are rarely observed in free-living bacteria.
396 Importantly, many EUGENs from intracellular bacteria produce candidate or validated effector
397 proteins that are translocated into host cells through dedicated type III or type IV secretion
398 systems (27). Thus, EUGENs are predicted to play an important role in the establishment of
399 parasitic or symbiotic bacterial lifestyles.

400 In this study, bioinformatics analysis combined with translocation assays led to the
401 identification of 7 *C. burnetii* effector proteins encoding eukaryotic-like domains involved in
402 protein/protein interactions, protein/chromatin interactions and post-translational modifications.
403 CBU0447 and CBU0175 are conserved among *C. burnetii* strains whereas the remaining 5
404 EUGENs present some degree of polymorphism (7). Upon ectopic expression in epithelial cells
405 of translocated ankyrin repeats-containing proteins, AnkA (CBU0072) was largely cytoplasmic,
406 whereas AnkF (CBU0447) seemed to associate with membranes that partially co-localised with
407 the lysosomal marker LAMP1. AnkG (CBU0781), which was previously reported to localise at
408 mitochondria and translocate to the nucleus upon staurosporine treatment of transfected cells
409 (21), partially localised to the nucleus even in the absence of staurosporine in our hands. It is thus
410 possible that other Ank proteins modify their intracellular localisation at different stages of
411 infection.

412 Here, we have focused our study on CBU1217, which encodes 4 RCC repeats in its C-
413 terminal domain (aa 196-497). RCC repeats are found in the Regulation of Chromosome

414 Condensation 1 (RCC1) eukaryotic protein (28). In eukaryotes, the RCC domain consists of
415 seven homologous repeats of 51-68 amino acid residues, arranged in a β -propeller fold (15). A
416 single RCC domain constitutes the majority of the protein in the case of the RCC1 subgroup of
417 the RCC1 superfamily, whereas multiple RCC domains can be found, either alone or in
418 combination with other functional domains the other subgroups of the superfamily (13). As such,
419 RCCs are versatile domains that can be involved in protein/protein or protein/chromatin
420 interactions, guanine nucleotide exchange factor (GEF) and post-translational modifications
421 including ubiquitination and phosphorylation (13). RCC1 is primarily found in association with
422 histones H2A and H2B on chromatin (29) and acts as a GEF for the small GTPase Ran, a master
423 regulator of nucleocytoplasmic transport during interphase and mitotic spindle assembly during
424 mitosis (30).

425 Among vacuolar bacterial pathogens, the *L. pneumophila* effector protein LegG1 encodes
426 an RCC-like domain (RLD) consisting of 3 out of the 7 RCC repeats typically found in
427 eukaryotes (31). Of note, LegG1 localises at *Legionella*-containing vacuoles (LCVs) where it
428 recruits and activates Ran to promote microtubule polymerisation and LCV mobility (32).
429 Differently from LegG1, *C. burnetii* NopA encodes an additional RCC repeat and, despite the
430 lack of typical nuclear or nucleolar localisation signals, exclusively localises at nuclei with a
431 strong enrichment in the chromatin fraction, which is consistent with RCC1 localisation. NopA
432 RCC repeats are necessary and sufficient to target the protein to nucleoli and exert its functions.
433 Moreover, the first RCC repeat seems to be critical for targeting NopA to the nucleus as removal
434 of this repeat from NopA_{C-term} displaces the protein to the cytoplasm. Interestingly, the first 2
435 RCC repeats (aa 196-310) alone localise at promyelocytic leukaemia (PML) bodies. This
436 localisation remains unchanged with the addition of the 3rd RCC repeat and it is only the

437 expression of the complete NopA_{C-term} that triggers protein localisation at nucleoli, suggesting the
438 presence of a nucleolar-targeting motif in the 4th RCC repeat.

439 Similarly to the eukaryotic protein RCC1, NopA interacts with Ran, with preferential
440 affinity for the GDP-bound form and promotes the activation of Ran. Differently from RCC1
441 however, NopA also triggers a nucleolar accumulation of Ran. Thus, the observed increase in the
442 intracellular levels of GTP-bound Ran may result from either a GEF activity of NopA (which has
443 been reported for RCC1), or via the observed sequestration of Ran at nucleoli, which would
444 prevent GTP-bound Ran to recycle back to the cytoplasm, where Ran GTPase activating proteins
445 (GAPs) stimulate GTP to GDP conversion. As we were unable to purify sufficient amounts of
446 either full length NopA or NopA_{C-ter} we could not assess for the moment whether NopA has
447 intrinsic GEF activity. Interestingly, a residual increase in the intracellular levels of Ran-GTP
448 was still observed in cells challenged with the *nopA::Tn* mutant strain, as compared to infections
449 with the Dot/Icm-defective *dotA::Tn* strain. This may suggest that other *C. burnetii* effectors may
450 have a role in the modulation of Ran activity.

451 Of note, mutations in *nopA* do not affect *C. burnetii* intracellular replication (10).
452 However, increasing the intracellular levels of Ran-GTP results in an global alteration in the
453 nucleocytoplasmic transport of proteins (24). It has been reported that during infections,
454 *C. burnetii* requires Dot/Icm activity to downmodulate the NF- κ B pathway by perturbing the
455 nuclear translocation of the p65 transcription factor (9). Here we demonstrate that NopA is one of
456 the effector proteins involved in this process, as indicated by the strong inhibition of nuclear
457 translocation of p65 upon treatment of cells with TNF- α or infection. The modulation of the NF-
458 κ B signalling pathway has been reported for a number of bacterial pathogen and viruses (1, 2). In
459 most cases, bacteria interfere with the degradation of I κ B α and the release of p65 or by

460 triggering the proteasomal degradation of p65 itself. Other bacteria, including *L. pneumophila*
461 and *Shigella flexneri* may also inhibit the innate immune response downstream of p65 nuclear
462 translocation, at the level of transcription and mRNA processing, respectively (2). Finally, an
463 emerging number of bacterial effectors inhibit NF- κ B activation by modulating the nuclear
464 translocation and/or accumulation of p65, by interfering with nucleocytoplasmic protein
465 transport. The *Salmonella* SPI-2 T3SS effector protein SpvD accumulates importin- α in the
466 nucleus by binding exportin Xpo2, thereby preventing p65 nuclear import (4). *O. tsutsugamushi*
467 uses Ankyrin repeats-containing effector proteins Ank1 and 6 by co-opting the function of both
468 importin- β and exportin 1, thus accelerating p65 nuclear export (5). Here we show that the NF-
469 κ B pathway is readily activated upon *C. burnetii* infections as shown by efficient I κ B α
470 degradation. However, NopA perturbs nuclear accumulation of p65 by triggering the nuclear
471 accumulation of GTP-bound Ran, resulting in an imbalanced Ran gradient across cells. In turn,
472 this leads to a defective nuclear import of proteins, as also demonstrated by challenging cells
473 ectopically expressing NopA with leptomycin B to block nuclear export.

474 Considering that these bacterial effectors manipulate common adaptors and GTPases
475 involved in nucleocytoplasmic transport, it would be of interest to monitor their effect on a
476 broader panel of proteins and investigate how infected cells respond to these perturbations. For
477 example, other *C. burnetii* effector proteins have been described to localise at the nucleus of
478 infected cells (21, 33, 34). In this perspective, it is important to note that nuclear translocation of
479 p65 is not completely ablated during *C. burnetii* infections, and that the strongest phenotypes are
480 observed at 48 and 72 hours post infection, which is compatible with a reduced, but still
481 detectable translocation of protein to the nucleus at earlier time points. Here we show that indeed
482 the perturbation of nuclear import by NopA affects a broader class of proteins, also outside the

483 context of *C. burnetii* infections, as indicated by the perturbation of nuclear translocation of IRF3
484 in response to Sendai Virus infection, in cells ectopically expressing NopA.

485 To monitor the downstream effects of inhibiting the nuclear accumulation of transcription
486 factors involved in immune sensing, we challenged differentiated THP-1 cells with wt *C. burnetii*
487 or strains carrying mutations either in the Dot/Icm secretion system or in *nopA*. As expected,
488 infections by the wt strain elicited a minor response in the expression of a panel of cytokines,
489 including TNF- α , interleukins and interferons, in agreement with the observation that *C. burnetii*
490 is a stealth pathogen. Evasion of the innate immune response was unmasked by infections with
491 the Dot/Icm-defective strain *dotA::Tn*, which triggered a significant cytokine response.
492 Interestingly, infections by the *nopA::Tn* mutant strain largely phenocopied the *dotA::Tn*
493 mutation, suggesting that NopA is critical for the down-modulation of the innate immune
494 response.

495 Together, this work highlighted a number of *C. burnetii* eukaryotic-like effector proteins
496 and showed that one of them, NopA, is responsible for evading the host innate immune response
497 by interfering with nucleocytoplasmic transport.

498

499 **Materials and Methods**

500
501 Antibodies, reagents, bacterial strains, cell lines and growth conditions used in this study are
502 listed in SI Appendix.

503
504 *Plasmids.*
505 Plasmids used in this study are listed in Table S4. DNA sequences were amplified by PCR using
506 Phusion polymerase (New England Biolabs) and gene-specific primers (Sigma).

