



29 **Summary**

30 Activation of transcription factor NF- $\kappa$ B is a hallmark of infection with the gastric pathogen  
31 *Helicobacter pylori* and associated with inflammation and carcinogenesis. Genome-wide RNAi screening  
32 revealed numerous hits involved in *H. pylori*-, but not IL-1 $\beta$ - and TNF- $\alpha$ - dependent NF- $\kappa$ B regulation.  
33 Pathway analysis including CRISPR/Cas9-knockout and recombinant protein technology,  
34 immunofluorescence microscopy, immunoblotting, mass spectrometry and mutant *H. pylori* strains,  
35 identified the *H. pylori* metabolite D-glycero- $\beta$ -D-manno-heptose 1,7-bisphosphate ( $\beta$ HBP) as a *cagPAI*  
36 type IV secretion system (T4SS)-dependent effector of NF- $\kappa$ B activation in infected cells. Upon pathogen-  
37 host cell contact, TIFA forms large complexes (TIFAsomes) including interacting host factors, such as  
38 TRAF2. NF- $\kappa$ B activation, TIFA phosphorylation as well as TIFAsome formation depended on a functional  
39 ALPK1 kinase, highlighting the ALPK1-TIFA axis as core of a novel innate immune pathway. ALPK1-TIFA-  
40 mediated NF- $\kappa$ B activation was independent of CagA protein translocation, indicating that CagA  
41 translocation and HBP delivery to host cells are distinct features of the pathogen's T4SS.

42

43 **Keywords**

44 *Helicobacter pylori*, NF- $\kappa$ B signaling, genome wide RNAi screen, innate immune response, inflammation.  
45 alpha kinase 1 (ALPK1), TRAF interacting protein with forkhead associated domain (TIFA), D-glycero- $\beta$ -D-  
46 manno-heptose 1,7-bisphosphate (HBP), Type IV secretion system (T4SS)

47

## 48 **Introduction**

49 *H. pylori* chronically colonizes the gastric mucosa of about half the world's population (Parkin, 2006).  
50 Infections can lead to ulcers, lymphoma of the mucosa-associated lymphoid tissue (MALT) and gastric  
51 adenocarcinoma (reviewed in (Salama et al., 2013)). It appears in two major strains, defined by the  
52 absence or presence of a type IV secretion system (T4SS), encoded by the *cag* pathogenicity island  
53 (*cagPAI*) that functions in the translocation of *H. pylori*'s only known effector protein, CagA, into host  
54 cells, where it is phosphorylated (Backert et al., 2000). The *cagPAI* positive strains exhibit particular  
55 strong inflammatory potential and increased pathogenicity (Blaser et al., 1995; Crabtree et al., 1991).

56 Activation of the transcription factor family nuclear factor kappa B (NF- $\kappa$ B) plays a central role in  
57 inflammation and carcinogenesis (DiDonato et al., 2012; Pasparakis, 2009). It can be triggered by various  
58 stimuli, such as the inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), and  
59 infections by various pathogens. The NF- $\kappa$ B family is comprised of five members, including p65 (RelA),  
60 which in the inactivated state is sequestered in the cytoplasm by its inhibitor, inhibitor of kappa B alpha  
61 (I $\kappa$ B $\alpha$ ). Upon activation, the I $\kappa$ B kinase (IKK) complex phosphorylates I $\kappa$ B $\alpha$ , which is subsequently  
62 degraded to release p65 for translocation into the nucleus, where it guides the transcription of target  
63 genes linked to inflammation and anti-apoptosis (Hacker and Karin, 2006; Hoffmann and Baltimore,  
64 2006). Its dual function in activation of inflammation and cell survival makes NF- $\kappa$ B a lynchpin in disease  
65 development. These features are of particular importance in the gastrointestinal system, where chronic  
66 inflammation and tissue damage act as tumor promoters (Quante and Wang, 2008), but our  
67 understanding of the underlying regulatory network is still incomplete for many inducers. Several RNAi  
68 loss-of function studies have identified new factors involved in NF- $\kappa$ B pathway activation including  
69 stimuli of the nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2 receptors  
70 (Bielig et al., 2014; Lipinski et al., 2012; Warner et al., 2013; Yeretssian et al., 2011), Epstein Barr virus  
71 (Gewurz et al., 2012), *Neisseria gonorrhoeae* (Gaudet et al., 2015), and *Shigella flexneri* infections

72 (Milivojevic et al., 2017). These studies have shown that NF- $\kappa$ B signaling, although sharing core elements,  
73 is highly complex and diverse.

74 Here we report on a high content cell-based RNAi screening approach, addressing *H. pylori*-induced NF-  
75  $\kappa$ B activation. Amongst several host factors, we identified  $\alpha$ -kinase 1 (ALPK1) and TRAF-interacting  
76 protein with FHA domain (TIFA) as key mediators of *H. pylori* induced NF- $\kappa$ B activation. Upon *H. pylori*  
77 infection, TIFA undergoes the formation of large protein complexes (TIFAsomes), including TRAF2 and  
78 additional host factors involved in NF- $\kappa$ B signaling. We demonstrate that the bacterial metabolite  
79 heptose-1,7-bisphosphate (HBP) triggers this particular route of NF- $\kappa$ B activation in a T4SS-dependent  
80 manner. Our findings reveal important mechanistic insight into the ability of the innate immune system  
81 to discriminate between less and highly virulent *H. pylori* traits (Koch et al., 2016) and the process of NF-  
82  $\kappa$ B activation that depends on the pathogen's T4SS (Backert and Naumann, 2010).

83

## 84 **Results**

### 85 **New regulators of *H. pylori*-induced NF- $\kappa$ B activation**

86 As a read-out system for the identification of factors involved in NF- $\kappa$ B pathway activation we used  
87 nuclear translocation of a p65-GFP fusion protein which is amenable for analysis by automated  
88 microscopy (Bartfeld et al., 2010) (Figure 1A). Besides *H. pylori*, we used TNF- $\alpha$  and IL-1 $\beta$  as stimuli for  
89 NF- $\kappa$ B activation. Robustness of the system was assessed by targeting known factors of the NF- $\kappa$ B  
90 pathway. Accordingly, knockdown of TNF- $\alpha$  receptor 1 (TNFR1) and of the adaptor protein myeloid  
91 differentiation primary response protein 88 (MYD88) blocked the signals by TNF $\alpha$  and IL-1 $\beta$ , respectively.  
92 Moreover, combined IKK- $\alpha$  and IKK- $\beta$  knock-down abolished activation by TNF $\alpha$ , IL-1 $\beta$  as well as by *H.*  
93 *pylori* (Figure 1B). Details of three screens performed are summarized in Figure S1A-C. The first screen  
94 using a human kinase siRNA library yielded 15 primary hits with increased responsiveness to *H. pylori*  
95 infection, 4 of which were confirmed by at least 2 independent siRNAs. These included 2 genes not  
96 previously implicated in NF- $\kappa$ B signaling: ALPK1 and CDC2-related kinase, arginine/serine rich (CRKRS)  
97 (Bartfeld, 2009) (Table S1). To extend our search, a second screen was carried out with only *H. pylori*  
98 infection as inducer, using two siRNA libraries targeting the whole and the druggable genome. This  
99 analysis yielded 347 hits: 235 positive and 112 negative regulators. Out of these, 200 of the positive and  
100 100 of the negative regulators were further validated using additional siRNAs and all three inducers,  
101 which yielded 43 positive (21.5%) and 33 negative *H. pylori*-induced regulators (33.0%). Comparison to  
102 NF- $\kappa$ B induction by IL-1 $\beta$  or TNF- $\alpha$  indicated that 21 of the positive and 24 of the negative regulators  
103 were strongly biased to *H. pylori* as an inducer (Figure 1C, Table S2).

104 STRING analysis (Szklarczyk et al., 2011) of our datasets revealed prominent protein interaction networks  
105 (Figure 1D and 1E). Notably, one obvious sub-network was formed by known general NF- $\kappa$ B key  
106 regulators, consisting of IKK- $\alpha$  (Chuk), IKK- $\beta$  (IKBKB), TAK1 (MAP3K7) and p65 (RelA) itself, corroborating  
107 the functional robustness of our approach (Figure 1D). The central roles of ubiquitination and

108 proteasomal degradation in NF- $\kappa$ B signaling was well reflected by the data set (Oeckinghaus et al., 2011).  
109 Apart from major members of the proteasome (PSMA1, PSMB3, PSMA3 and PSMC4), we identified RBX1,  
110 NEDD8 and Cullin-1 as necessary for ubiquitination (Figure 1D). The RING finger-like domain-containing  
111 protein RBX1 is part of cullin-RING-based E3 ubiquitin-protein ligase (CRLs) complexes, which are  
112 regulated by NEDD8 ligation (Scott et al., 2011). We also identified RNF31 as a positive regulator for  
113 *H. pylori*, TNF- $\alpha$  and IL-1 $\beta$  NF- $\kappa$ B signaling. A prominent negative regulator, for example, included the  
114 deubiquitinase tumor necrosis factor alpha induced protein 3 (TNFAIP3), known to downregulate NF- $\kappa$ B  
115 (Harhaj and Dixit, 2011) (Figure 1E). Several of the identified hits, although not uniquely involved in  
116 *H. pylori*-induced signaling, had only a weak effect on TNF- $\alpha$ -induced and none in IL-1 $\beta$ -induced NF- $\kappa$ B  
117 signaling. Of these, ARD1 and NARG1, which form an acetyltransferase complex connected with the  
118 ubiquitylation machinery (Arnesen et al., 2005), were further validated (Figure S1D). The regulators  
119 most specific for *H. pylori* also included  $\alpha$ -kinase 1 (ALPK1) (Figure S1E, Table S2), an atypical kinase  
120 previously implicated in apical protein transport and phosphorylation of myosin IA (Heine et al., 2005).  
121 Confirmation by Western blotting showed that ALPK1 was required for degradation of I $\kappa$ B $\alpha$  after  
122 infection with *H. pylori* but not after stimulation with TNF $\alpha$  or IL-1 $\beta$  (Figure S1E). In addition, we included  
123 the TRAF-interacting protein with FHA domain (TIFA) in our validation rounds, as it had the highest score  
124 of any gene in our genome-wide RNAi screen, albeit for only one siRNA (Table S2).

125

### 126 **Upstream regulators of ALPK1**

127 Notably, our analyses revealed a cluster of histone-modifiers as *H. pylori* specific hits (Figure 1D),  
128 including host cell factor C1 (HCF1), absent, small or homeotic 2 like (ASH2L), lysine K-specific  
129 methyltransferase 2B (MLL4), and SIN3 transcription regulator family member A (SIN3A) (Dou et al.,  
130 2005; Smith et al., 2005; Tyagi et al., 2007; Wysocka et al., 2003; Yokoyama et al., 2004). With HCF1 as a  
131 known co-regulator of transcription (Tyagi et al., 2007), we hypothesized that it may indirectly affect

132 expression of other determinants detected in the screens. To our surprise, quantitative RT-PCR (qRT-  
133 PCR) guided by initial microarray analysis, indicated that ALPK1 expression was abrogated after HCF1  
134 knockdown (Figure 1F). Similarly, knockdown of ASH2L or MLL4, known to act in concert with HCF1,  
135 turned out to abrogate ALPK1 expression as well (Figure 1F). Thus, methylation events mediated by the  
136 HCF1 methyltransferase complex appeared to be required for housekeeping expression of ALPK1 and the  
137 effects of the HCF1, ASH2L and MLL4 on *H. pylori*-induced NF- $\kappa$ B activation are likely caused indirectly via  
138 their influence on ALPK1 gene expression. These findings nominated ALPK1 as a central component of  
139 NF- $\kappa$ B signal transduction.

