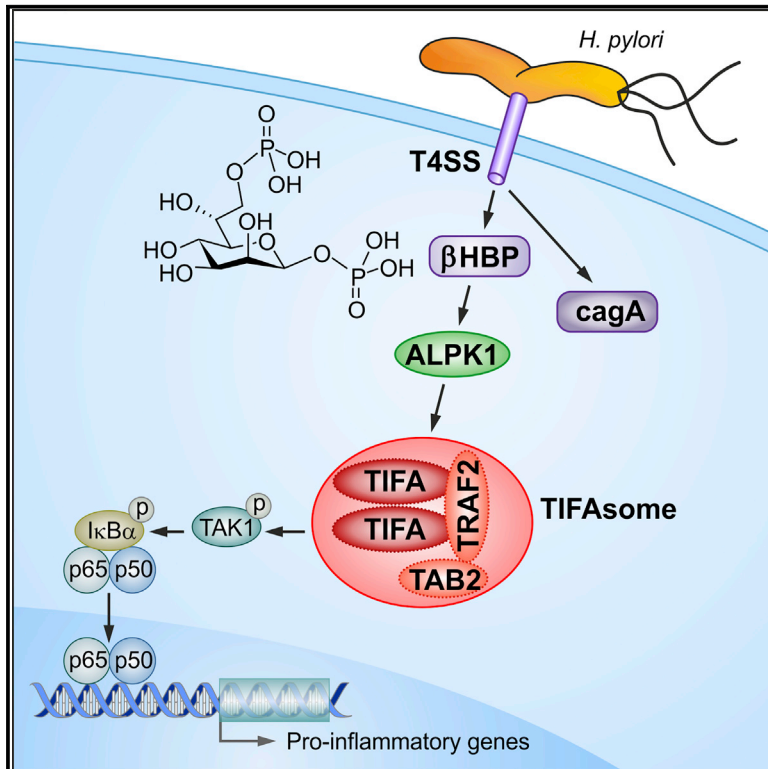


ALPK1- and TIFA-Dependent Innate Immune Response Triggered by the *Helicobacter pylori* Type IV Secretion System

Graphical Abstract



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In Brief

Zimmermann et al. identify pathogen and host factors involved in NF-κB activation after infection with type IV secretion-proficient *Helicobacter pylori*. Central hits are ALPK1 and TIFA. ALPK1 is necessary for phosphorylation-dependent formation of TIFA complexes (TIFAsomes) with TRAF2. This yields HBP-ALPK1-TIFA-TRAF2-NF-κB as the core-regulon of *H. pylori*-induced innate immune activation.

Highlights

- Genome-wide RNAi screen identifies key innate immune pathway induced by *H. pylori*
- Release of LPS metabolite HBP via *H. pylori*'s type IV secretion activates NF-κB
- The ALPK1-TIFA axis constitutes a core pathogen-specific pattern recognition system



ALPK1- and TIFA-Dependent Innate Immune Response Triggered by the *Helicobacter pylori* Type IV Secretion System

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SUMMARY

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

INTRODUCTION

H. pylori chronically colonizes the gastric mucosa of about half the world's population (Parkin, 2006). Infections can lead to ulcers, lymphoma of the mucosa-associated lymphoid tissue (MALT), and gastric adenocarcinoma (reviewed in Salama

et al., 2013). It appears in two major strains, defined by the absence or presence of a type IV secretion system (T4SS), encoded by the *cag* pathogenicity island (*cagPAI*) that functions in the translocation of its only known effector protein, CagA, into host cells, where it is phosphorylated (Backert et al., 2000). The *cagPAI* positive strains exhibit increased inflammatory potential and pathogenicity (Blaser et al., 1995; Crabtree et al., 1991).