507
508 *Plasmid design for secretion assay in C. burnetii.*
509 Selected genes from Table S2 were amplified from *C. burnetii* RSA439 NMII genomic DNA
510 using primer pairs indicated in Table S5. PCR products were cloned into the pXDC61-BLAM
511 plasmid to generate N-terminal-tagged fusion version of all candidate effector proteins.

512
513 *Plasmid design for mammalian cells transfection.*
514 Effector-coding genes were amplified from *C. burnetii* RSA439 NMII genomic DNA using
515 primer pairs indicated in Table S5. PCR products were cloned either into pLVX-mCherry-N2 or
516 pRK5-HA plasmids to generate N-terminal-tagged mCherry or HA fusion versions of all effector
517 proteins, respectively. For tripartite split-GFP assay, pCMV_GFP1-9-OPT, pcDNA3-GFP10-
518 zipper-GFP11, pcDNA3-GFP10-zipper and pcDNA3-zipper-GFP11 were kindly provided by Dr.
519 Stephanie Cabantous. For cloning of Ran in pcDNA3-GFP10-zipper, Ran was amplified using
520 forward primers Ran-BspEI and reverse primers Ran-XbaI-rev. For cloning of NopA, RCC1 and
521 Fibrillarin in pcDNA3-zipper-GFP11, genes were amplified using forward primers NopA-NotI,

522 RCC1-NotI or FBL-NotI and reverse primers NopA-ClaI-rev, RCC1-ClaI-rev or FBL-ClaI-rev.
523 pGBKT7 containing eukaryotic sequence of Ran WT, RanT24N/Q69L/N122I mutants were
524 kindly provided by Prof. Aymelt Itzen. Ran WT and mutants were amplified from pGBKT7-Ran-
525 WT, pGBKT7-Ran-T24N, pGBKT7-Ran-Q69L, pGBKT7-Ran-N122I using primers pairs XhoI-
526 Ran-F and Ran-XmaI-rev, and the PCR products were cloned into pLVX-GFP-N2.

527

528 *Plasmid design for nopA complementation in C. burnetii.*

529 For *nopA* complementation, the *nopA* sequence was amplified, together with its putative
530 promoter, using NheI-prom1217-F and PstI-prom1217-R (see Table S5) and the PCR products
531 were cloned into pUC18R6K-miniTn7T-Kan-tetRA-4HA. Plasmids were electroporated in the
532 *nopA::Tn* mutant strain *Tn227* (10).

533

534 *Beta-Lactamase translocation assay.*

535 For *C. burnetii* effector translocation assays, cells were cultured in black, clear- bottomed, 96-
536 well plates and infected with the appropriate *C. burnetii* strain (MOI of 100) for 24 and 48 h.
537 *C. burnetii* expressing BLAM alone or BLAM-tagged CBU0021 were used as negative and
538 positive controls, respectively. Cell monolayers were loaded with the fluorescent substrate
539 CCF4/AM (LiveBLAzer-FRET B/G loading kit; Invitrogen) in a solution containing 20 mM
540 HEPES, 15 mM probenecid (Sigma) pH 7.3, in HBSS. Cells were incubated in the dark for 1 h at
541 room temperature and imaged using an EVOS inverted fluorescence microscope. Images were
542 acquired using DAPI and GFP filter cubes. The image analysis software CellProfiler was used to
543 segment and count total cells and positive cells in the sample using the 520 nm and 450 nm
544 emission channels, respectively, and to calculate the intensity of fluorescence in each channel.

545 Following background fluorescence subtraction using negative control samples, the percentage of
546 positive cells was then calculated and used to evaluate effector translocation. A threshold of 20%
547 of positive cells was applied to determine efficient translocation of bacterial effector proteins.

548

549 *Immunofluorescence staining and microscopy.*

550 Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS solution at room temperature for 20
551 min. Samples were then rinsed in PBS solution and incubated in blocking solution (0.5% BSA,
552 50 mM NH₄Cl in PBS solution, pH 7.4). Cells were then incubated with the primary antibodies
553 diluted in blocking solution for 1 h at room temperature, rinsed five times in PBS solution, and
554 further incubated for 1 h with the secondary antibodies diluted in the blocking solution. To
555 visualize HA-tagged NopA or nuclear/nucleolar proteins, cells were fixed as previously described
556 in 4% (wt/vol) paraformaldehyde in PBS solution. Then, cells were permeabilized with 0.5%
557 Triton X-100 in PBS solution for 3 min at room temperature. Sample were then rinsed in PBS
558 solution and incubated with blocking solution [0.1% Triton X-100, 5% (wt/vol) milk in PBS
559 solution] for 1 h at room temperature. Cells were then incubated with the primary antibodies
560 diluted in blocking solution for 1 h at 37 °C, rinsed five times in PBS solution, and incubated
561 with the secondary antibodies for 1 h at room temperature. For all conditions, coverslips were
562 mounted by using Fluoromount mounting medium (Sigma) supplemented with Hoechst 33258
563 for DNA staining. Samples were imaged with a Zeiss Axio Imager Z1 epifluorescence
564 microscope (Carl Zeiss) connected to a CoolSNAP HQ² CCD camera (Photometrics). Images
565 were acquired alternatively with 100x, 63× or 40× oil immersion objectives and processed with
566 MetaMorph (Universal Imaging). ImageJ and CellProfiler software were used for image analysis
567 and quantifications.

568

569 *Immunoprecipitations and pull-down assays.*

570 For coimmunoprecipitation experiments, pLVX-GFP-N2-tagged wt Ran, RanT24N/Q69L/N122I
571 mutants or vector control were co-transfected with pRK5-HA-NopA_{Cter} in U2OS cells. 24 h post
572 transfection, cells were lysed in lysis buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5 mM
573 EDTA, 1% NP-40) supplemented with a protease inhibitor tablet (Complete; Roche) and
574 incubated with 25 µl of GFP-Trap magnetic beads (Chromotek) for 2 h at 4° C with rotation. The
575 beads were then washed 3 times with wash buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5
576 mM EDTA), resuspended in Laemmli buffer 4X and analysed by Western blot.

577

578 *Tripartite split-GFP assay.*

579 U2OS were grown in DMEM supplemented in 10% (v/v) foetal calf serum (FCS) at 37°C and
580 5% CO₂. For the interaction assay, U2OS cells were co-transfected with Lipofectamine 2000
581 (Gibco, Invitrogen Co.) with plasmids encoding for GFP1-9, GFP10 and GFP11 fusions. At 24h
582 post transfection, cells were fixed in 4% paraformaldehyde in PBS solution and processed for
583 immunofluorescence. Protein-protein interactions were scored by calculating the percentage of
584 GFP-positive cells over the total number of cells positive for the anti-GFP antibody.

585

586 *Cell fractionation.*

587 U2OS cells were grown to 60% confluence in 100-mm Petri dishes before being transfected with
588 10 µg of pRK5-HA- NopA_{N-ter} or pRK5-HA- NopA_{C-ter} in JetPEI reagent (PolyplusTransfection)
589 according to the manufacturer's recommendations. 24 h after transfection, cells were washed in
590 PBS and pelleted at 4°C. U2OS cells cultured in 100-mm dishes were infected with the *nopA::Tn*
591 mutant or the corresponding complemented strain (*nopA::Tn Comp.*) expressing a 4HA-tagged

592 version of NopA. After 24h of infection, cells were washed in PBS and pelleted at 4°C.
593 Transfected or infected cell pellets were subjected to cell fractionation as previously described
594 (37). Where appropriate, cytoplasmic, nuclear and chromatin fractions were subjected to
595 immunoprecipitation using 40 µl of anti-HA magnetic beads (Sigma) for 2 h at 4° C with
596 rotation. Bound proteins were eluted using 80 µl of 100 µg/ml⁻¹ HA-peptide (Sigma), then
597 resuspended in Laemmli buffer 4X and analysed by Western blot.

598

599 *Ran activation assay.*

600 For the analysis of enzymatic activity of NopA, U2OS cells were either infected or transfected
601 and lysed with lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1
602 mM EDTA, 2% Glycerol) containing a protease inhibitor tablet (Complete; Roche). Cell lysates
603 were then centrifuged for 10 min at 14,000g at 4° C. For Ran-GTP immunoprecipitation, 40 µl of
604 RanBP1 beads (Cell Biolabs, Inc.) were incubated with cell lysates for 1 h at 4° C, and then
605 washed 3 times with lysis buffer, subjected to SDS-PAGE and visualised by Western blotting
606 using an anti-Ran antibody (1:4000, Sigma). GTP-bound Ran levels were determined by
607 calculating the signal ratio of GTP-bound Ran over the total amount of Ran.