140

#### 141 **Role of ALPK1 in NF- $\kappa$ B activation**

142 To further consolidate the function of ALPK1 in *H. pylori*-specific NF- $\kappa$ B activation, an ALPK1 deficient cell  
143 line was generated using CRISPR/Cas9 technology (Figure S2A and S2B). Knockout of ALPK1 in several  
144 independent cell lines fully prevented p65 translocation after infection (Figure 2A), more strikingly than  
145 any of the siRNAs did. Yet, ALPK1 knockout did not affect the response to TNF- $\alpha$  or IL-1 $\beta$  (Figure 2B).  
146 Consistently, ALPK1 deficiency abrogated infection-induced TAK1 and I $\kappa$ B- $\alpha$  phosphorylation and  
147 subsequent I $\kappa$ B- $\alpha$  degradation but did not influence the response to TNF- $\alpha$  treatment (Figure 2C). Cells  
148 lacking ALPK1 were unable to mount an IL-8 response upon infection, as evidenced by significantly  
149 reduced IL-8 mRNA levels in infected ALPK1 deficient cells (Figure 2D). To test if p65 translocation could  
150 be rescued, ALPK1 deficient cells were transfected with recombinant Myc-Flag-ALPK1. Indeed, only  
151 rescued cells exhibited NF- $\kappa$ B activation upon *H. pylori* infection (Figure 2E, upper panel), whereas TNF- $\alpha$   
152 treatment triggered p65 translocation also in ALPK1 deficient cells (Figure 2E, lower panel). These results  
153 identify ALPK1 as a strictly essential mediator of NF- $\kappa$ B activation in *H. pylori* infection.

154

155 **TIFA is crucial for *H. pylori*-induced NF- $\kappa$ B activation acting downstream of ALPK1**

156 Simultaneously, we also tested CRISPR/Cas9-generated TIFA knockout cells to illuminate its role in  
157 *H. pylori*-specific NF- $\kappa$ B activation (Figure S2C and D). Interestingly, the TIFA knockout line failed to show  
158 any sign of *H. pylori*-specific NF- $\kappa$ B activation, even after extended monitoring for 3 h (Figure 2A), while  
159 p65 translocation readily occurred upon treatment with IL-1 $\beta$  or TNF- $\alpha$  (Figure 2B). Further, knockout of  
160 TIFA specifically abrogated *H. pylori*-induced phosphorylation of TAK1 and I $\kappa$ B $\alpha$ , as well as the  
161 subsequent degradation of I $\kappa$ B $\alpha$  (Figure 2C) but had no effect on TNF $\alpha$ -induced TAK1 and I $\kappa$ B $\alpha$   
162 phosphorylation. Consequently, TIFA knockout cells did not show IL-8 mRNA expression upon infection,  
163 as measured by qRT-PCR 3 h p.i. (Figure 2D). Next, we sought to determine the cellular localization of  
164 TIFA by confocal immunofluorescence microscopy. Non-infected AGS SIB02 cells transfected with Myc-  
165 Flag-TIFA revealed diffuse TIFA fluorescence (red) throughout the cytoplasm and the nucleus whereas  
166 p65 location (green) was restricted to the cytoplasm and excluded from the nucleus (Figure S3A upper  
167 panel). Infection with wild type *H. pylori* or TNF- $\alpha$  treatment induced p65 nuclear translocation by 30 min  
168 (middle and lower panel). Consistent with NF- $\kappa$ B activation, only infection with wild type *H. pylori*, but  
169 not TNF- $\alpha$  treatment, induced the formation of so-called TIFAsomes (Gaudet and Gray-Owen, 2016)  
170 (Figure S3A). To further define the importance and specificity of TIFA oligomer formation upon infection,  
171 TIFA deficient cells were transfected with Myc-Flag-TIFA or a Myc-Flag-TIFA mutant for T9, an important  
172 tyrosine phosphorylation site for TIFA activation (Huang et al., 2012). In cells that remained non-  
173 transfected, only TNF- $\alpha$  treatment but not *H. pylori* infection induced p65 translocation (Figure 3A, arrow  
174 head upper panel and lower panel). Successful transfection with wild type, but not with the TIFA mutant  
175 T9A, restored both p65 translocation and TIFAsome formation upon *H. pylori* infection (arrow upper  
176 panel and middle panel). To monitor TIFAsome formation in more detail, we transfected TIFA deficient  
177 AGS cells with Tomato-TIFA and infected them with *H. pylori*. Time-lapse video microscopy of live cells  
178 confirmed infection-induced TIFAsome formation (from 7 min p.i.) and p65 translocation (from 12 min



179 p.i.) only in transfected cells (Movie S1). TIFAsome formation could not be observed in ALPK1 deficient  
180 cells but was rescued by co-transfection with ALPK1 (Figure 3B) indicating that ALPK1 acts upstream of  
181 TIFA and thus plays a crucial role in the phosphorylation of TIFA T9 required for TIFAsome formation.

182

### 183 **TIFAsomes are multifactorial protein complexes**

184 To identify protein–protein interactions (PPI) of TIFAsomes by mass spectrometry (MS) we used a Myc-  
185 Flag-TIFA fusion, which was transiently overexpressed in AGS cells for pull-down of TIFA associated  
186 proteins (Table S3). MS discovered a total of 77 TIFAsome associated proteins that were enriched after  
187 *H. pylori* infection were identified by MS. The identified TIFA-binding proteins were used to generate PPI  
188 network graphs using STRING (Figure S3B). Interestingly, our data revealed that TIFAsomes contain  
189 several classical NF- $\kappa$ B key regulators, including TAB2 and TRAF2. Moreover, TRIM21, a factor involved in  
190 ubiquitination and proteasomal degradation, was enriched in this complex, consistent with its known  
191 role in NF- $\kappa$ B regulation (Oeckinghaus et al., 2011). Other prominent components of the TIFAsomes  
192 included the microtubule- and actin-associated proteins KIF11 and myosin IIA. Intriguingly, myosin IIA  
193 has been reported to be a target for ALPK1 in patients with gout (Lee et al., 2016). We also identified  
194 annexin A2, which was previously shown to interact with the p50 subunit of NF- $\kappa$ B to regulate p50  
195 complex translocation into the nucleus (Jung et al., 2015). TRAF2 was chosen as an example for  
196 validating the MS data by immunoblotting and, evidently, co-precipitated with TIFA confirming the  
197 presence of TRAF2 in the *H. pylori*-induced TIFAsomes, consistent with the known interaction of TIFA and  
198 TRAF2 (Kanamori et al., 2002) (Figure S3C).

199

### 200 **CagA translocation and ALPK1-TIFA activation constitute independent pathways**

201 In line with a number of previous studies (reviewed in Backert and Naumann (2010) p65 translocation,  
202 TAK1 activation and IL-8 mRNA upregulation in our system were dependent on the T4SS, but not on CagA  
203 (Figure S4A-S4C). To test whether activation of the ALPK1-TIFA axis itself also depends solely on T4SS  
204 function, we transfected AGS SIB02 cells with Tomato-TIFA for monitoring TIFAsome formation after  
205 infection (Figure 4A). As anticipated, TIFAsomes quickly formed after infection with wild type and CagA-  
206 deficient *H. pylori* but not the T4SS-deficient *cagPAI* mutant (Figure 4A). To test whether ALPK1 or TIFA in  
207 turn have an impact on CagA translocation, we determined CagA phosphorylation. Interestingly, CagA  
208 phosphorylation readily occurred in TIFA- or ALPK1-deficient cells (Figure 4B), suggesting that neither of  
209 the two factors is required for CagA translocation. To extend this analysis to other factors previously  
210 associated with CagA translocation, we generated a CRISPR/Cas9 knock out of the carcinoembryonic  
211 antigen-related cell adhesion molecule 1 (CEACAM1), recently identified as a receptor of HopQ (Javaheri  
212 et al., 2016; Koniger et al., 2016), an adhesin critical for T4SS function (Belogolova et al., 2013). In  
213 agreement with the proposed pleiotropic receptor recognition by HopQ, we observed partial  
214 dependency of both CagA translocation and NF- $\kappa$ B activation on the presence of CEACAM1 (Figure S4D-  
215 F). This places the branching point of the two processes, CagA translocation and activation of the ALPK1-  
216 TIFA axis, immediately downstream of the HopQ-CEACAM interaction.

217

#### 218 **T4SS required for release of HBP and ALPK1-TIFA dependent NF- $\kappa$ B activation**

219 We speculated that formation of TIFAsomes and subsequent activation of NF- $\kappa$ B could be triggered by a  
220 bacterial component secreted in a T4SS-dependent fashion. The pathogen-associated molecular pattern  
221 (PAMP) heptose-1,7-bisphosphate (HBP), which has recently been shown to activate TIFA (Gaudet et al.,  
222 2015; Milivojevic et al., 2017), appeared a likely candidate. HBP is a monosaccharide produced in the  
223 synthesis pathway of Gram-negative lipopolysaccharides (Valvano et al., 2002). We thus prepared  
224 bacterial lysates as previously described (Gaudet et al., 2015) from wild type *H. pylori*, as well as the

225 corresponding  $\Delta cagA$ ,  $\Delta cagL$  and  $\Delta cagPAI$  mutant strains, and used them to transfect wild type, as well  
226 as TIFA- and ALPK1-deficient AGS SIB02 cells. qRT-PCR analysis showed that within 3 h IL-8 expression  
227 was upregulated in control cells transfected with lysates from both T4SS competent and T4SS deficient  
228 bacteria, as well as with lysate prepared from Gram-negative *N. gonorrhoeae* but not from Gram-positive  
229 *L. monocytogenes* (Figure 5A). By contrast, in ALPK1- or TIFA-deficient cells no increase in IL-8 expression  
230 was observed upon transfection of any lysates. Moreover, we observed TIFAsome formation and nuclear  
231 translocation of p65 in NF- $\kappa$ B reporter cells upon transfection of all *H. pylori*- (wild type,  $\Delta cagA$ ,  $\Delta cagL$   
232 and  $\Delta cagPAI$ ) and *N. gonorrhoeae*- but not *L. monocytogenes*-derived lysates (Figure 5B and data not  
233 shown). Translocation of p65 in NF- $\kappa$ B reporter cells transfected with *H. pylori* wild type lysate was also  
234 monitored by time-lapse video microscopy and, in contrast to the activation by live *H. pylori*, occurred in  
235 a non-synchronized fashion (Movie S2).

236 IL-8 was also upregulated upon transfection with lysates from the Gram-negative *S. paratyphi* and *E. coli*  
237 (Figure 5C), further supporting the idea that the NF- $\kappa$ B-inducing factor is a molecule expressed  
238 specifically by Gram-negative bacteria such as HPB. Thus, we chemically synthesized D-glycero- $\beta$ -D-  
239 manno-heptose 1,7-bisphosphate ( $\beta$ HBP), as well as its anomer D-glycero- $\alpha$ -D-manno-heptose 1,7-  
240 bisphosphate ( $\alpha$ HBP), and the monophosphate D-glycero-D-manno-heptose 7-phosphate (heptose 7-P)  
241 (Figure S5A-C). Transfecting AGS cells with increasing amounts of  $\beta$ HBP, as compared to  $\alpha$ HBP or heptose  
242 7-P, induced IL-8 expression (Figure 5D) and formation of TIFAsomes (Figure 5B, lower panel), an  
243 observation further confirmed by time-lapse video microscopy of p65 translocation (Movie S3). This  
244 suggests NF- $\kappa$ B activation can be induced by translocation of the HBP  $\beta$ -anomer via the ALPK1-TIFA axis.  
245 Generation of a *H. pylori rfaE* knockout mutant corroborated the action of HBP as the specific inducer of  
246 NF- $\kappa$ B. RfaE catalyzes the phosphorylation of D-glycero-D-manno-heptose-7-phosphate at the C-1  
247 position to generate HBP. Infection with this mutant still allowed for CagA translocation but not NF- $\kappa$ B  
248 activation (Fig. 5E and F). To demonstrate that the observed ALPK1-TIFA axis-dependent induction of NF-

249  $\kappa$ B is not a cell line specific phenomenon, we tested gastric primary cells isolated from patient samples,  
250 expanded as organoids and seeded in 2D (Schlaermann et al., 2016) prior to transfection with  
251 recombinant TIFA. Consistent with our previous observations we found that *H. pylori* wild type, but not  
252 the *cagPAI* mutant, infection caused formation of TIFAsomes in these authentic gastric cells. This  
253 indicates that the ALPK1-TIFA signaling route is active in human primary cells (Figure 6).