Activation of the transcription factor family nuclear factor κ B (NF- κ B) plays a central role in inflammation and carcinogenesis (DiDonato et al., 2012; Pasparakis, 2009). It can be triggered by stimuli such as the inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), and infections by various pathogens. The NF- κ B family comprises five members, including p65 (RelA), which in the inactivated state is sequestered in the cytoplasm by inhibitor of kappa B alpha (I κ B α). Upon activation, the I κ B kinase (IKK) complex phosphorylates I κ B α , which is subsequently degraded to release p65 for translocation into the nucleus, where it guides the transcription of target genes linked to inflammation and anti-apoptosis (Häcker and Karin, 2006; Hoffmann and Baltimore, 2006). Its dual function in activation of inflammation and cell survival makes NF- κ B a lynchpin in disease development. These features are of particular importance in the gastrointestinal system, where chronic inflammation and tissue damage act as tumor promoters (Quante and Wang, 2008), but our understanding of the underlying regulatory network is still incomplete for many inducers. Several RNAi loss-of-function studies have identified factors involved in NF- κ B pathway activation, including stimuli of the nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2 receptors (Bielig et al., 2014; Lipinski et al., 2012; Warner et al., 2013; Yeretssian et al., 2011), Epstein-Barr virus (Gewurz et al., 2012), *Neisseria gonorrhoeae* (Gaudet et al., 2015), and *Shigella flexneri* infections (Milivojevic et al., 2017). These studies have shown that NF- κ B signaling, although sharing core elements, is highly complex and diverse.