608

609 *NF-κB/IRF3 translocation assays.*

610 To analyse NF-κB translocation, U2OS cells were grown to 60% confluence before being
611 transfected as previously described. At 24h post transfection, cells were incubated with media
612 containing 10 ng/ml TNFα for 30min at 37° C. Alternatively, cells were preincubated with media
613 containing 5 nM LMB for 4 hours at 37° C, followed by a TNFα treatment as indicated above
614 where needed. For *C. burnetii* infection assays, cells were infected with *C. burnetii* and incubated

615 at 37° C for 1 to 3 days. Cells were then fixed in 4% paraformaldehyde in PBS solution and
616 processed for NF-κB immunostaining. The image analysis software CellProfiler was used to
617 segment all nuclei using the Hoechst staining and cell contours using nuclei as seeds and the p65
618 labelling. Cytoplasm was segmented by subtracting nuclei from cell objects. Next, mCherry
619 signal was used to identify and isolate the subpopulation of transfected cells, and single cell
620 measurements of the ratio of the mean p65 fluorescence in the nucleus versus cytoplasm were
621 calculated for each condition. For infection assays, CellProfiler was used to identify and isolate
622 the population of infected cells based on the GFP fluorescence associated with the strains of
623 *C. burnetii* used in this study and nuclear p65 fluorescence was specifically measured as
624 described above in the subpopulation of infected cells. To analyse IRF3 translocation, pLVX-
625 mCherry-N2-tagged NopA, CvpB or empty vector were co-transfected with pcDNA3-3xFLAG-
626 tagged IRF-3 in U2OS cells. At 24h post transfection, cells were infected with a defective-
627 interfering H4 Sendai Virus (38) provided by D. Garcin (Department of Microbiology and
628 Molecular Medicine, University of Geneva, Switzerland) and used at 50 hemagglutination units
629 HAU/ml for 18h at 37°C. Cells were then fixed in 4% paraformaldehyde in PBS solution and
630 processed for FLAG immunostaining. IRF3 nuclear translocation was measured as described
631 above for p65.

632
633 *Densitometry.*
634 Regions of Interest (ROIs) were obtained from each band of interest and the intensity was
635 measured using ImageJ. For each band, the same ROI was used for background calculation and
636 removal from areas adjacent to each band. For the experiments illustrated in Figure 5, the
637 intensity of bands from samples treated with TNF were normalised for the intensity of the

638 corresponding untreated sample. For the experiments illustrated in Figure 6, the intensity of
639 bands from samples challenged with *C. burnetii* or treated with TNF were normalised for the
640 intensity of the non-infected (NI) sample.

641

642 *Real-time quantitative RT-PCR (qRT-PCR) analysis of cytokine mRNA.*

643 Total RNA was extracted from THP-1 cells using the RNeasy Micro kit and was submitted to
644 DNase treatment (Qiagen), following manufacturer's instructions. RNA concentration and purity
645 were evaluated by spectrophotometry (NanoDrop 2000c, Thermo Fisher Scientific). 500 ng of
646 RNA were reverse transcribed with both oligo-dT and random primers, using PrimeScript RT
647 Reagent Kit (Perfect Real Time, Takara) in a 10 ml reaction. Real time PCR reactions were
648 performed in duplicates using Takyon ROX SYBR MasterMix blue dTTP (Eurogentec) on an
649 Applied Biosystems QuantStudio 5, using the following program: 3 min at 95°C followed by 40
650 cycles of 15 s at 95°C, 20 s at 60°C and 20 s at 72°C. Ct values for each transcript were
651 normalised to the geometric mean of the expression of RPL13A, B2M and ACTB (i.e. reference
652 genes) and the fold-changes were determined by using the $2^{-\Delta\Delta Ct}$ method. Primers used for
653 quantification of transcripts by real time quantitative PCR are indicated in Table S5.

654

655 *SCID mouse infections.*

656 SCID (C.B-17/LcrHsd-Prkdcscid) mice were purchased from Envigo (Indianapolis, IN, USA)
657 and housed in the TAMHSC animal facility. All animal procedures were done in compliance with
658 Texas A&M University IACUC (AUP#2016-0370). Infections were performed as described
659 previously (36). Briefly, 6-8 week old female mice (SCID or C57BL/6) were infected with 1 x
660 10^6 viable *C. burnetii* phase II strain via intra-peritoneal (IP) injection. Inoculum concentrations
661 were confirmed by serial dilution spot plating on ACCM-D agarose as described previously (39).

662
663 *Mouse tissue collection, processing, and DNA purification.*
664 At 10 days (competitive infections) or 14 days post-infection (single infections), the mouse
665 spleens were removed and weighed at necropsy to determine splenomegaly (spleen weight/body
666 weight). Each spleen was added to 1 mL PBS and homogenized using an Omni (TH) equipped
667 with plastic tips (Kennesaw, GA, USA). 100 μ L of homogenate was added to 400 μ L of TriZol
668 LS (Invitrogen) for RNA extraction. For DNA extraction, 100 μ L of homogenate was added to
669 900 μ L tissue lysis buffer (Roche) plus 100 μ L of proteinase K and incubated at 55°C overnight.
670 The following day 100 μ L of 10% SDS (w/v) was added and incubated at room temperature for 1
671 hour. Lysed tissue samples were then processed using Roche High Pure PCR template
672 preparation kit according to manufacturer's instructions (Indianapolis, IN, USA).

673
674 *Enumeration of Coxiella in Mouse Spleens.*
675 DNA purified from infected organs was used as template for TaqMan real time PCR using
676 primers and probe for *com1* or primers and probe of *IS1111* as described previously (36).
677 Quantitative PCR was performed in 20 μ L reactions with ABI TaqMan universal PCR mastermix
678 run on an ABI StepOne Plus machine. The replication index reported for each mouse was
679 calculated by dividing the number of genome copies recovered from spleens by the number of
680 genome copies in the original inoculum.

681
682 *Flow cytometry.*
683 For intracellular human TNF- α /IFN- α 4 staining, 5×10^4 THP-1 cells differentiated in PMA (200
684 ng/mL) for 2 day seeded in 24-well plates were infected with the indicated *C. burnetii* strain for

685 72 and 96 h. Cells were then treated with 1 µg/ml of brefeldin A (BFA) for the last 24 hours. The
686 following day, cells were fixed using 2% paraformaldehyde in PBS solution for 20 min at 4° C.
687 After washing with FACS buffer (1% BSA in PBS solution), cells were permeabilized in FACS
688 buffer supplemented with 0.1% saponin for 30 min at 4° C and then stained with anti-TNF-α-PE
689 and IFN-α-PE antibodies for 1 hour at 4° C. Infected cells were analysed based on the GFP
690 fluorescence associated with the strains of *C. burnetii*. Flow cytometry analyses were performed
691 on a BD FACSCalibur flow cytometer using flow cytometry (CellQuest software, BD
692 Biosciences, San Jose, CA). FlowJo software (Tree Star, Ashland, OR) was used to analyze data.

693

694 Data Availability Statement: All data discussed in the paper will be made available to readers.

695

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706

707 **Declaration of interest statement**

708 The authors declare no conflicts of interest.

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712

713 **Figure Legends**

714 **Figure 1. Identification of *C. burnetii* EUGENS.** **A**, U2OS cells were challenged with *C.*
715 *burnetii* strains expressing BLAM-tagged versions of candidate EUGENS for 6, 12, 24, 48 and 72
716 hours. The percentage of BLAM-positive, infected cells was automatically calculated using
717 CellProfiler over the total number of infected cells per each condition. Empty = BLAM empty
718 vector. The Dot/Icm-dependent translocation of the effectors that were efficiently secreted was
719 validated in the *dotA::Tn* mutant strain at 72 hours post challenge. **B**, Representative images of
720 positive (blue) cells treated as in **A**. **C**, Non-infected or GFP-expressing *C. burnetii*-infected
721 U2OS cells were transfected with plasmids encoding N-terminally tagged mCherry versions of
722 the effector proteins validated in **A** (red). 24 hours after transfection cells were fixed and labelled
723 with Hoechst (blue) and an anti-LAMP1 antibody (white). White arrows point at CBU1217 sub-
724 nuclear localisation. Scale bars are 10 μ m.

725
726 **Figure 2. Intracellular localisation of CBU1217/NopA and role in *C. burnetii* replication**
727 **during infection.** U2OS cells were challenged either with wt GFP-tagged *C. burnetii* (white)
728 transformed with plasmids encoding 4HA-tagged CBU1217/NopA (**A**, **B**, red) or the 4HA tag
729 alone (**C**, red), or with the GFP-tagged *dotA::Tn* mutant (**D**, white), transformed with plasmids
730 encoding 4HA-tagged CBU1217/NopA, all under the control of an IPTG-inducible promoter. 72
731 hours post-infection cells were fixed and labelled with Hoechst (blue) and anti-fibrillarin
732 antibodies (green). I= IPTG-induced; NI= non-induced. Arrow points at 4HA-CBU1217/NopA
733 localisation in infected cell. U2OS cells were transfected with plasmids encoding HA-tagged
734 versions of either full length (**E**), or the indicated deletion mutants (**F** and **G**) of HA-tagged
735 NopA. 24 hours after transfection cells were either fixed and labelled with Hoechst (blue), an

736 anti-fibrillarin antibody (green) and an anti-HA antibody (red, centre panels) or lysed and
737 processed for cell fractionation (right panels). Cell fractions were analysed by Western blotting
738 using anti-fibrillarin and anti-GAPDH antibodies as nuclear and chromatin (Nu, Ch) and
739 cytoplasmic (Cy) markers, respectively, and anti-HA antibodies to reveal NopA localisation.
740 Scale bars are 10 μm . **H**, Genome Equivalents (GE) calculated using TaqMan real-time PCR with
741 DNA purified from infected spleens of 5 SCID mice per group on day 14 after challenge with
742 1×10^6 GE of the strains shown. **I**, Replication index calculated as the ratio between spleen GE at
743 the time of necropsy and the input GE of the strains listed in the figure legend. **J**, Spleen weight
744 as a percentage of total body weight at the time of necropsy on day 14 after infection with 1×10^6
745 GE of the strains listed in the figure legend. Values are the mean of three independent infections,
746 with error bars indicating standard deviations from the mean.