254

255 **Discussion**

256 Discriminating pathogenic from non-pathogenic microbial traits is a prime function of the innate immune  
257 system and the epithelial barrier is equipped with exquisite sensory means to accomplish this task. We  
258 were interested in understanding the basis of the innate recognition of highly pathogenic strains of  
259 *H. pylori*, known to elicit particularly strong NF- $\kappa$ B-based immune reactions in the gastric mucosa  
260 depending on their *cagPAI*-encoded T4SS (Backert and Naumann, 2010; Koch et al., 2016). Our extensive  
261 screening program now provides a list of host factors involved in nuclear translocation of p65 selectively  
262 stimulated by *H. pylori* as compared to IL-1 $\beta$  and TNF- $\alpha$ . Of the main hits, ALPK1 identified in the initial  
263 kinome screen (Bartfeld, 2009) appeared to be furthest upstream in the pathway, triggered by the T4SS-  
264 dependent infusion of HBP to the host cell cytosol. ALPK1, either directly or indirectly, then causes TIFA  
265 phosphorylation and complex formation with an array of host factors, leading to activation of classical  
266 NF- $\kappa$ B signaling (Figure 7). This uncovers the HPB-ALPK1-TIFA axis as the key regulator of *H. pylori* T4SS-  
267 dependent NF- $\kappa$ B activation.

268 ALPK1 is a member of the atypical kinase family alpha kinases that recognize phosphorylation sites  
269 within  $\alpha$ -helices (Ryazanov et al., 1999). Until recently, the only substrates suggested for ALPK1 were  
270 myosin IA and IIA (Heine et al., 2005; Lee et al., 2016). Indeed, we found myosin IIA to be part of the  
271 TIFAsomes but whether it is phosphorylated by ALPK1 or plays a role in the activation of innate immunity  
272 during *H. pylori* infection has to be elucidated. A recent report also identified ALPK1 as necessary for TIFA  
273 oligomerization after infection with *S. flexneri* (Milivojevic et al., 2017), connecting it to the innate  
274 immune response. A role for ALPK1 and TIFA in inflammation was first predicted from an analysis of  
275 major genetic susceptibility loci in the experimental IL10<sup>-/-</sup> mouse model of inflammatory bowel disease  
276 (Bleich et al., 2010). ALPK1 was later found to be upregulated in patients with gout and to modulate the  
277 expression of inflammatory cytokines after the addition of urate crystals in an experimental gout model  
278 (Wang et al., 2011). The role of ALPK1 in these inflammatory scenarios is still unknown, but the

279 importance in innate immune sensing of *S. flexneri* (Milivojevic et al., 2017) together with the data  
280 presented here point to a general role of ALPK1 in HBP sensing, making it a key regulator of  
281 inflammation.

282 TIFA is the direct neighbor of ALPK1 on human chromosome four. It is a ubiquitously expressed  
283 cytoplasmic protein first identified as a TRAF2 binding protein that activates the NF- $\kappa$ B pathway  
284 (Kanamori et al., 2002). TIFA phosphorylation at T9 promotes NF- $\kappa$ B activation through self-  
285 oligomerization and interaction with TRAF (Huang et al., 2012) and has recently been shown to be  
286 triggered by HBP (Gaudet et al., 2015) or by oxidative stress via Akt kinase (Lin et al., 2016). The kinase  
287 activity of ALPK1 is required for TIFA oligomerization after infection with *S. flexneri* (Milivojevic et al.,  
288 2017) and *H. pylori*, as we show here. However, whether ALPK1 directly phosphorylates TIFA at T9, which  
289 is not within the  $\alpha$ -helix classically recognized by atypical kinases, remains to be resolved.

290 Both our RNAi screening and mass spectrometry analyses point towards a complex array of host factors  
291 involved in NF- $\kappa$ B activation by *H. pylori* whose functions remain largely unknown. However, amongst  
292 the positive regulators in the RNAi screens we identified HCF1, ASL2, and MLL4 as important for ALPK1  
293 transcriptional activation. Future downstream target analysis might provide insights into genetic  
294 programs co-regulated with ALPK1. Specificity for *H. pylori* was also seen for two acetyltransferases,  
295 ARD1 and NARG1. Moreover, immune precipitation of TIFA revealed a large protein complex consisting  
296 of factors that likely integrate signals upstream from ALPK1 and guide them towards the more classical  
297 NF- $\kappa$ B route. In fact, TIFAsomes contained a number of core NF- $\kappa$ B pathway components, including  
298 TRAF2 and TAB2, as well as many more, whose contribution to NF- $\kappa$ B signaling (and potentially also  
299 additional pathways) awaits future analysis. Interestingly, ALPK1 itself did not co-localize with  
300 TIFAsomes, as evidenced by both immune precipitation/mass spectrometry and immunofluorescence of  
301 transfected cells, pointing towards a transient or indirect phosphorylation mechanism of this kinase.

302 As the *H. pylori* component responsible for NF- $\kappa$ B activation, we here identify the small molecule  
303 metabolite  $\beta$ HBP, which is a precursor of LPS previously recognized as a PAMP of several Gram-negative  
304 bacteria, including *N. gonorrhoeae* (Gaudet et al., 2015) and *S. flexneri* (Milivojevic et al., 2017). We  
305 demonstrate the strict dependency of NF- $\kappa$ B activation on the pathogen's functional T4SS, suggesting  
306 that it delivers  $\beta$ HBP to the inside of host cells. Accordingly, mutants of not only the T4SS, but also the  
307 respective LPS biosynthetic pathway (*rfaE*), fail to induce NF- $\kappa$ B and downstream IL-8. Consistently, we  
308 found no evidence in support of the previously proposed function of CagA in NF- $\kappa$ B activation (Lamb et  
309 al., 2009), nor of the proposed role of small peptidoglycans supposedly translocated by *H. pylori* (Viala et  
310 al., 2004). Moreover, no significant hits in our screens pointed to a role of NOD1, NOD2, or respective  
311 downstream factors (Lipinski et al., 2012; Warner et al., 2013; Yeretssian et al., 2011).

312 The T4SS machinery is thought to be activated by the interaction of certain *cagPAI*-encoded ligands  
313 (CagL, CagA, CagY, CagI) with members of the integrin family (Kwok et al., 2007, Jiménez-Soto et al.,  
314 2009, Rohde et al., 2003). In addition, we have identified HopQ as a further factor critical in this process  
315 (Belogolova et al., 2013) and subsequent work in other laboratories has shown that HopQ interacts with  
316 members of the CEACAM family of receptors (Koniger et al., 2016, Javaheri et al., 2016). Both types of  
317 interaction appear to be crucial for translocation of the CagA effector protein (Belogolova et al., 2013,  
318 Oleastro and Ménard, 2013). Interestingly, activation of the ALPK1-TIFA-NF- $\kappa$ B axis is independent of  
319 CagA, and the *rfaE* mutant still supports CagA translocation. These findings mechanistically separate  
320 CagA translocation from  $\beta$ HBP delivery, although both depend on the functional integrity of T4SS. While  
321 we show that this striking pathway functions readily in authentic normal human gastric primary cells,  
322 and that TIFA activation occurs in the very first minutes of *H. pylori* infection of the epithelium, it  
323 constitutes the initial trigger of a broader inflammatory cascade initiated by this pathogen, to which  
324 additional autocrine and paracrine cellular pathways contribute (Backert et al., 2016; Velin et al., 2016).  
325 Here, we have identified a novel mechanistic principle of how pathogenic *H. pylori* bacteria are

326 recognized by innate immune defense at the stage of initial epithelial cell contact. This finding will guide

327 future work towards a better understanding of pathogen defense and gastric pathogenesis.

328



329 **Experimental Procedures**

330

331 **Ethical permissions.** For Primary cell preparation, gastric tissue samples from individuals undergoing  
332 gastrectomy or sleeve resection were obtained from the Clinics for General, Visceral and Transplant  
333 Surgery, and the Center of Bariatric and Metabolic Surgery, Charité University Medicine, Berlin,  
334 Germany. Usage of the pseudonymized samples for experimental purposes was approved by the ethics  
335 committee of the Charité University Medicine, Berlin (EA1/058/11 and EA1/129/12).

336

337 **Bacterial strains and cultivation.** The following *H. pylori* strains were used in this study: P1 and P12 wild  
338 type (strain collection no. P213 and P511) and the mutant strains P12 $\Delta$ cagPAI, P12 $\Delta$ cagA and P12 $\Delta$ cagL  
339 and P12 $\Delta$ rfaE (strain collection no. P387, P378, P454 and 588). *H. pylori* cultivation was carried out as  
340 described before (Backert et al., 2000). If not otherwise mentioned MOI 100 was used.

341

342 **Cell Culture.** AGS cells stably transfected with a p65-GFP construct (AGS SIB02, NF- $\kappa$ B reporter) (Bartfeld  
343 et al., 2010), AGS SIB02 CRISPR-Cas9 control and knockout cells (AGS STZ001, AGS STZ003 and AGS  
344 STZ004) and the parental AGS cells (ATCC CRL 1739, human gastric adenocarcinoma epithelial cell line)  
345 were cultivated in RPMI medium (Gibco) supplemented with 10% heat inactivated fetal calf serum and 2  
346 mM L-glutamate. Cultures were kept at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. AGS cell infections  
347 were carried out after serum starvation in serum free medium.

348

349 **Primary cell culture.** Culture of gastric primary glands was performed as described by Schlaermann et al.  
350 (2016) Briefly, cells from freshly isolated glands were seeded in Matrigel for the formation of organoids

351 and expanded. Cells were sheared every 10 days and for experimental use seeded on collagen coated  
352 coverslips and grown for 1-2 days in 2D medium.

353  
354 **p65 translocation assay.** AGS SIB02 cells were seeded into 12-well plates and infected with *H. pylori*  
355 (MOI 100, 30min) or stimulated by IL-1 $\beta$  (10 ng/ml, Peprotech) or TNF- $\alpha$  (20 ng/ml, Peprotech) for 30  
356 min under serum-free conditions, fixed with ice-cold Methanol, nuclei counterstained with Hoechst  
357 33342 (2  $\mu$ g/ml, Molecular Probes). Images were acquired using automated microscopy (Olympus) NF- $\kappa$ B  
358 translocation was analyzed using an automated cell-based assay.

359  
360 **Screening Procedure.** For the kinome screen we used the Qiagen “Human Kinase siRNA Set”, containing  
361 1,292 siRNAs targeting 646 kinase and kinase-associated genes. To reduce sample size, two siRNAs for  
362 each gene were pooled and tested in six conditions: *H. pylori* (45 min and 90 min), TNF $\alpha$  (30 min and 75  
363 min) and IL 1 $\beta$  (45 min and 90 min) using four independent biological replicates. AGS SIB02 cells were  
364 seeded onto 96-well plates and transfected using the RNAiFect Transfection Kit (Qiagen). One day after  
365 transfection, cells were split into new wells (96-well-plate or 12-well-plate according to experimental  
366 setting). Experiments were conducted after a minimum of 60 h to allow reduction of target protein  
367 levels. Data was normalized (either using plate-median or, in comparison, z-score) and significance was  
368 assessed with Welch’s t-test. Primary hits were identified combining statistical significance (p-value  
369  $\leq 0.05$ ). For the validation screen the 5% top candidates were taken into account for further analysis and  
370 an additional 4 siRNAs were tested and hits were confirmed with at least two siRNAs each with a z-score  
371 of  $\leq -1$  or  $\geq 1$ .