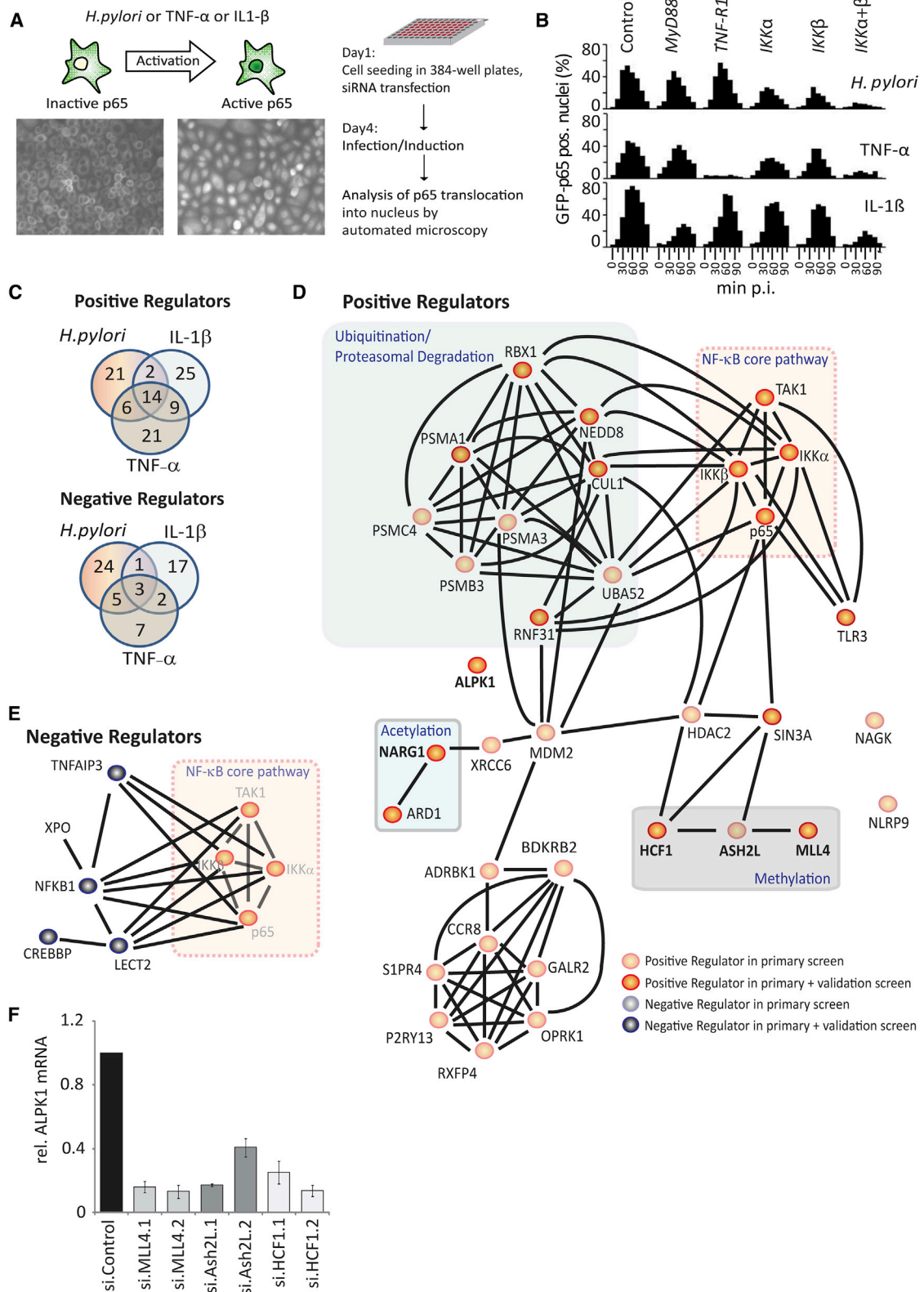


Figure 1. Genome-wide RNAi Screen for *H. pylori*-Induced NF- κ B Activation

(A) Screen setup: 72 hr post-transfection with siRNAs AGS SIB02 cells (GFP-p65) were infected with *H. pylori* or induced with IL-1 β or TNF- α and analyzed for p65 translocation into the nucleus.

(legend continued on next page)

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

RESULTS

Regulators of *H. pylori*-Induced NF- κ B Activation

As a readout for identifying factors involved in NF- κ B pathway activation we used nuclear translocation of a p65-GFP fusion protein, which is amenable for analysis by automated microscopy (Bartfeld et al., 2010) (Figure 1A). Besides *H. pylori*, we used TNF- α and IL-1 β as stimuli for NF- κ B activation. Robustness of the system was assessed by targeting known factors of the NF- κ B pathway. Accordingly, knockdown of TNF- α receptor 1 (TNFR1) and of the adaptor protein myeloid differentiation primary response protein 88 (MYD88) blocked the signals by TNF- α and IL-1 β , respectively. Moreover, combined IKK- α and IKK- β knockdown abolished activation by TNF- α , IL-1 β , and by *H. pylori* (Figure 1B). Details of three screens performed are summarized in Figures S1A–S1C. The first screen using a human kinase small interfering RNA (siRNA) library yielded 15 primary hits with increased responsiveness to *H. pylori* infection, four of which were confirmed by at least two independent siRNAs. These included two genes not previously implicated in NF- κ B signaling: ALPK1 and CDC2-related kinase, arginine/serine rich (CRKRS) (Bartfeld, 2009) (Table S1). To extend our search, a second screen was carried out with only *H. pylori* as inducer, using two siRNA libraries targeting the whole and the druggable genome. This analysis yielded 347 hits: 235 positive and 112 negative regulators. Of these, 200 positive and 100 negative regulators were further validated using additional siRNAs and all three inducers, yielding 43 positive and 33 negative *H. pylori*-induced regulators. Comparison to NF- κ B induction by IL-1 β

or TNF- α indicated that 21 of the positive and 24 of the negative regulators were strongly biased to *H. pylori* as an inducer (Figure 1C; Table S2).

STRING analysis (Szklarczyk et al., 2011) of our datasets revealed prominent protein interaction networks (Figures 1D and 1E). Notably, one obvious sub-network was formed by known general NF- κ B key regulators, consisting of IKK- α (Chuk), IKK- β (IKKBK), TAK1 (MAP3K7) and p65 (RelA) itself, confirming the functional robustness of our approach (Figure 1D). The central roles of ubiquitination and proteasomal degradation in NF- κ B signaling were well reflected by the dataset (Oeckinghaus et al., 2011). Apart from major members of the proteasome (PSMA1, PSMB3, PSMA3 and PSMC4), we identified RBX1, NEDD8 and Cullin-1 as necessary for ubiquitination (Figure 1D). The RING finger-like domain-containing protein RBX1 is part of cullin-RING-based E3 ubiquitin-protein ligase (CRLs) complexes, which are regulated by NEDD8 ligation (Scott et al., 2011). We also identified RNF31 as a positive regulator for *H. pylori*, TNF- α , and IL-1 β NF- κ B signaling. A prominent negative regulator included the deubiquitinase tumor necrosis factor- α -induced protein 3 (TNFAIP3), known to downregulate NF- κ B (Harhaj and Dixit, 2011) (Figure 1E). Several of the identified hits had only a weak effect on TNF- α - and none on IL-1 β -induced NF- κ B signaling. Of these, ARD1 and NARG1, which form an acetyltransferase complex connected with the ubiquitylation machinery (Arnesen et al., 2005), were further validated (Figure S1D). The regulators most specific for *H. pylori* also included ALPK1 (Figure S1E; Table S2), an atypical kinase previously implicated in apical protein transport and phosphorylation of myosin IA (Heine et al., 2005). Confirmation by western blotting showed that ALPK1 was required for degradation of I κ B α after infection with *H. pylori* but not after stimulation with TNF- α or IL-1 β (Figure S1E). In addition, we included the TRAF-interacting protein with FHA domain (TIFA) in our validation, as it had the highest score of any gene in our genome-wide RNAi screen, albeit for only one siRNA (Table S2).