747
748 **Figure 3. NopA interacts with the small GTPase Ran.** **A**, U2OS cells challenged for 24 hours
749 with either the *C. burnetii* *nopA* transposon mutant (*nopA::Tn*) or the corresponding
750 complemented strain (*nopA::Tn* Comp.) were lysed and processed for cell fractionation. Whole
751 cell lysates (WCL) were probed with the indicated antibodies, as well as anti-GAPDH and anti-
752 fibrillarin antibodies as cytoplasmic (Cy), and nuclear/chromatin (Nu, Ch) markers, respectively.
753 Following immunoprecipitation with anti-HA-coated magnetic beads, the presence of Ran and
754 that of fibrillarin (as a negative control) was assessed using specific antibodies (IP HA). **B**, U2OS
755 cells transfected with HA-tagged versions of either the N-terminal domain (NopA_{N-ter}), the C-
756 terminal domain (NopA_{C-ter}) of NopA or CvpF as negative control were lysed and processed for
757 cell fractionation. Whole cell lysates (WCL) were probed with the indicated antibodies, as well as
758 anti-GAPDH and anti-fibrillarin antibodies as cytoplasmic (Cy), nuclear/chromatin (Nu, Ch)

759 markers, respectively. Following immunoprecipitation with anti-HA-coated magnetic beads, the
760 presence of candidate interacting proteins was assessed using specific antibodies (IP HA). **C**,
761 U2OS cells were transfected with plasmids encoding GFP1-9 in combination with plasmids
762 encoding either the GFP10 and GFP11 tags alone as negative control (top row), GFP10 and
763 GFP11 linked by a leucine zipper motif (GFP10-zip-GFP11) as positive control (middle row) or
764 GFP10-Ran and GFP11-NopA (bottom row). 24 hours after transfection cells were fixed and
765 labelled with Hoechst (blue) and anti-GFP antibodies (red) to reveal nuclei and the expression of
766 GFP1-9, respectively. Protein-protein interaction was assessed following the reconstitution of
767 GFP (Reconst. GFP, green). **D**, the percentage of cells presenting GFP reconstitution over the
768 total number of GFP1-9-positive cells was calculated. Values are means \pm SD from 2
769 independent experiments. Asterisks indicate statistically significant variations (n.s. = non-
770 significant, **** = $P < 0.0001$, one-way ANOVA, Dunnett's multiple comparison test). Scale bars
771 are 20 μm .

772

773 **Figure 4. NopA increases the intracellular levels of Ran-GTP.** **A**, The GFP-trap assay was
774 carried out in U2OS cells expressing HA-tagged NopA_{C-ter} in combination with either GFP alone,
775 GFP-Ran, GFP-Ran_{T24N} (GDP-locked), GFP-Ran_{Q69L} (GTP-locked) or GFP-Ran_{N122I} (guanosine
776 free). Whole cell lysates (WCL, upper panels) were probed with anti-GFP and anti-HA antibodies
777 to assess the expression of the GFP-tagged proteins and HA-tagged NopA_{C-ter}, and anti-tubulin
778 antibodies as loading control. Protein-protein interactions were assessed using anti-GFP and anti-
779 HA antibodies following GFP capture (GFP-trap, lower panels). **B**, GTP-bound Ran was pulled
780 down using RanBP1-coated beads from cell lysates of U2OS cells challenged for 24 hours with
781 either wt *C. burnetii* (wt), a *nopA* transposon mutant (*nopA::Tn*), the corresponding

782 complemented strain (*nopA::Tn Comp.*) or the Dot/Icm-defective mutant (*dotA::Tn*). Non-
783 infected cells were used as control. Whole cell lysates (WCL) were probed with anti-*C. burnetii*
784 (NMII), anti-Ran and anti- β -tubulin antibodies. GTP-bound Ran was revealed using an anti-Ran
785 antibody (IP RanBP1). **C**, GTP-bound Ran was pulled down using RanBP1-coated beads from
786 cell lysates of U2OS cells expressing either the HA tag alone, HA-tagged versions of either full
787 length (NopA_{FL}), the N-terminal domain (NopA_{N-ter}), the C-terminal domain (NopA_{C-ter}) of NopA
788 or CvpB. Whole cell lysates (WCL) were probed with anti-HA antibodies to assess the
789 expression of the HA-tagged versions of NopA and anti-Ran and anti-tubulin antibodies as
790 loading controls. GTP-bound Ran was revealed using an anti-Ran antibody (IP RanBP1). The
791 signal ratio of GTP-bound Ran over the total amount of Ran is indicated for experiments
792 illustrated in **B** and **C**. Values are mean \pm SD from 3 independent experiments. n.s = non-
793 significant, **** = $P < 0.0001$, ** = $P < 0.007$, * = $P < 0.02$, one-way ANOVA, Dunnett's multiple
794 comparison test.

795
796 **Figure 5. Overexpression of NopA interferes with the nuclear translocation of p65.**
797 Representative Western blot of U2OS cells expressing either the HA tag alone, HA-NopA or HA-
798 CvpB left untreated or incubated with 10 ng/ml TNF- α for 30 min, lysed and processed for cell
799 fractionation. Whole cell lysates (**A**, WCL) were used to assess the overall levels of p65 and
800 I κ B α and nuclear fractions (**B**) to monitor p65 translocation to the nucleus (Nuclear Fraction).
801 The signal ratio of p65 over tubulin or fibrillarin and of I κ B α over tubulin is indicated for
802 experiments illustrated in **A** and **B**. Values are mean \pm SD from 3 independent experiments. **C**,
803 Representative images of U2OS cells expressing mCherry-NopA or mCherry-CvpB and treated
804 as in **A**. The localisation of p65 was monitored using an anti-p65 antibody and Hoechst staining

805 of nuclei. Asterisks indicate transfected cells. **D**, CellProfiler was used to identify mCherry-
806 expressing U2OS cells and measure the median of the ratios of p65 fluorescence intensity at
807 nuclei versus cytoplasm. Values are means \pm SEM from 2 independent experiments where a
808 minimum of 200 nuclei were measured per condition. Asterisks indicate statistically significant
809 variations (n.s. = non-significant, **** = $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, one-
810 way ANOVA, Dunnett's (A & B) and Bonferroni (D) multiple comparison test). Scale bars are
811 10 μm .

812
813 **Figure 6. NopA interferes with the nuclear translocation of p65 during *C. burnetii***
814 **infections.** Representative Western blot of U2OS cells challenged for 72 hours with GFP-tagged
815 strains of wt *C. burnetii* (wt), the Dot/Icm-defective *dotA* transposon mutant (*dotA::Tn*), the *nopA*
816 transposon mutant (*nopA::Tn*) or the corresponding complemented strain (*nopA::Tn Comp.*).
817 Non-infected cells (NI) and cells treated with 10 ng/ml TNF- α (TNF- α) for 30 min were used as
818 negative and positive controls, respectively. Cells were lysed and fractionated to isolate nuclear
819 fractions. Whole cell lysates (**A**, WCL) were used to assess the overall levels of p65 and I κ B α
820 and nuclear fractions (**B**) to monitor p65 translocation to the nucleus (Nuclear Fraction).
821 Normalised densitometry of indicated protein ratios was calculated. Values are means \pm SD from
822 2 independent experiments. **C**, Representative images of U2OS cells treated as in **A**. The
823 localisation of p65 (red) was monitored using an anti-p65 antibody and Hoechst staining of nuclei
824 (blue). White arrows indicate nuclei of infected cells. **D**, U2OS cells were treated as in **A** for 24,
825 48 and 72 hours. CellProfiler was used to identify infected and total U2OS cells and measure the
826 median of the ratios of p65 fluorescence intensity at nuclei versus cytoplasm. Values are means \pm
827 SEM from 2 independent experiments where a minimum of 400 nuclei were measured per

828 condition (n.s. = non-significant, *** = $P < 0.0001$, one-way ANOVA, Dunnett's multiple
829 comparison test). Scale bars are 10 μm .

830

831 **Figure 7. NopA inhibits cytokines production.** **A**, Differentiated THP-1 cells were challenged
832 either with GFP-expressing wt *C. burnetii* (wt), the Dot/Icm-defective *dotA* transposon mutant
833 (*dotA::Tn*), the *nopA* transposon mutant (*nopA::Tn*) or the corresponding complemented strain
834 (*nopA::Tn Comp.*) for 24, 48, 72 and 96 hours. The expression of TNF- α and IFN- α 4 cytokines
835 was assessed by RT-qPCR for the indicated time points. **B**, Dot plots from a representative
836 experiment showing intracellular staining of TNF- α and IFN- α in cells infected for 72 and 96
837 hours and treated with brefeldin A (BFA) for the last 24 hours. Infected cells were first gated on
838 GFP+ population and the percentage of cells expressing TNF- α and IFN- α was assessed. Flow
839 cytometry data are presented on graphs as fold relative to wt. Values are means \pm SD from three
840 independent experiments. n.s. = non-significant, **** = $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$,
841 * = $P < 0.1$. Full statistical analysis for the 72 hours time point illustrated in **A** is available at
842 Figure S7.