372

373 For the primary screen the Qiagen Human Druggable Genome siRNA Set V2.0 Library containing 4 siRNAs  
374 per gene and the Qiagen Human Genome 1.0 Library containing 2 siRNAs per gene were used.  
375 Altogether 24,000 genes were targeted by 62,000 individual siRNAs. For the validation screen 4  
376 individual siRNAs were purchased from Qiagen. AGS SIB02 cells were seeded into 384-well plates, siRNAs  
377 were transfected with HiperFect transfection reagent (Qiagen), 72 h post transfection cells were  
378 stimulated for 45 min by infecting with *H. pylori* (MOI 100) or in the validation screen cells were  
379 additionally induced by IL-1 $\beta$  (10 ng/ml, Miltenyi Biotec.) or TNF- $\alpha$  (10 ng/ml, Becton Dickinson) for 45  
380 min under serum-free conditions, fixed with ice-cold methanol, nuclei counterstained with Hoechst  
381 33342 (2  $\mu$ g/ml, Sigma Aldrich). Images were acquired using automated microscopy (Olympus) NF- $\kappa$ B  
382 translocation was analysed using an automated cell-based assay as recently described (Bartfeld et al.,  
383 2010). In short, cell nuclei were detected and the surrounding cytoplasmic area set using image analysis  
384 software (Scan<sup>^</sup>R, Olympus) following quantification of translocation of p65-GFP. Experiments were  
385 performed in triplicates. Data analysis primary screen: First, plate normalization was performed using  
386 CellHTS2, then Redundant siRNA Analysis (RSA) was used for defining the hit candidates. Positive  
387 regulators are defined by an RSA-score  $\geq -2$ , negative regulators by an RSA-score of  $\geq +2$ . Hit validation  
388 screen was performed for 300 selected primary hit candidates (200 positive and 100 negative regulators)  
389 using 4 siRNAs per gene. Cells were transfected for 72 h and NF- $\kappa$ B activated with either *H. pylori*  
390 infection, TNF- $\alpha$  or IL-1 $\beta$ . Cells were fixed after 45 min and p65-GFP translocation automatically  
391 quantified. Data were analyzed using CellHTS2. Hit genes were defined according to their Z-score:  $< -1$  for  
392 positive and  $> 1$  for negative regulators. As a positive control for all screening experiments the  
393 combinatorial knockdown of IKK $\alpha$  and  $\beta$  was used.

394

395 **Immunoblotting.** Cells were directly lysed in 2x Laemmli buffer, separated by 10% SDS-PAGE, transferred  
396 to PDVF membranes, blocked in TBS buffer supplemented with 0.1% Tween 20 and 5% milk, and probed

397 against primary antibodies at 4°C overnight. Membranes were probed with matching secondary horse-  
398 radish peroxidase-conjugated antibodies (Amersham, 1:3000) and detected with ECL reagent (Perkin  
399 Elmer). Primary antibodies: Anti I $\kappa$ B $\alpha$  (44D4) #4812, p-TAK1 (Thr 184/187) #4508 and p-I $\kappa$ B $\alpha$  (Ser32)  
400 (14D4) #2859, pNF- $\kappa$ B p65 (Ser536) #3033 from Cell Signaling; anti phospho-tyrosine 99 (pY99) sc-7020,  
401 anti CagA (b300) sc-25766, and  $\beta$ -actin A5441 from Sigma Aldrich.

402  
403 **qRT-PCR.** qRT-PCR was performed using Power SYBR Green RNA-to-C<sub>T</sub> 1-Step Kit (Applied Biosystems)  
404 according to the manufacturer's recommendations using the following primers: GAPDH  
405 5'-GGTATCGTGGAAGGACTCATGAC-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3', IL-8  
406 5'-ACACTGCGCCAACACAGAAAT-3' and 5'-ATTGCATCTGGCAACCCTACA-3', TIFA  
407 5'-TGGTAAACCGTCATCTGGAG-3' and 5'-GAGTTCCTGACTCCCCAGC -3'. ALPK1  
408 5'-CACCAAGAACAACAATAGCCG-3' 5'-ACCTGAAGGATGTGATTGGC-3'

409  
410 **Plasmids.** For overexpression of *N*-terminal wild type Myc-Flag-TIFA we used full length human TIFA  
411 cDNA from Origene #RC204357 where we introduced a silent TGG to TCG point mutation encoding L103  
412 to destroy the PAM site preventing CAS9 cutting of TIFA when overexpressed in TIFA deficient cells  
413 (strain collection no. pMW930). Mutation of the PAM site and TIFA T9A mutation (strain collection no.  
414 pMW931) were introduced by using PfuTurbo DNA Polymerase (Agilent Technologies) complying with  
415 the protocol of Stratagene's single-site QuikChange *Site-Directed Mutagenesis* Kit. For generating *N*-  
416 terminal Tomato-TIFA, TIFA from pMW930 was amplified and cloned into ptdTomato N1 (modified from  
417 Clontech #6085-1) (strain collection no. pSTZ006). Human ALPK1 was amplified and cloned into pLenti-  
418 *N*-Myc-DDK (strain collection no. pMW909).

419 Myc-Flag-ALPK1 5'- CTGCCGCCGCGATCGCCATGAATAATCAAAAAGTGGTAGC-3' and  
420 5'- TTGCGGCCGCGTGCATGGTTTCTCCATTGAAG-3', Tomato-TIFA  
421 5'- CGGCTAGCCGCCATGACCAGTTTTGAAG -3'and 5'- CGGAATTCCGGTTGACTCATTTTCATCCATTCT -3'

422  
423 **Confocal microscopy.** AGS, AGS SIB02 or primary cells were seeded on poly-l-lysine or collagen coated  
424 glass coverslips and transfected for 24h with recombinant ALPK1, recombinant TIFA or its functional  
425 mutants using FuGENE6 (Promega). Cells were left uninfected or infected with *H. pylori* (MOI 100),  
426 treated with TNF- $\alpha$  (20 ng/ml) or transfected for 3 h with bacterial lysates using lipofectamine 2000  
427 (Thermo Fisher Scientific) under serum free conditions. For visualization of MYC-tagged protein, cells  
428 were fixed with 4 % PFA, blocked with 3% BSA, 5%FCS, 0.3Tritx100 for 1 h at RT and incubated with  
429 primary antibodies (anti-MYC (CST, Cat. No 9B11, 1:6000), anti-tubulin (Abcam, Cat. No. ab 6160, 1:100))  
430 overnight at 4°C. Secondary staining was performed using fluorescently coupled antibodies (donkey anti  
431 rabbit-Cy3 (1:100, Cat. No. 711-166-020), goat anti rat-Dylight® 649 (1:100 Cat. No. 112-486-062), DAPI  
432 (1:300, Roche, Cat. No. H1840-10)). Samples were mounted with Moviol, analyzed by laser scanning  
433 microscopy using a Leica SP8. Images were processed using ImageJ.

434  
435 **Bacterial lysate preparation and transfection.** Bacterial lysates were prepared as described in Gaudet et  
436 al., 2015 (Gaudet et al., 2015). In short, *H. pylori* and *N. gonorrhoeae* were grown overnight on GC agar  
437 plates, *E. coli* was grown overnight on LB agar plates, *L. monocytogenes* was grown overnight in brain  
438 heart infusion medium and *S. paratyphi* was grown overnight in LB media. Bacteria from overnight  
439 cultures were collected in PBS and resuspended in water at OD<sub>600</sub> 1 and boiled for 15 min at 400 rpm  
440 shaking. Bacterial debris was removed by centrifuged at 4000 x g for 3 min and the supernatant treated  
441 with 10  $\mu$ g/mL RNase A (New England Biolabs), 1 U/mL DNase 1 (Qiagen) and 100  $\mu$ g/mL ProteinaseK  
442 (New England Biolabs) for 5 min at RT. Samples were boiled again for 5 min and passed through a 0,22

443  $\mu\text{m}$  filter. For lysate transfection of cells in a 24-well format, 5  $\mu\text{l}$  lysate was mixed with 5  $\mu\text{l}$   
444 lipofectamine 2000 (Thermo Fisher Scientific) and 30  $\mu\text{l}$  OptiMEM (Gibco), incubated for 30 min at RT  
445 and added to AGS cells at 70% confluence.

446  
447 **Statistical analysis.** All graphs were prepared using GraphPad Prism 7 software (GraphPad Software Inc.,  
448 CA, USA). Data are presented as mean  $\pm$  SEM. Data was considered significant if  $p < 0.05$ .

449

450 **Additional Supplemental Files**

451 **Table S1 Results of kinome screen.**

452 Names of genes are listed alphabetically with gene identification number (Gene ID). accession numbers  
453 (Accession #), mean numbers of cells counted in four pictures of a well (Cell #) and z-scores, which give  
454 indication of the strength of the effect. Negative z-scores indicate inhibition and positive z-scores  
455 indicate promotion of p65-translocation. In the primary screen as well as in the hit validation, each siRNA  
456 was tested in six conditions: H1 = *H. pylori* 45 min; H2 = *H. pylori* 90 min; T1 = TNF $\alpha$  30 min; T2 = TNF $\alpha$   
457 75 min; I1 = IL-1 $\beta$  45 min; I2 = IL-1 $\beta$  90 min. In the primary screen. two siRNAs per gene were pooled and  
458 the mean z-score of four experiments using this pool is shown. To identify primary hits. bold colored font  
459 marks the z-score in the condition in which the gene scored as a primary hit during the primary screen  
460 (red for inhibition, blue for promotion of p65-translocation). In the hit validation four siRNAs per gene  
461 were tested separately and for each siRNA. a mean z-score of four experiments was calculated. Here, the  
462 sum of the z-scores of four siRNAs is shown. To identify z-score sums composed of at least two effective  
463 siRNAs with z-scores of  $\leq -1$  or  $\geq 1$ . Bold colored font marks the z-score-sum in the condition in which at  
464 least two siRNAs were effective (red/blue as above). Colored cells and gene names in bold colored font  
465 indicate confirmed hits, defined as genes where at least two siRNAs were effective in the hit validation in  
466 the same condition as it was selected for in the screen (red/blue as above).

467  
468 **Table S2 Results of primary and validation screen.** Primary screen: 24,000 genes were silenced using  
469 Qiagen siRNA libraries. NF- $\kappa$ B activation was induced with *H. pylori*. Experiments were done in triplicates.  
470 CellHTS2 and Redundant siRNA Analysis (RSA) was used for defining the hit candidates. According to the  
471 corresponding RSA-score, 235 candidates were found to positively regulate and 112 factors to negatively  
472 regulate *H. pylori* NF- $\kappa$ B pathway. Validation screen: 300 candidate genes (200 positive and 100 negative  
473 regulators from the primary screen) were tested. *H. pylori* and in addition IL-1 $\beta$  and TNF $\alpha$  were used as  
474 inducers for NF- $\kappa$ B activation. Data analysis based on CellHTS2. The Venn diagram shows the validated

475 factors. Apart from general factors functioning for all inducers, 21 positive regulators and 24 negative  
476 regulators unique for the *H. pylori* NF- $\kappa$ B pathway were found.

477

478 **Table S3 Results of mass spectrometry analysis of protein–protein interactions of TIFAsomes.**

479 Composition of 78 candidate proteins identified upon *H. pylori* infection as analyzed by UHPLC/MS/MS.

480

481 **Movie S1 *H. pylori* infection activates NF- $\kappa$ B via TIFAsome formation.** AGS SIB02 TIFA knockout cells

482 were seeded on 8 $\mu$ -well dishes (Ibidi) and transfected with pdtTomato-TIFA. Cells were infected with

483 *H. pylori* (MOI 100) and imaged on a Zeiss Axiovert 200M wide field microscope with an incubator at

484 37°C and 5% CO<sub>2</sub> using a Hamamatsu Orca camera. Images for Tomato-TIFA and DIC were recorded every

485 15 s. The setup was controlled by the Volocity software (Perkin Elmer) which was also used to generate

486 the video.

487

488 **Movie S2 Translocation of p65 in NF- $\kappa$ B reporter cells transfected with *H. pylori* lysate.** AGS SIB02 cells

489 were seeded on 8 $\mu$ -well dishes (Ibidi) and transfected with lysates from *H. pylori*. Live cell imaging was

490 performed using a Leica TCS-SP5 inverted confocal microscope with an incubator at 37°C and 5% CO<sub>2</sub>.