Upstream Regulators of ALPK1

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

(B) Knockdown of NF- κ B pathway activators leads to reduced p65 translocation in cells treated as in (A) and fixed at 0, 15, 30, 45, 60, 75, 90, or 105 min followed by quantification via automated microscopy. Inhibitory siRNAs used are IKK α and IKK β for *H. pylori*, TNF-R1 for TNF- α , or MyD88 for IL-1 β . Control siRNA is Allstars.

(C) Venn diagram showing the validated factors. Apart from factors functioning for all inducers, 21 positive and 24 negative regulators unique for the *H. pylori*-induced NF- κ B pathway were found.

(D and E) Selected protein-protein interactions among (D) positive or (E) negative regulators from the screen in connection to the NF- κ B core pathway, identified with a confidence score of ≥ 0.4 from the STRING database. Hits found to be unique for *H. pylori* NF- κ B signaling are shown in bold. Ubiquitination and proteasomal degradation is known to be heavily interconnected with the NF- κ B core pathway. Additionally, we identified an acetylation complex formed by ARD1 and NARG1 and a methylation complex consisting of HCF1, MLL4, and Ash2L to be important positive regulators in *H. pylori* NF- κ B pathway. In contrast, the identified kinase ALPK1 is not connected to the NF- κ B pathway. Negative regulators included the known NF- κ B inhibitors TNFAIP3, CREBBP, and NFKB1.

(F) siRNA-mediated loss of MLL4, Ash2L, or HCF1 strongly reduced the level of ALPK1 mRNA, as identified by real-time qPCR. Data represent the mean \pm SD of two independent experiments.

See also Figure S1 and Tables S1 and S2.