843

844 Supplementary figure legends are available in SI Appendix

845

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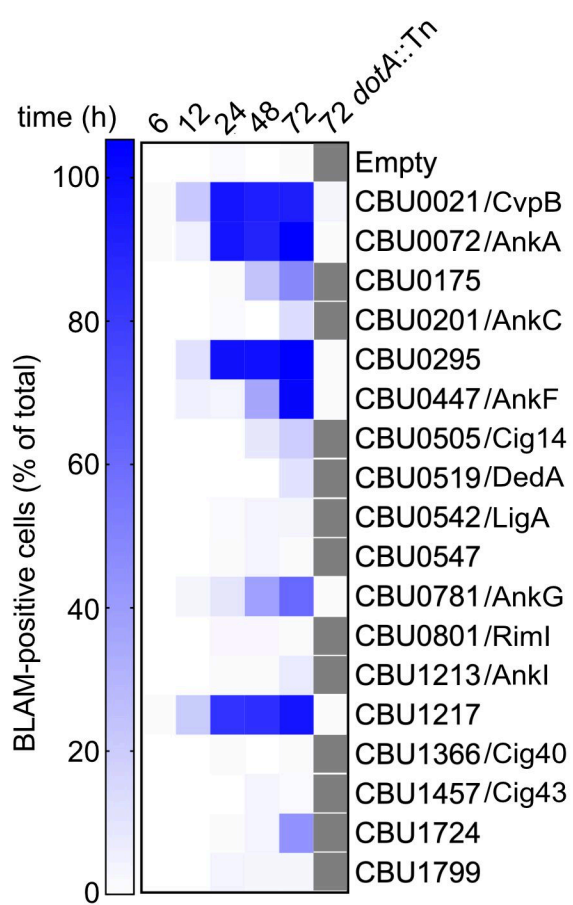
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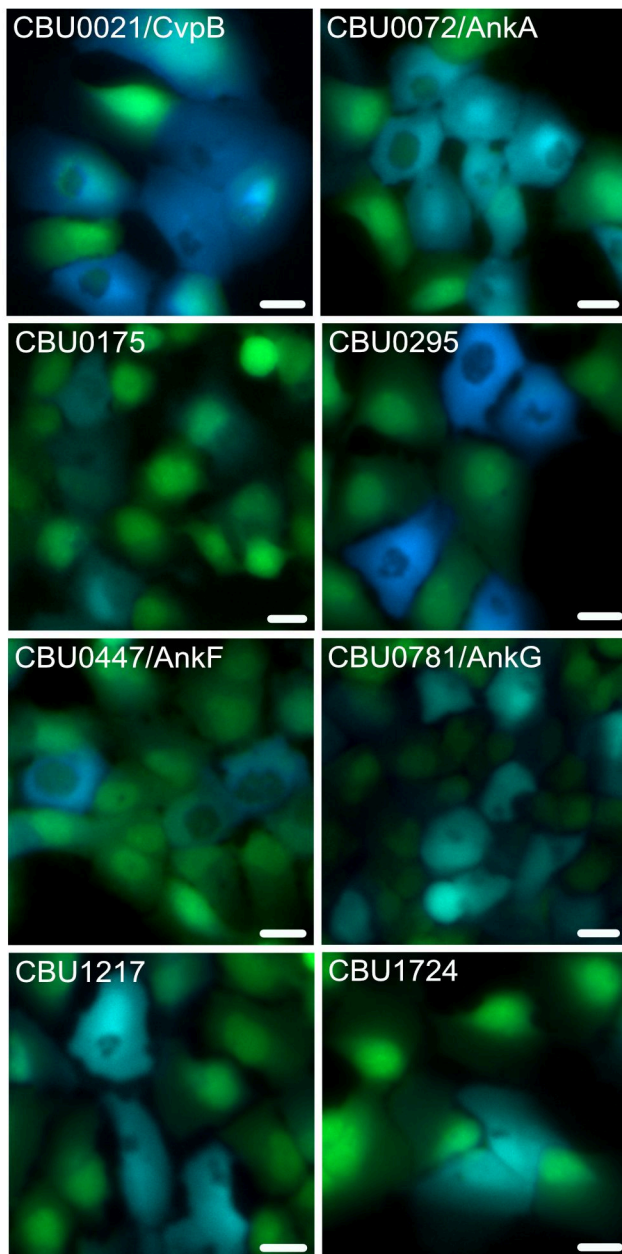
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