491 Frames for GFP and DIC were recorded every 30 s and merged into a video using Volocity software

492 (Perkin Elmer).

493

494 **Movie S3  $\beta$ HBP induces p65 translocation.** AGS SIB02 cells were seeded on 8 $\mu$ -well dishes (Ibidi). Each

495 well of the plate was transfected with a defined concentration of HPB. Wells were imaged in succession

496 by constantly moving the stage from well to well. Images were recorded on a Zeiss Axiovert 200M wide

497 field microscope with an incubator at 37°C and 5% CO<sub>2</sub> using a Hamamatsu Orca camera. Images for



498 eGFP and DIC were recorded every 2 mins. The setup was controlled by the Volocity software (Perkin  
499 Elmer) which was also used to generate the videos.

500

#### 501 **Author contributions**

502 S.B., N.M. performed kinome screen and revealed ALPK1; J.L., N.M. carried out the genome-wide screen;  
503 A.M., K.P.P., M.R. performed bioinformatics analyses; M.K., F.G. M.R. recognized factors including TIFA;  
504 C.R., M.R., S.B., O.S., M.N. performed NF-kB pathway analysis; S.Z. validated factors using CRISPR/Cas9;  
505 S.Z., M.A.Z., L.P. characterized HBP-ALPK1-TIFA interactions and performed cell assays; M.S. generated  
506 genetic constructs; A.Z., P.K. synthesized HBP; M.S. performed mass spectrometry; V.B., M.A.Z., L.P.  
507 confocal microscopy; T.F.M. conceived and supervised the project; S.Z., M.A.Z., L.P., S.B., T.F.M. wrote  
508 manuscript;

509

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514

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647

648 **Figure legends**

649 **Fig. 1: Genome-wide RNAi screen for *H. pylori* induced NF- $\kappa$ B activation.** (A) Screen setup: 72 h post-  
650 transfection with siRNAs AGS SIB02 cells (GFP-p65) were infected with *H. pylori* or induced with IL-1 $\beta$  or  
651 TNF- $\alpha$ , fixed and analyzed for p65 translocation into the nucleus. (B) Knockdown of NF- $\kappa$ B pathway  
652 activators leads to reduced p65 translocation in cells treated as in (A) and fixed at 0, 15, 30, 45, 60, 75, 90  
653 or 105 min followed by quantification via automated microscopy. Inhibitory siRNAs used are IKK $\alpha$  and  
654 IKK $\beta$  for *H. pylori*, TNF-R1 for TNF- $\alpha$  or MyD88 for IL-1 $\beta$ . Control siRNA is Allstars. (C) Venn diagram  
655 showing the validated factors. Apart from factors functioning for all inducers, 21 positive and 24 negative  
656 regulators unique for the *H. pylori*-induced NF- $\kappa$ B pathway were found. (D, E) Selected protein-protein  
657 interactions among (D) positive or (E) negative regulators from the screen in connection to the NF- $\kappa$ B  
658 core pathway, identified with a confidence score of  $\geq 0.4$  from the STRING database. Hits found to be  
659 unique for *H. pylori* NF- $\kappa$ B signaling are shown in bold. Ubiquitination and proteasomal degradation is  
660 known to be heavily interconnected with the NF- $\kappa$ B core pathway. Additionally, we identified an  
661 acetylation complex formed by ARD1 and NARG1 and methylation complex consisting of HCF1, MLL4 and  
662 Ash2L to be important positive regulators in *H. pylori* NF- $\kappa$ B pathway. In contrast, the identified kinase  
663 ALPK1 is not connected to the NF- $\kappa$ B pathway. Among negative regulators we found the known NF-  
664  $\kappa$ B inhibitors TNFAIP3, CREBBP and NFKB1 (F) siRNA-mediated loss of MLL4, Ash2L or HCF1 strongly  
665 reduced the level of ALPK1 mRNA, as identified by qRT-PCR.

666  
667 **Fig. 2. ALPK1 and TIFA are essential for the *H. pylori*-induced activation of NF- $\kappa$ B.** CRISPR/Cas9-  
668 mediated silencing of ALPK1 or TIFA in AGS SIB02 cells (A) impedes NF- $\kappa$ B activation after infection with  
669 *H. pylori* MOI 100 for the indicated times. Shown is percentage of cells with nuclear p65 per well. (B)  
670 Silencing does not influence TNF- $\alpha$ - or IL-1 $\beta$ -induced NF- $\kappa$ B activation. (C) Loss of ALPK1 or TIFA blocks  
671 TAK1 phosphorylation at Thr-184/187 and I $\kappa$ B- $\alpha$  phosphorylation at Ser32. I $\kappa$ B- $\alpha$  degradation was

672 blocked specifically in ALPK1 or TIFA deficient cells at 30 min post *H. pylori* infection at MOI 100.  
673 Silencing ALPK1 did not inhibit TNF- $\alpha$ -dependent (20 ng/ml for 30 min) NF- $\kappa$ B activation. Blot  
674 representative of at least two independent experiments. (D) qRT-PCR analysis of IL-8 transcription in  
675 ALPK1 and TIFA depleted AGS cells infected with *H. pylori* at MOI 100 for 30 min, normalized to non-  
676 infected control cells. (E) ALPK1 rescues NF- $\kappa$ B activation upon *H. pylori* infection (MOI 100 for 30 min) in  
677 ALPK1 depleted AGS SIB02 cells expressing GFP-p65 (green) transfected with Myc-Flag-ALPK1 for 24 h.  
678 Cells were labelled for Myc (magenta). ALPK1-expressing cells show nuclear translocation of p65 (top).  
679 Cells activated instead with TNF- $\alpha$  (20 ng/ml, for 30 min) also show nuclear translocation of p65.  
680 Arrowheads: non transfected, not activated cells, arrows: transfected, activated cells. Scale bar: 20  $\mu$ m.  
681 Data in graphs represents mean  $\pm$  SEM of three independent experiments. Statistical analysis was  
682 performed using Student's *t*-test (\*\*\*)  $p \leq 0.0001$ .

683  
684 **Fig. 3: TIFA activates NF- $\kappa$ B upon *H. pylori* infection through formation of TIFAsomes dependent on**  
685 **ALPK1.** (A) AGS SIB02 were depleted for TIFA using CRISPR/Cas9 and were transfected for 24 h with wild  
686 type or T9A mutated Myc-Flag-TIFA, or an empty vector cDNA constructs prior to infection. Only wild  
687 type TIFA rescues TIFAsome formation and NF- $\kappa$ B activation upon *H. pylori* infection (MOI 100, 30 min).  
688 Cells activated with TNF- $\alpha$  (20 ng/ml, 30 min) did not show TIFAsome formation. TIFA labelled in red.  
689 Arrowheads: non transfected, not activated cells, arrows: transfected, activated cells. Scale bar: 20  $\mu$ m.  
690 (B) Lack of ALPK1 hinders TIFAsome formation in cells infected with *H. pylori* and can be rescued by  
691 transfection with ALPK1. AGS SIB02 were depleted for ALPK1 using CRISPR/Cas9 and transfected for 24 h  
692 with Myc-Flag-TIFA (red) or Tomato-TIFA (red) combined with Myc-Flag-ALPK1. *H. pylori* infection (MOI  
693 100 for 30 min) did not induce TIFAsomes or NF- $\kappa$ B activation unless cells were co-transfected with  
694 ALPK1 and TIFA. Scale bar: 20  $\mu$ m.

695  
30

696 **Fig. 4: NF- $\kappa$ B activation and TIFAsome formation via ALPK1 and TIFA is independent of CagA**  
697 **translocation but dependent of a functional T4SS** (A) *H. pylori* WT and the  $\Delta$ cagA but not the  $\Delta$ cagPAI  
698 deletion mutant induce TIFAsome formation and NF- $\kappa$ B translocation. AGS SIB02 cells were transfected  
699 for 24 h with Tomato-TIFA (red) and then infected with *H. pylori* WT,  $\Delta$ cagA or  $\Delta$ cagPAI (MOI 100, 30  
700 min). Scale bar: 20  $\mu$ m. (B) CagA translocation and phosphorylation is not affected in cells depleted for  
701 TIFA or ALPK1 via CRISPR/Cas9. Cells were infected with *H. pylori* WT at MOI 100 and analyzed by  
702 Western blot at 3 h p.i. for tyrosine phosphorylation and total CagA. Blots are representative of two  
703 independent experiments.

704  
705 **Fig. 5. Bacterial lysates (HBP) trigger NF- $\kappa$ B activation dependent on ALPK1-TIFA axis.** (A) Transfection  
706 with bacterial lysates derived from *H. pylori* T4SS competent (P12 WT and P12 $\Delta$ cagA) and T4SS deficient  
707 strains induce IL-8 mRNA expression in AGS control cells but not in cells depleted of TIFA- or ALPK1 by  
708 CRISPR/Cas9. Bacterial lysates were prepared as described (Gaudet et al., 2015) and transfected into  
709 cells using lipofectamine. Levels of IL-8 mRNA were measured using qRT-PCR 3 h post transfection and  
710 normalized to mock transfected control cells. Data represent the mean  $\pm$  SEM of three independent  
711 experiments. Statistical analysis was performed using Student's *t*-test (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.001$ ;  
712 \*\*\*,  $p \leq 0.0001$ ; ns = not significant). (B) TIFAsome formation induced by *H. pylori* lysates from wild type  
713 and T4SS mutant bacteria and by  $\beta$ HBP. AGS SIB02 cells were transfected for 24 h with Tomato-TIFA  
714 (red), then mock transfected or transfected with lysates derived from *H. pylori* WT, the  $\Delta$ PAI mutant, *L.*  
715 *monocytogenes* or  $\beta$ HBP for 3 h, fixed and analyzed by microscopy. Scale bar: 20  $\mu$ m. (C) Bacterial lysates  
716 from Gram-negative bacteria but not from Gram-positive *Listeria* induce IL-8 mRNA expression in AGS  
717 cells treated as in (A). Data represent the mean  $\pm$  SEM of three independent experiments. (D) D-Glycero-  
718  $\beta$ -D-manno-heptose 1,7-bisphosphate ( $\beta$ HBP) but not D-glycero- $\alpha$ -D-manno-heptose 1,7-bisphosphate  
719 ( $\alpha$ HBP) or D-glycero-D-manno-heptose 7-phosphate (heptose 7-P) induce IL-8 mRNA expression in AGS

720 cells 3 h post transfection, normalized to mock transfected control cells. (E) CagA phosphorylation can be  
721 detected in cells upon infection with a *H. pylori rfaE* mutant. Wild type cells and cells infected with *H.*  
722 *pylori* WT and  $\Delta rfaE$  mutant at MOI 100 and analyzed by Western blot 3 h p.i. for tyrosine  
723 phosphorylation and total amounts of CagA. Blots are representative of two independent experiments.  
724 (F) NF- $\kappa$ B activation by *H. pylori* is dependent on expression of *rfaE*. AGS SIB02 cells were infected with  
725 wild type P12,  $\Delta rfaE$ ,  $\Delta cagA$  and  $\Delta cagPAI$  *H. pylori* (MOI 100, 30 min) and nuclear p65 translocation  
726 analyzed by automated microscopy. Unless otherwise indicated, data represent the mean  $\pm$  SEM of two  
727 independent experiments. See also Movie S2 and S3.

728

729 **Fig. 6. TIFAsome formation in primary gastric epithelial cells.** Human gastric primary cells were grown as  
730 2D monolayers as previously described (Schlaermann et al., 2016) and transfected with Tomato-TIFA for  
731 24 h using FuGENE6. Cells were left uninfected or infected with *H. pylori* P12 WT and  $\Delta PAI$  (MOI 100, 30  
732 min) then fixed and counterstained for DNA (DAPI). As in AGS cells, infection with T4SS competent  
733 bacteria induced formation of TIFAsomes.