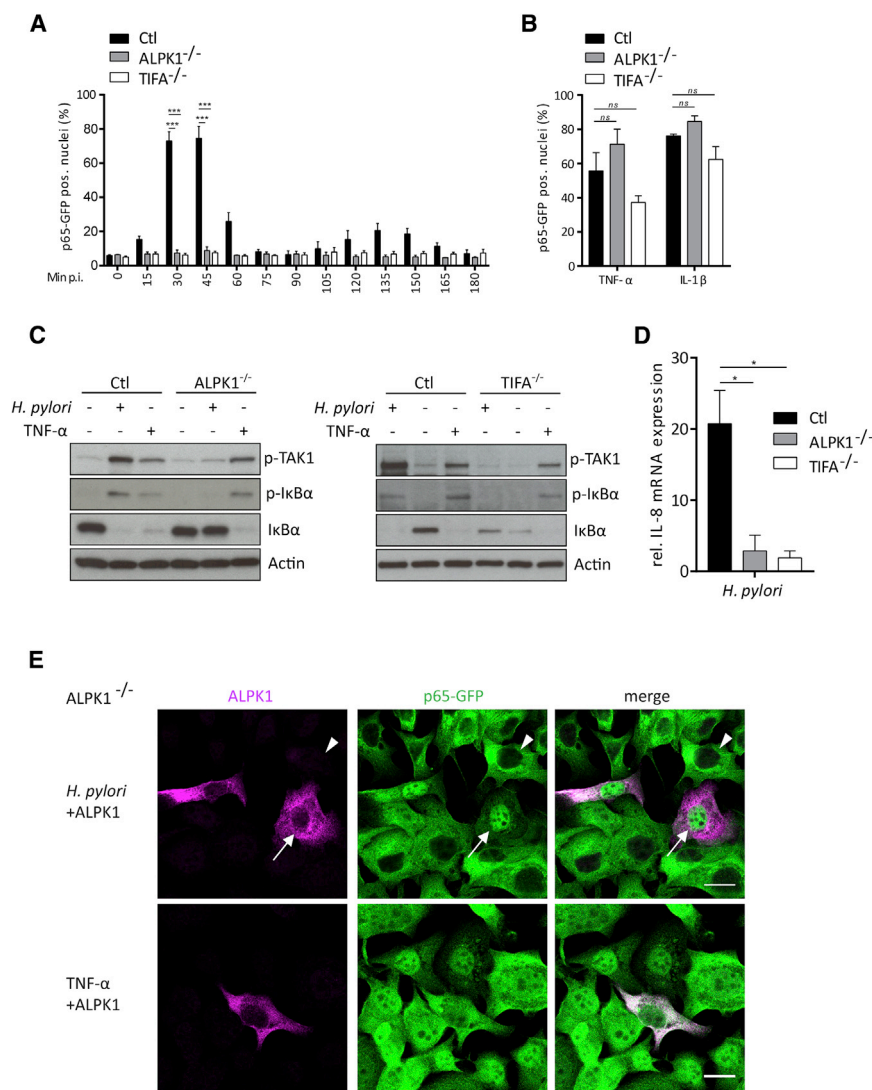


Figure 2. ALPK1 and TIFA Are Essential for *H. pylori*-Induced Activation of NF-κB

(A) CRISPR/Cas9-mediated silencing of ALPK1 or TIFA in AGS SIB02 cells impedes NF-κB activation after infection (MOI 100) for the indicated times.

(B) Silencing does not influence TNF-α- or IL-1β-induced NF-κB activation.

(C) Loss of ALPK1 or TIFA blocks TAK1 phosphorylation at Thr-184/187 and IκBα phosphorylation at Ser32. IκBα degradation was blocked specifically in ALPK1- or TIFA-deficient cells at 30 min p.i. (MOI 100). Silencing ALPK1 did not inhibit TNF-α-dependent (20 ng/mL for 30 min) NF-κB activation. Blot representative of at least two independent experiments.

(D) Real-time qPCR analysis of IL-8 transcription in ALPK1- and TIFA-depleted AGS cells infected for 30 min (MOI 100), normalized to non-infected control cells.

(E) ALPK1 rescues NF-κB activation upon *H. pylori* infection (MOI 100 for 30 min) in ALPK1-depleted AGS SIB02 cells expressing p65-GFP (green) transfected with ALPK1-Myc-Flag for 24 hr and labeled for Myc (magenta). ALPK1-expressing cells show nuclear translocation of p65 (top). Cells activated with TNF-α (20 ng/mL, for 30 min) also show nuclear translocation of p65. Arrowheads, non-transfected, not activated cells; arrows, transfected, activated cells. Scale bar, 20 μm. Graphs show mean ± SEM of three independent experiments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; ns, not significant, Student's t test.

See also Figures S2 and S3 and Movie S1.

(Tyagi et al., 2007), we hypothesized that it may indirectly affect expression of other hits. To our surprise, reverse transcription-quantitative PCR (RT-qPCR) indicated that ALPK1 expression was abrogated after HCF1 knockdown (Figure 1F). Similarly, knockdown of ASH2L or MLL4, known to act in concert with HCF1, abrogated ALPK1 expression as well (Figure 1F). Thus, methylation mediated by the HCF1 methyltransferase complex seems necessary for housekeeping expression of ALPK1, and the effects of the HCF1, ASH2L and MLL4 on *H. pylori*-induced NF-κB activation are likely caused indirectly via their influence on ALPK1 gene expression. These findings nominated ALPK1 as a central component of NF-κB signal transduction.