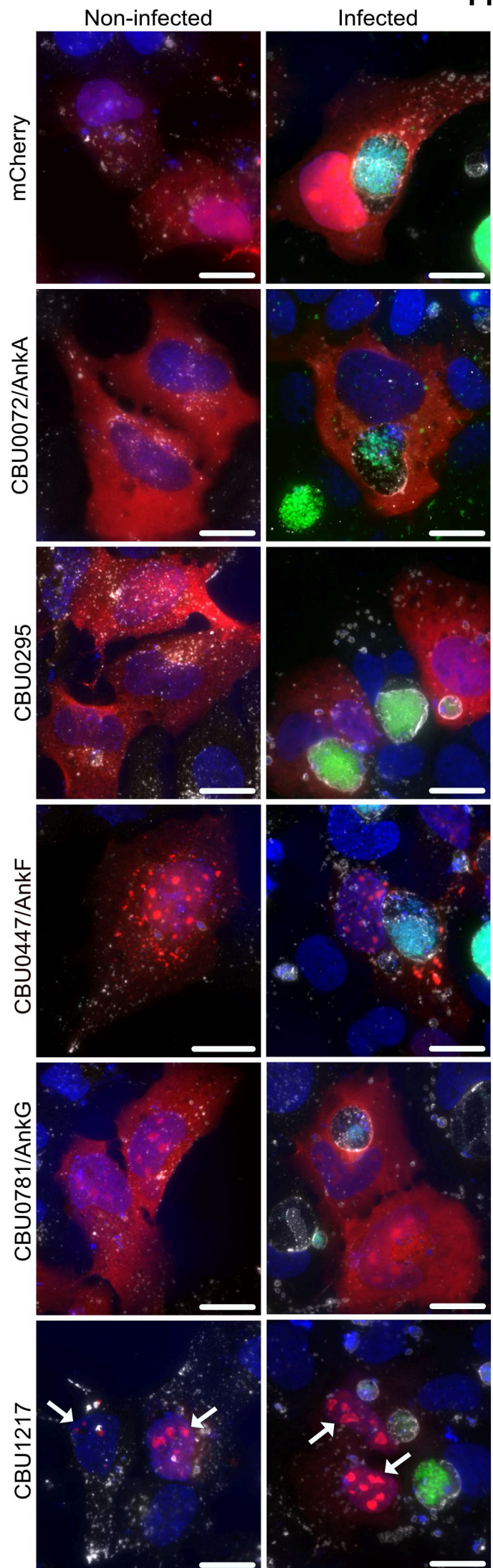
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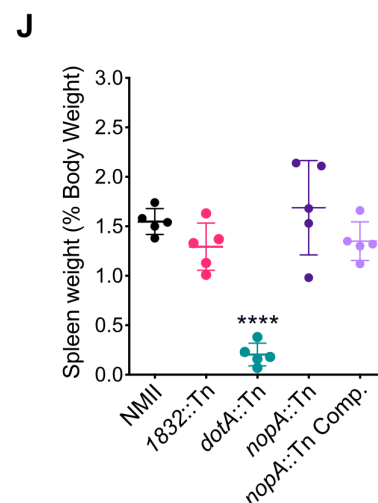
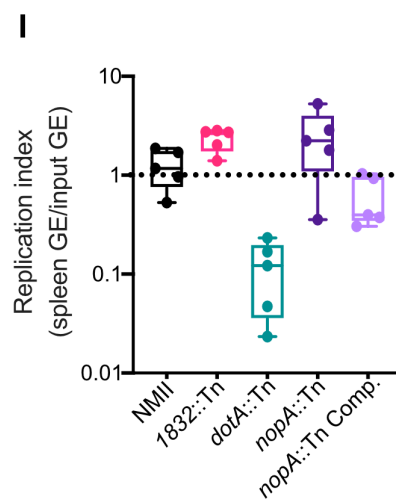
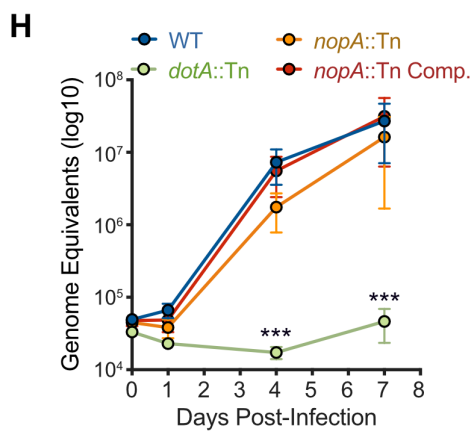
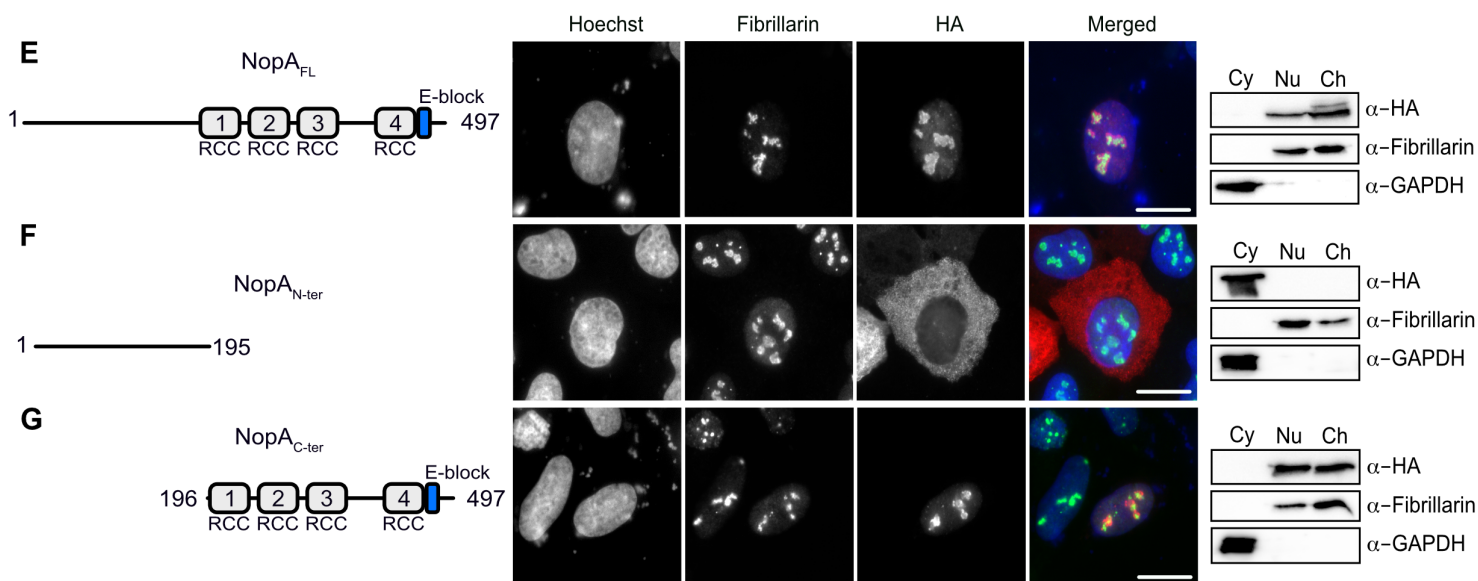
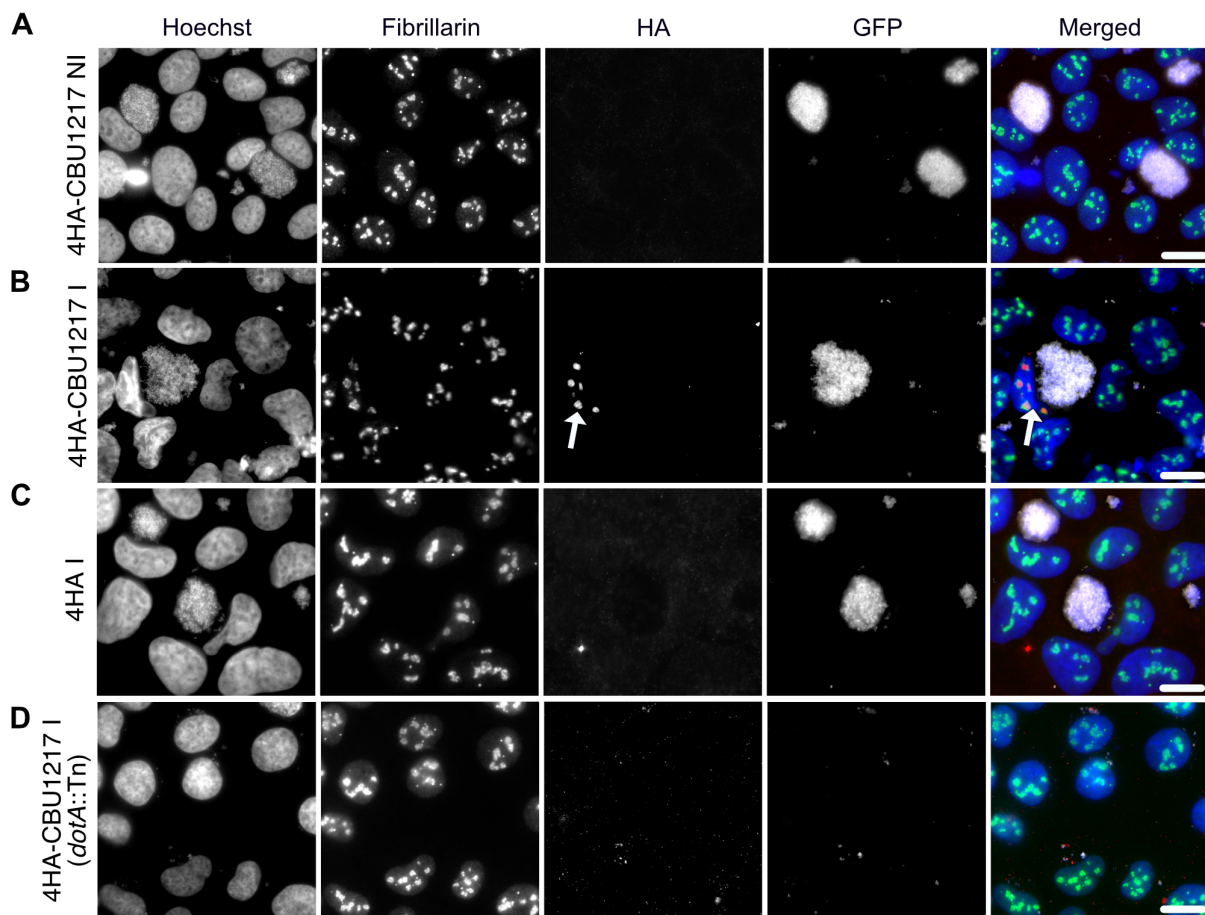