734

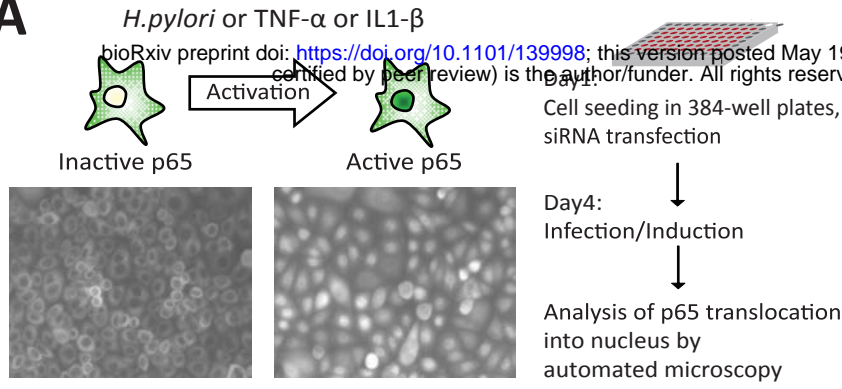
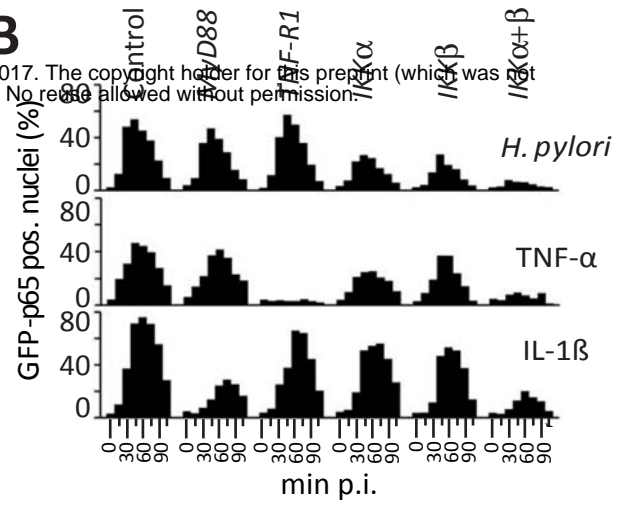
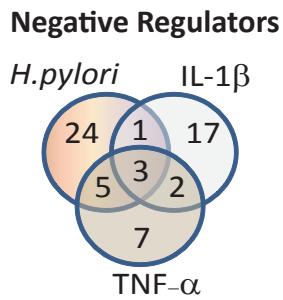
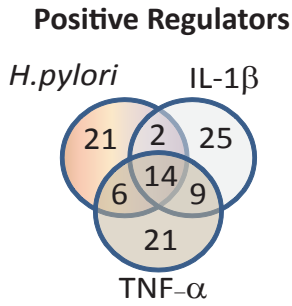
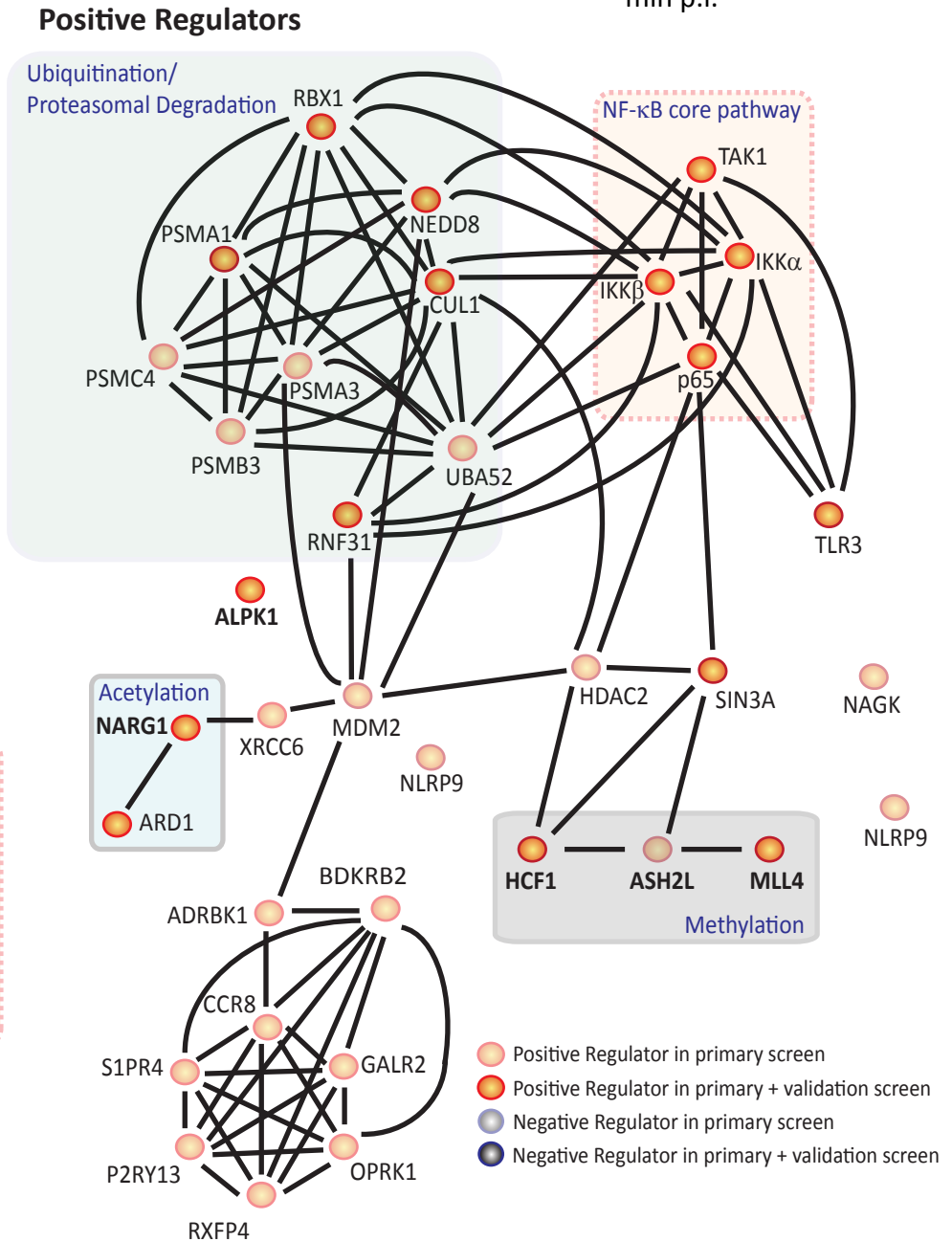
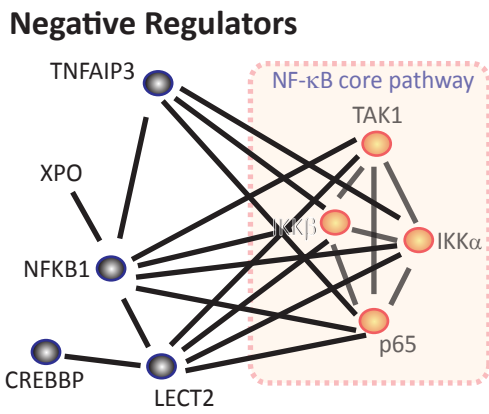
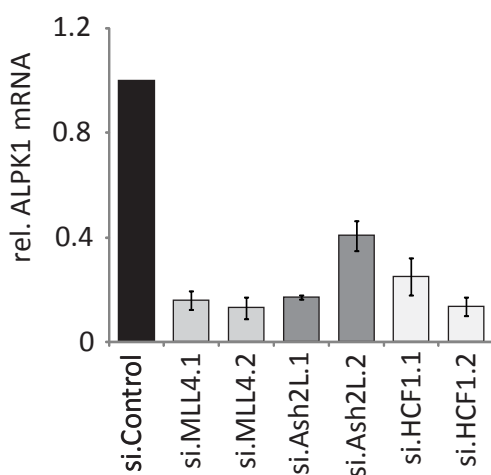
735 **Fig. 7. Schematic of *H. pylori* – host cell interaction leading to NF- $\kappa$ B pathway activation.** *H. pylori* T4SS  
736 function depends on binding mediated by HopQ-CEACAMs and T4SS –  $\alpha 1\beta 5$  integrins and facilitates the  
737 translocation of CagA and  $\beta$ HBP.  $\beta$ HBP in turn activates the ALPK1-TIFA-axis.