Role of ALPK1 in NF-κB Activation

To further consolidate its function in *H. pylori*-specific NF-κB activation, two independent ALPK1-deficient cell lines were generated using CRISPR/Cas9 technology (Figures S2A and S2B). Knockout fully prevented p65 translocation after infection

Knockout cells were unable to mount an IL-8 response upon infection (Figure 2D). To test whether p65 translocation could be rescued, ALPK1-deficient cells were transfected with recombinant ALPK1-Myc-Flag. Indeed, only rescued cells exhibited NF-κB activation upon *H. pylori* infection (Figure 2E, upper panel), while TNF-α triggered p65 translocation also in ALPK1-deficient cells (Figure 2E, lower panel). These results identify ALPK1 as an essential mediator of NF-κB activation in *H. pylori* infection.

TIFA Is Crucial for *H. pylori*-Induced NF-κB Activation Acting Downstream of ALPK1

Simultaneously, we also tested CRISPR/Cas9-generated TIFA knockout cells to illuminate its role in *H. pylori*-specific NF-κB activation (Figures S2C and S2D). Interestingly, knockout cells failed to show any sign of *H. pylori*-specific NF-κB activation, even after extended monitoring for 3 hr (Figure 2A), while p65 translocation readily occurred upon treatment with IL-1β or