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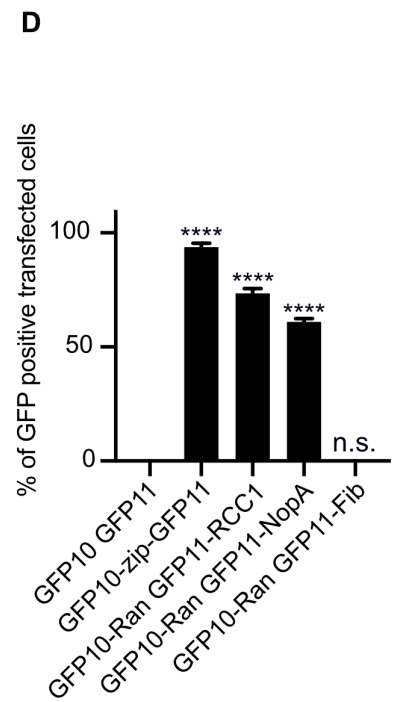
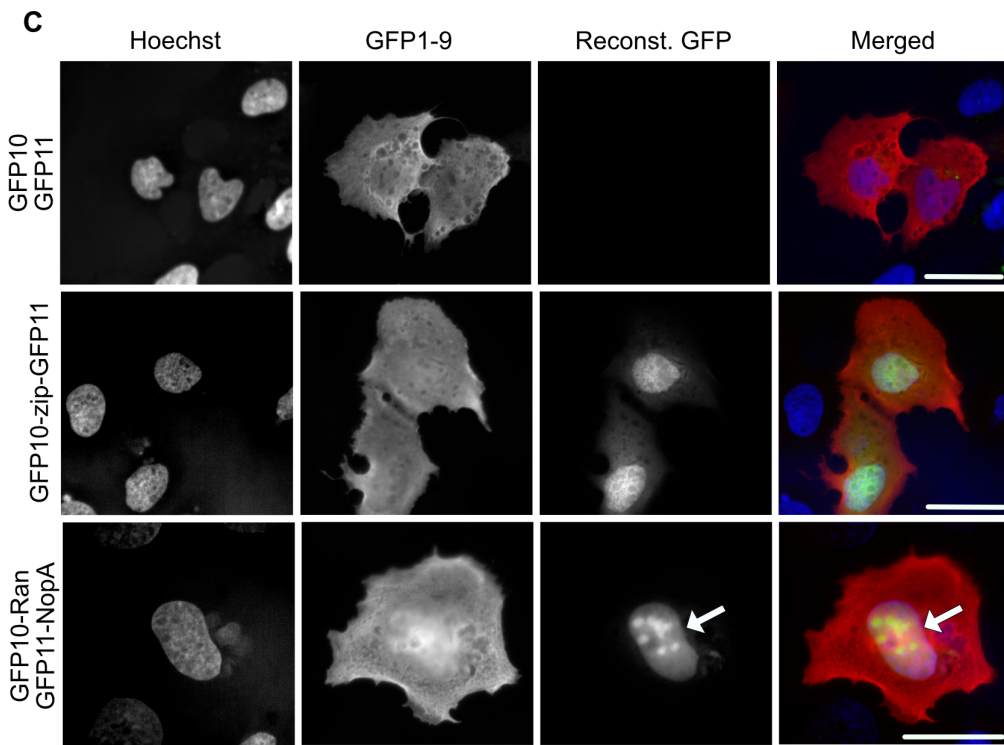
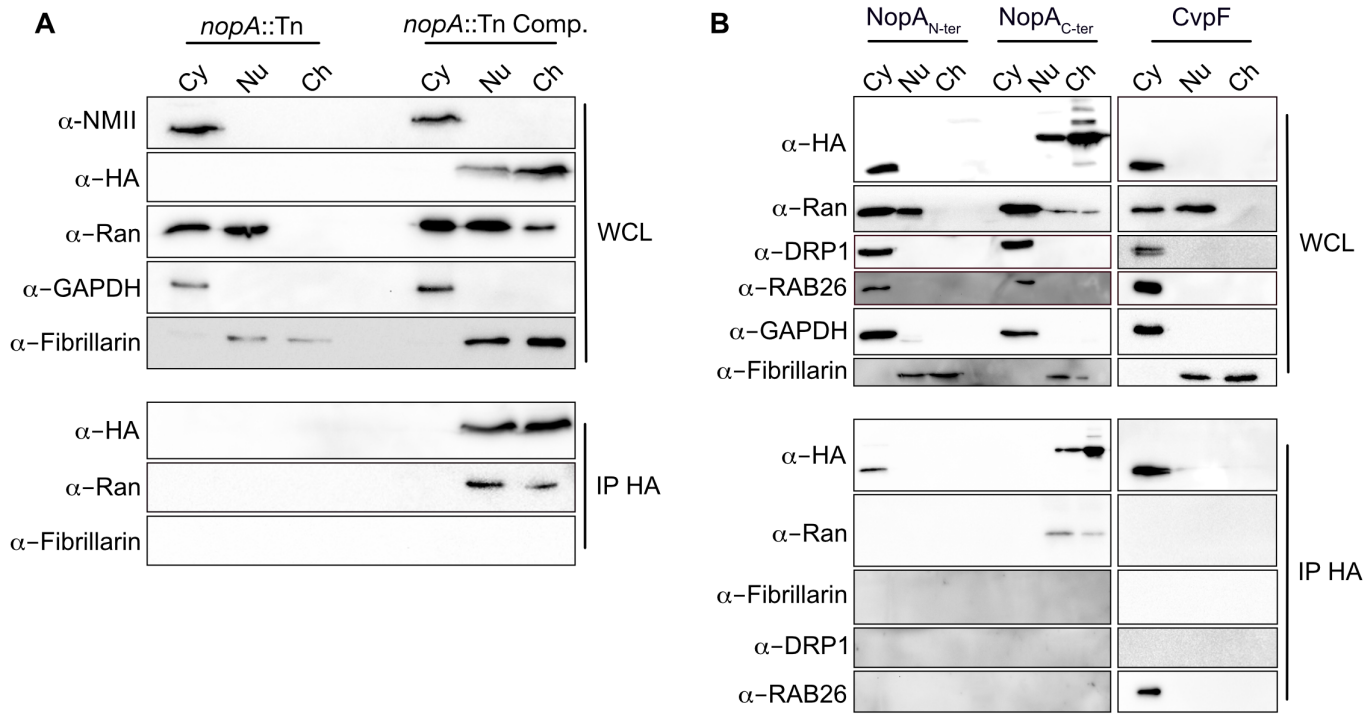