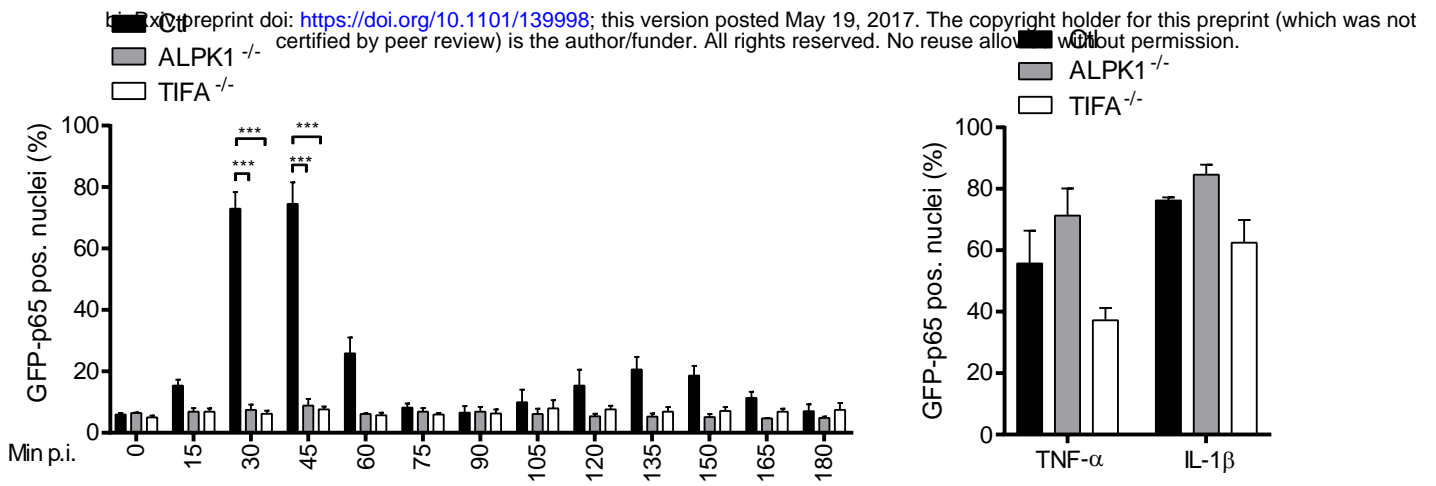
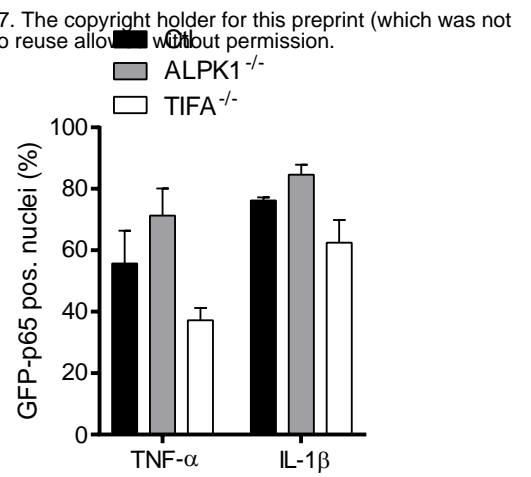
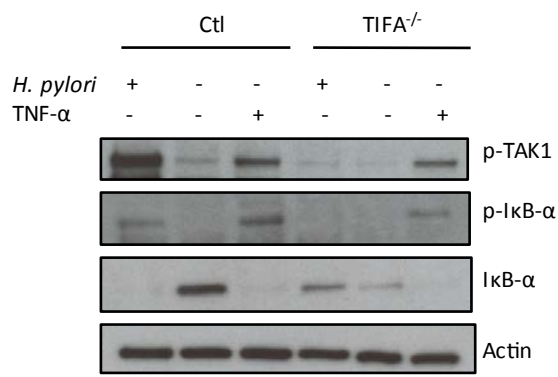
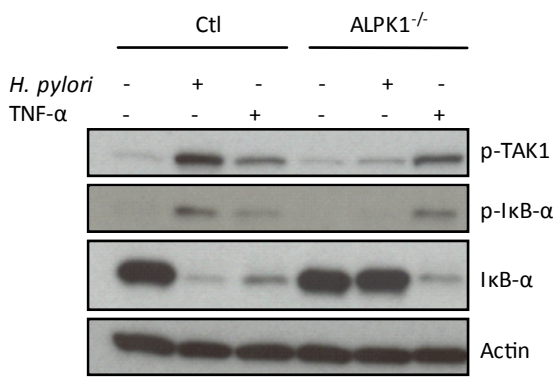
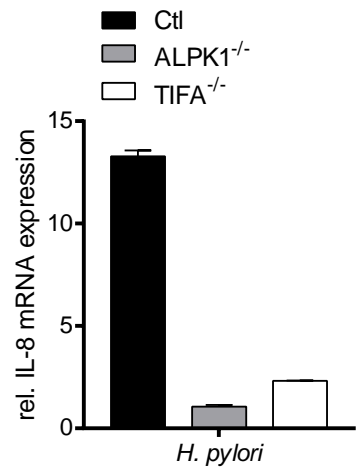
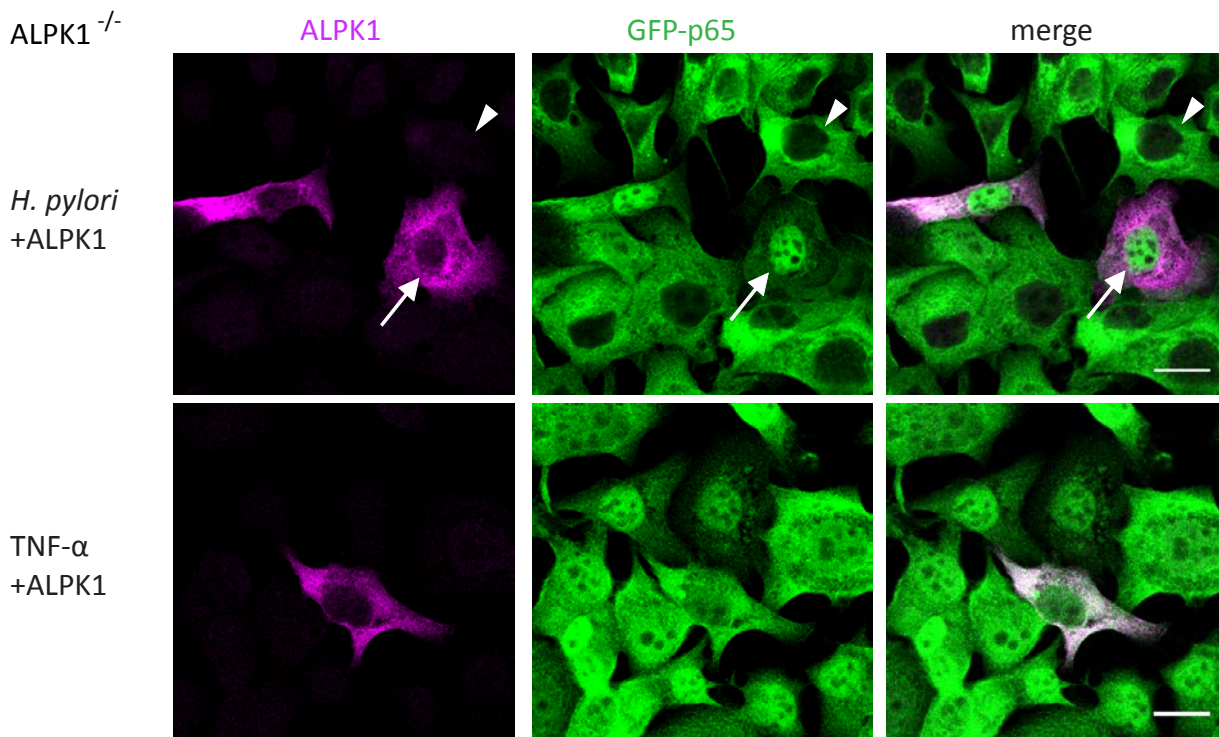


735 cells 3 h post transfection, normalized to mock transfected control cells. (E) CagA phosphorylation can be  
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738 phosphorylation and total amounts of CagA. Blots are representative of two independent experiments.  
739 (F) NF- $\kappa$ B activation by *H. pylori* is dependent on expression of *rfaE*. AGS SIB02 cells were infected with  
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741 analyzed by automated microscopy. Unless otherwise indicated, data represent the mean  $\pm$  SEM of two  
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743  
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752 translocation of CagA and  $\beta$ HBP.  $\beta$ HBP in turn activates the ALPK1-TIFA-axis.

**Fig 1****A****B****C****D****E****F**

**Fig 2****A****B****C****D****E**

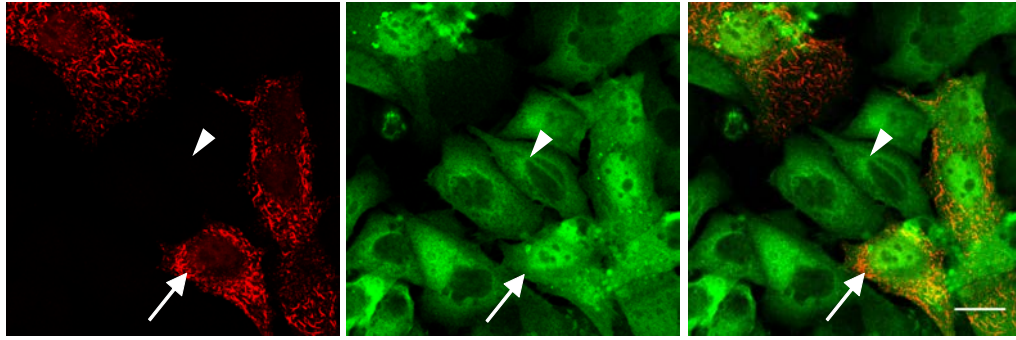
# Fig 3

## A

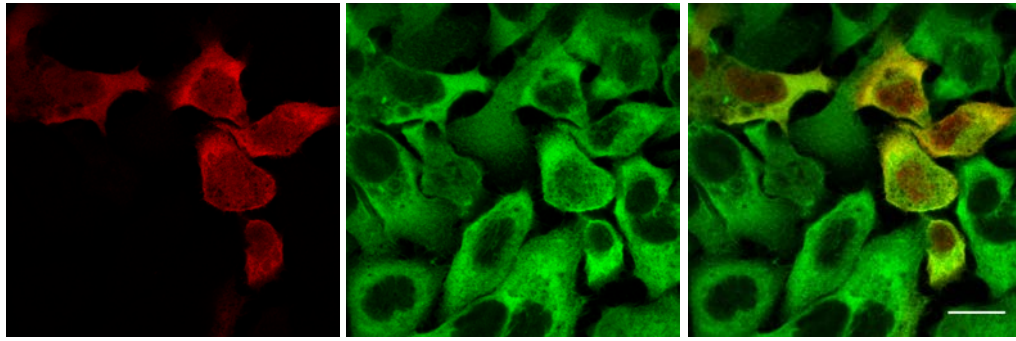
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TIFA<sup>-/-</sup>