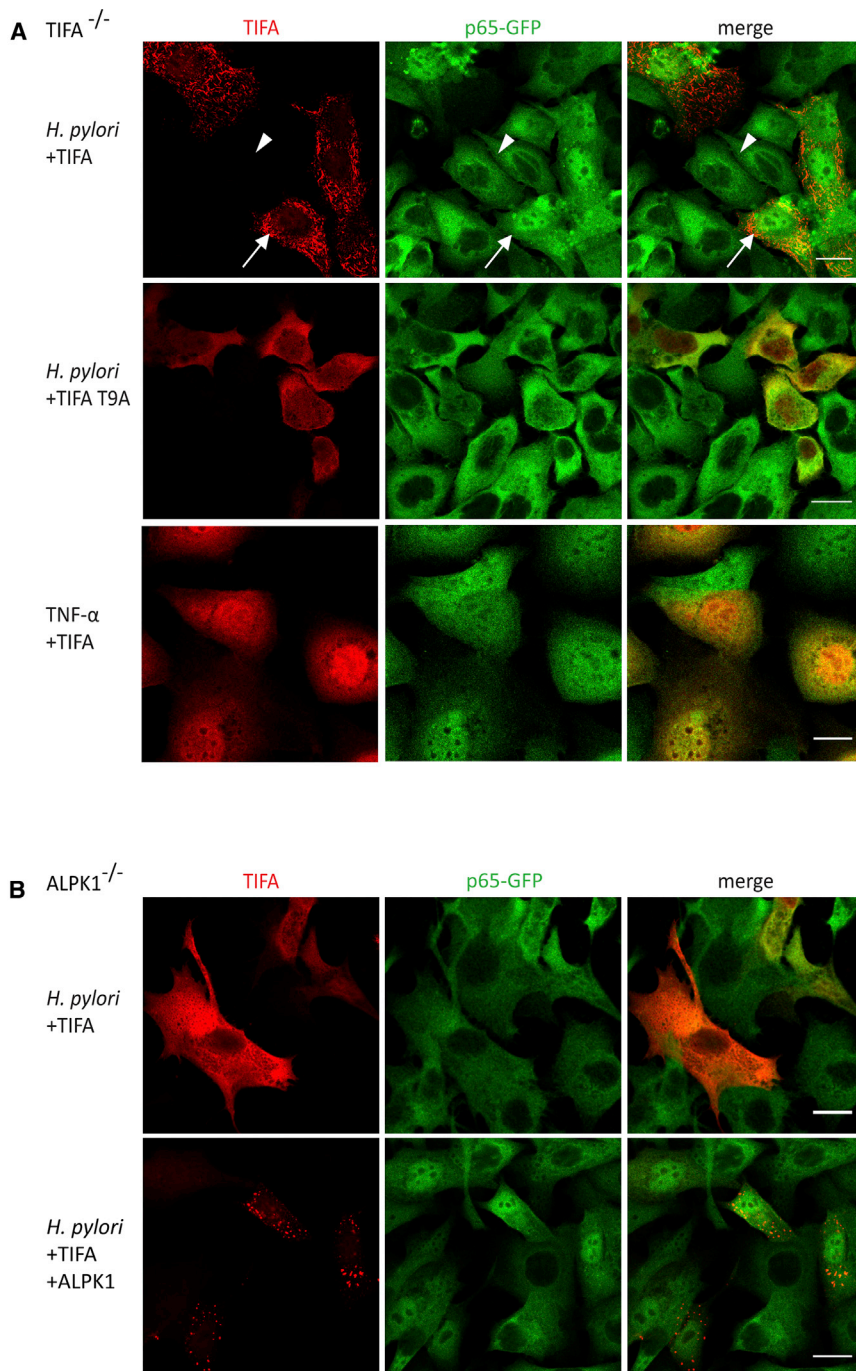


Figure 3. TIFA Activates NF- κ B upon *H. pylori* Infection through Formation of TIFAsomes Dependent on ALPK1

(A) AGS SIB02 depleted for TIFA using CRISPR/Cas9 were transfected for 24 hr with wild-type or T9A mutated TIFA-Myc-Flag, or an empty vector cDNA construct prior to infection. Only wild-type TIFA rescues TIFAsome formation and NF- κ B activation upon infection (MOI 100, 30 min). Cells activated with TNF- α (20 ng/mL, 30 min) did not show TIFAsome formation (red). Arrowheads, non-transfected, not activated cells; arrows, transfected, activated cells.

(B) Lack of ALPK1 hinders TIFAsome formation in infected AGS SIB02 cells and can be rescued by transfection with ALPK1. Cells were depleted for ALPK1 using CRISPR/Cas9 and transfected for 24 hr with TIFA-Myc-Flag (red) or TIFA-dtTomato (red) combined with ALPK1-Myc-Flag. *H. pylori* infection (MOI 100, 30 min) did not induce TIFAsomes or NF- κ B activation unless cells were co-transfected with ALPK1 and TIFA. Scale bars, 20 μ m.

See also Table S3.

revealed diffuse TIFA fluorescence (red) throughout the cytoplasm and the nucleus, while p65 location (green) was restricted to the cytoplasm and excluded from the nucleus (Figure S3A, upper panel). Infection with wild-type (WT) *H. pylori* or TNF- α treatment induced p65 nuclear translocation by 30 min (middle and lower panel). Consistent with NF- κ B activation, only infection with WT *H. pylori*, but not TNF- α treatment, induced the formation of so-called TIFAsomes (Gaudet and Gray-Owen, 2016) (Figure S3A). To further define the importance and specificity of TIFAsome formation upon infection, TIFA-deficient cells were transfected with TIFA-Myc-Flag or a TIFA-Myc-Flag mutant for T9, an important threonine phosphorylation site for TIFA activation (Huang et al., 2012). In cells that remained non-transfected, only TNF- α treatment but not *H. pylori* infection induced p65 translocation (Figure 3A, arrowhead in upper panel and lower panel). Successful transfection

with WT TIFA, but not with the TIFA mutant T9A, restored both p65 translocation and TIFAsome formation upon *H. pylori* infection (arrow upper panel and middle panel). To monitor TIFAsome formation in more detail, we transfected TIFA-deficient AGS cells with TIFA-dtTomato and infected them with *H. pylori*. Time-lapse video microscopy of live cells confirmed infection-induced TIFAsome formation (from 7 min p.i.) and p65 translocation (from 12 min p.i.) only in transfected cells (Movie S1). TIFAsome formation could not be observed in ALPK1-deficient cells but