Figure 4

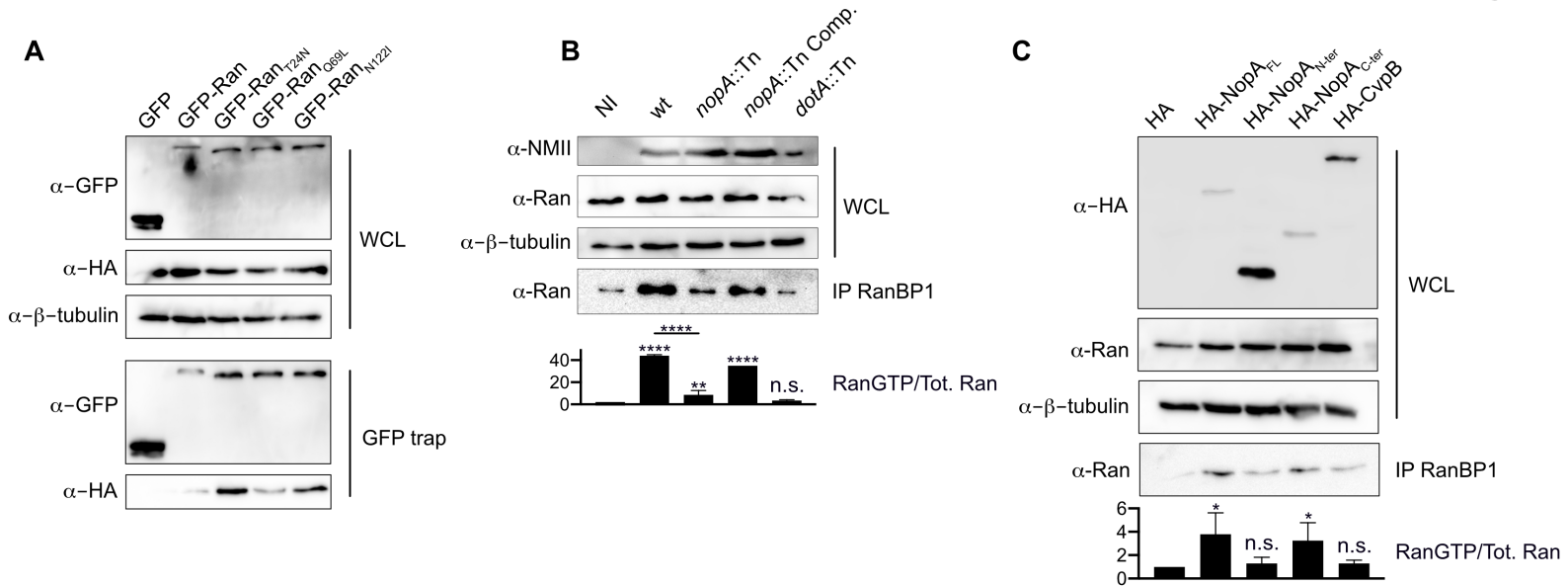
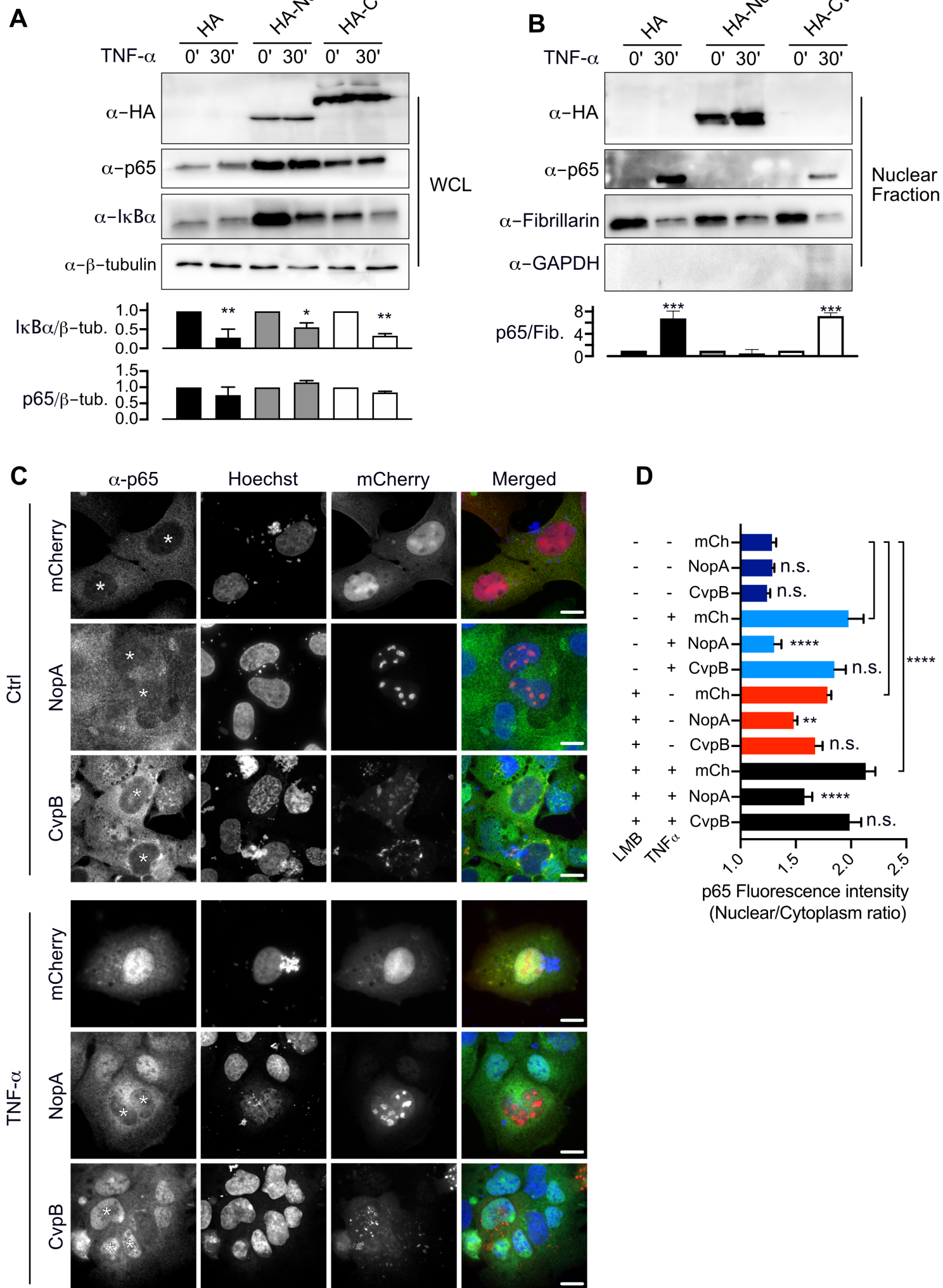
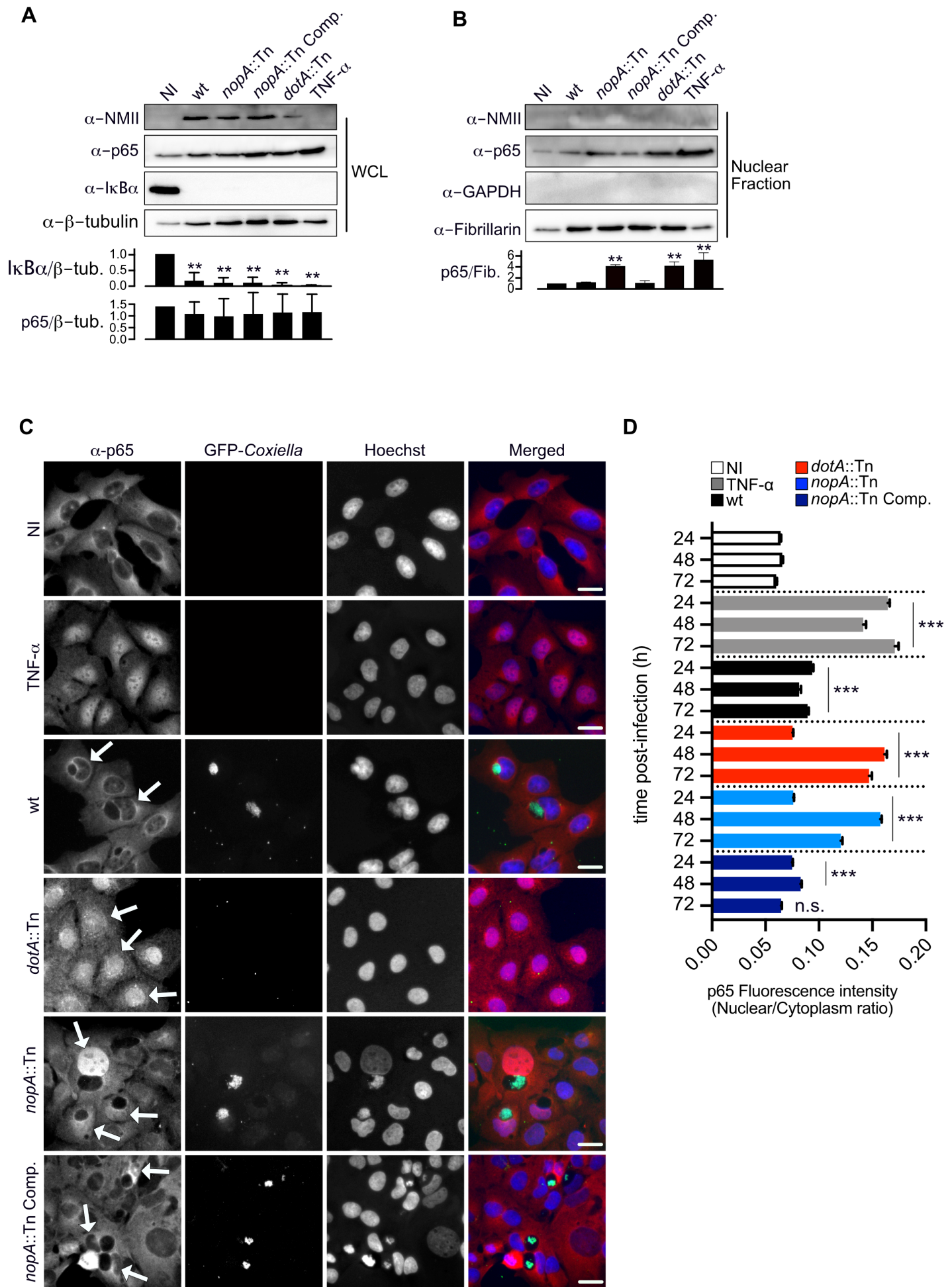
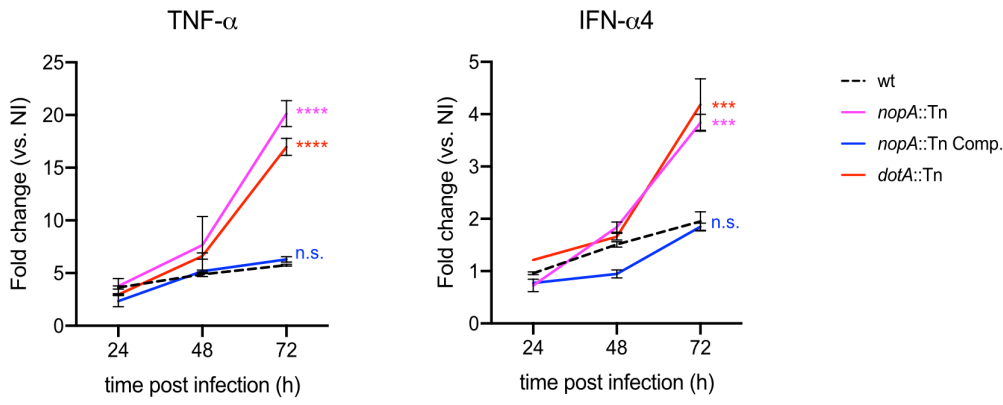


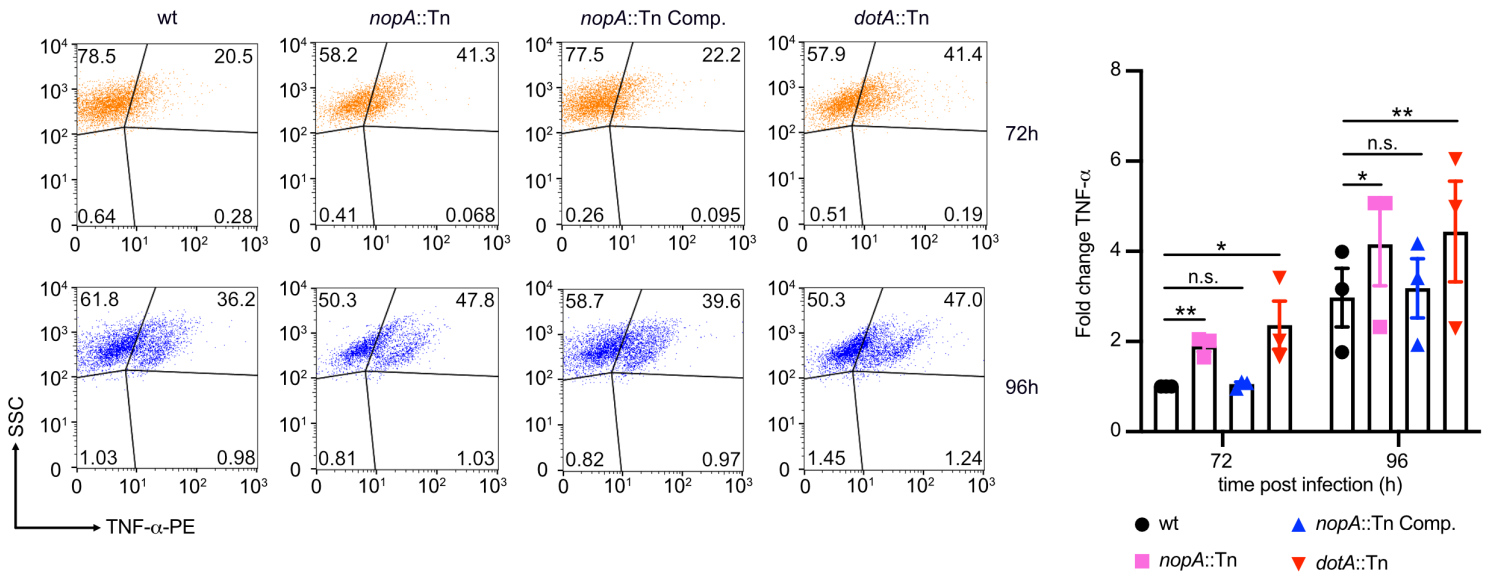
Figure 5



A



B



C