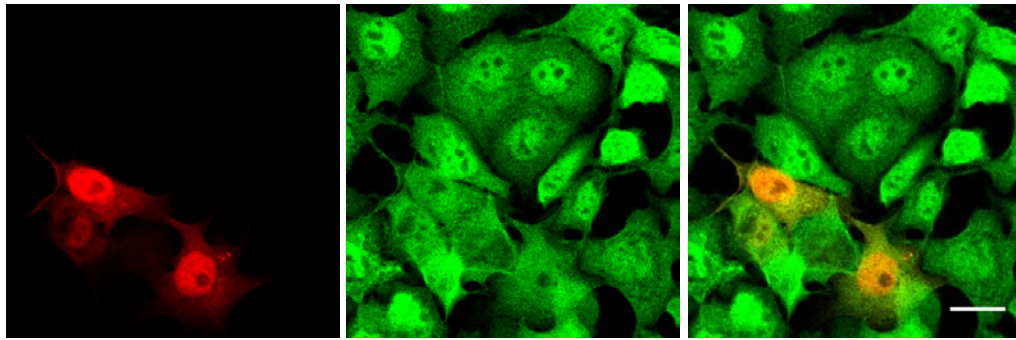
*H. pylori*  
+TIFA



*H. pylori*  
+TIFA T9A



TNF- $\alpha$   
+TIFA



## B

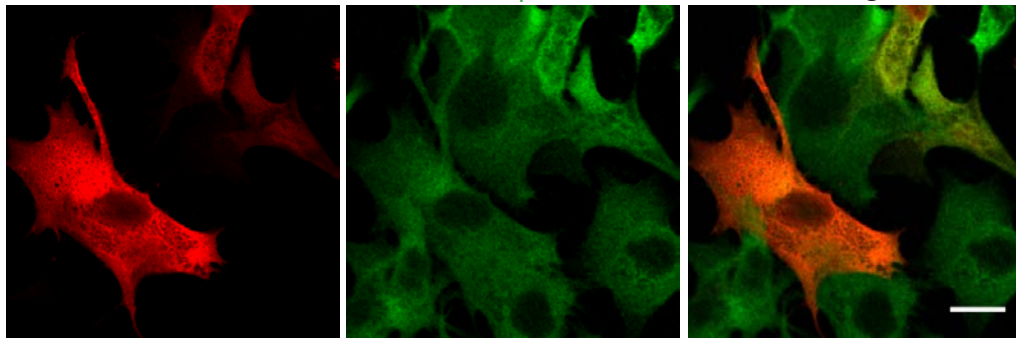
ALPK1<sup>-/-</sup>

TIFA

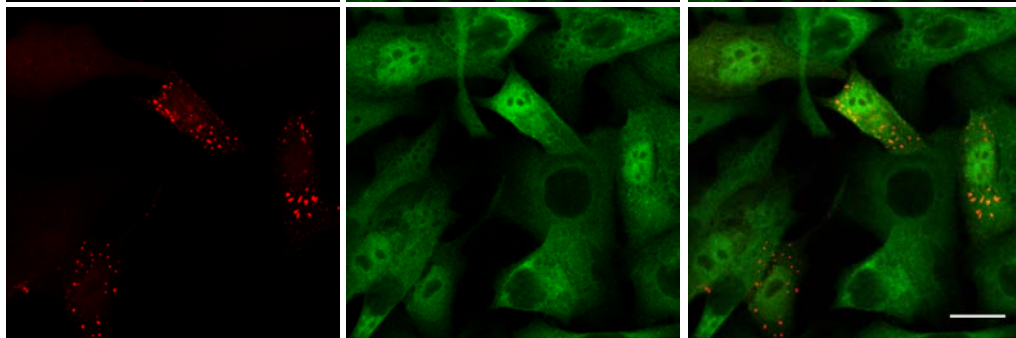
GFP-p65

merge

*H. pylori*  
+TIFA

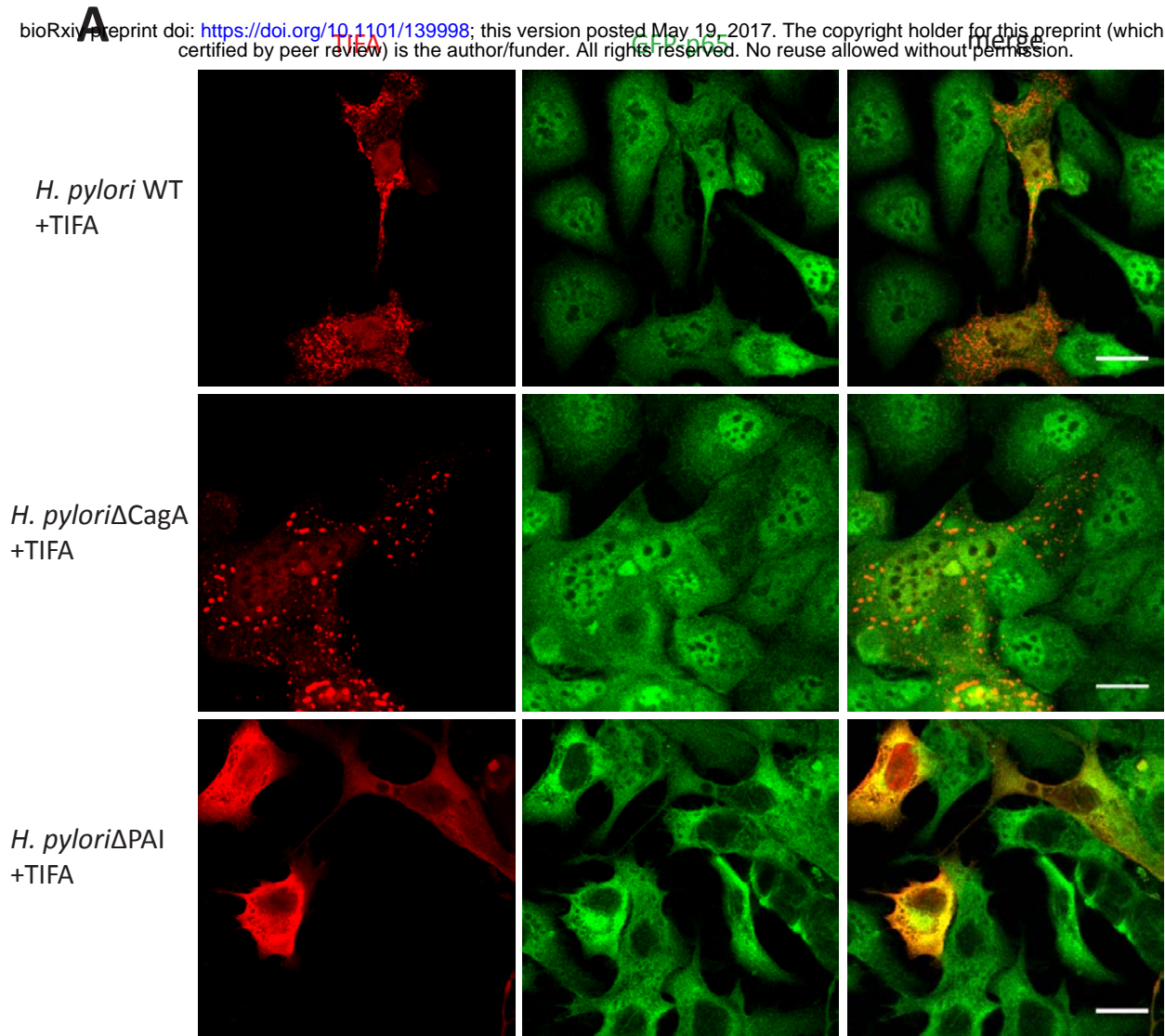


*H. pylori*  
+TIFA  
+ALPK1

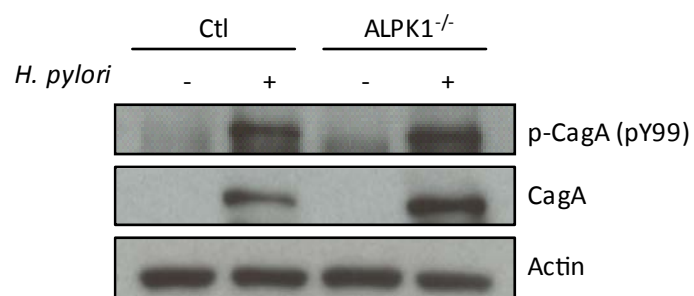
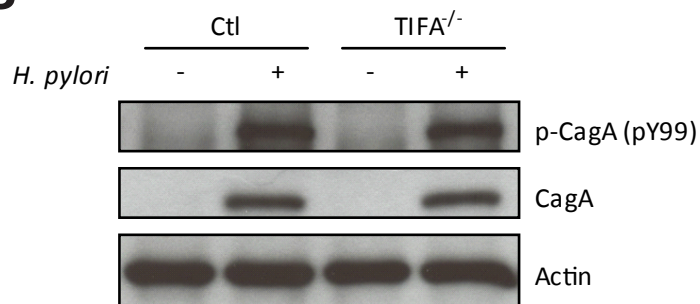


# Fig 4

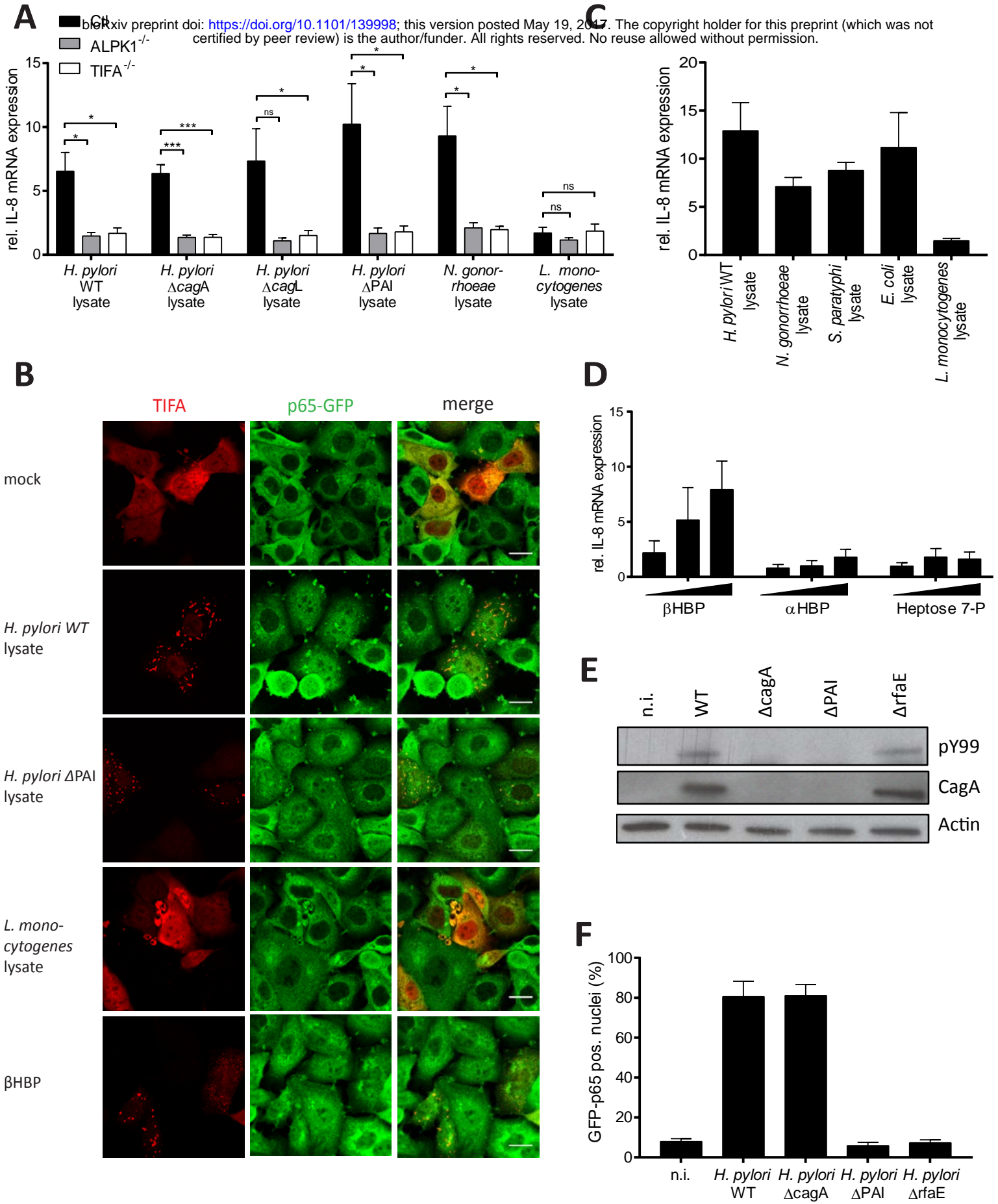
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## B



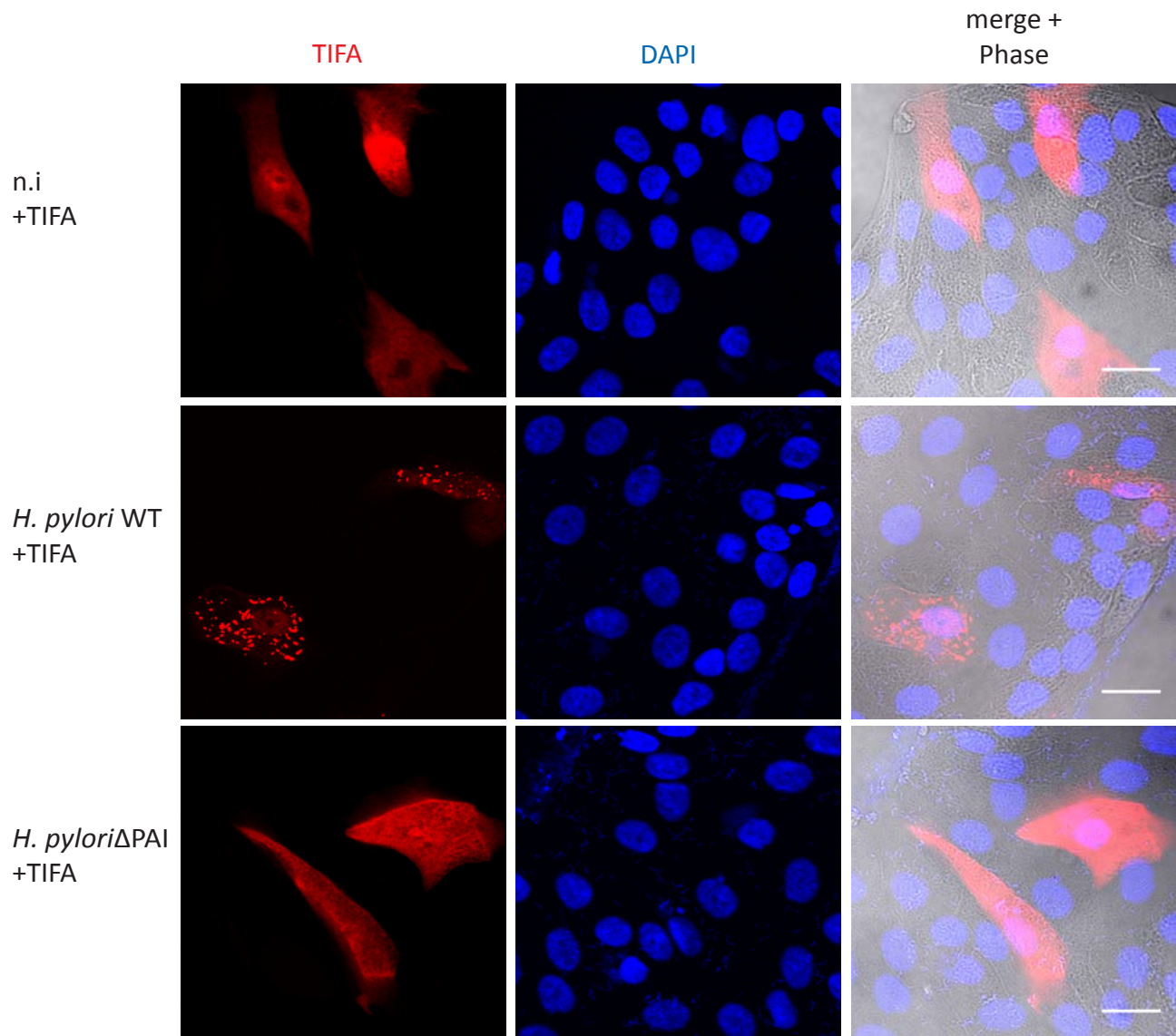
**Fig 5**



# Fig 6

## A

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# Fig 7

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