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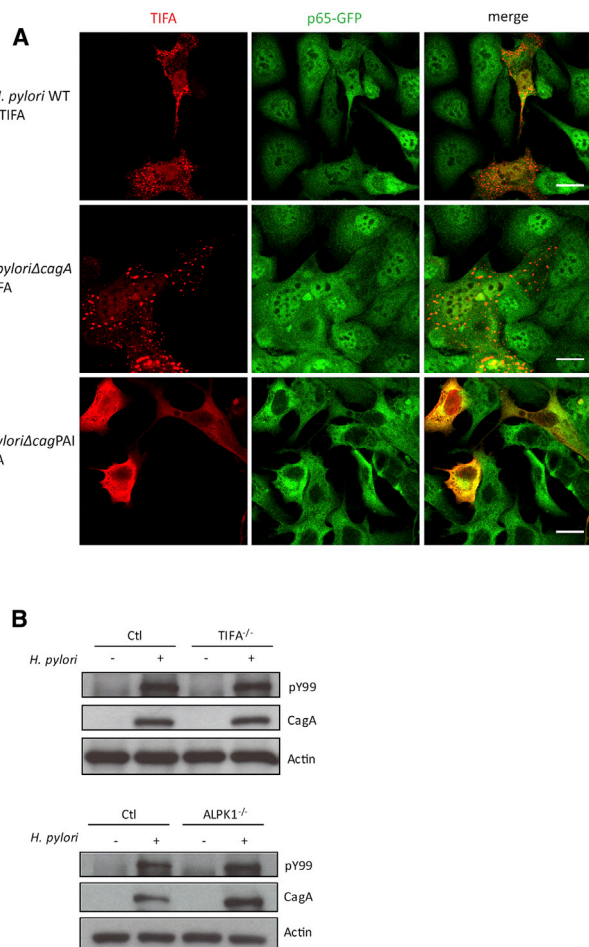


Figure 4. NF- κ B Activation and TIFAsome Formation via ALPK1 and TIFA Is Independent of CagA Translocation but Dependent on a Functional T4SS

(A) *H. pylori* WT and the Δ cagA but not the Δ cagPAI deletion mutant induce TIFAsome formation and NF- κ B translocation. AGS SIB02 cells were transfected for 24 hr with TIFA-dtTomato (red) and then infected with *H. pylori* WT, Δ cagA, or Δ cagPAI (MOI 100, 30 min). Scale bar, 20 μ m.

(B) CagA translocation and phosphorylation are not affected in cells depleted for TIFA or ALPK1 via CRISPR/Cas9. Cells were infected with *H. pylori* WT at MOI 100 and analyzed by western blot at 3 hr p.i. for tyrosine phosphorylation and total CagA. Blots are representative of two independent experiments.

See also Figure S4.

was rescued by co-transfection with ALPK1 (Figure 3B) indicating that ALPK1 acts upstream of TIFA and thus plays a crucial role in the phosphorylation of TIFA T9 required for TIFAsome formation. Consistent with previous publications and as described in the Human Protein Atlas (Gaudet et al., 2015; Huang et al., 2012; Uhlén et al., 2015), we observed a large proportion of TIFA in the nucleus, which upon infection appeared to be translocated to the cytoplasm to form TIFAsomes (Movie S1).

TIFAsomes Are Multifactorial Protein Complexes

To identify protein-protein interactions (PPIs) of TIFAsomes by mass spectrometry (MS), we used a TIFA-Myc-Flag fusion

transiently overexpressed in AGS cells for pull-down of TIFA-associated proteins (Table S3). A total of 77 TIFAsome-associated proteins enriched after *H. pylori* infection were identified by MS and used to generate PPI network graphs using STRING (Figure S3B). Interestingly, our data revealed that TIFAsomes contain classical NF- κ B key regulators, including TAB2 and TRAF2. Moreover, TRIM21, a factor involved in ubiquitination and proteasomal degradation, was enriched in this complex, consistent with its known role in NF- κ B regulation (Oeckinghaus et al., 2011). Other prominent components included the microtubule- and actin-associated proteins KIF11 and myosin IIA. Intriguingly, myosin IIA has been reported to be a target for ALPK1 in patients with gout (Lee et al., 2016). We also identified annexin A2, which is known to interact with the p50 subunit of NF- κ B to regulate p50 complex translocation into the nucleus (Jung et al., 2015). TRAF2 was chosen for validating the MS data by immunoblotting and, evidently, co-precipitated with TIFA confirming the presence of TRAF2 in the *H. pylori*-induced TIFAsomes, consistent with the known interaction of TIFA and TRAF2 (Kanamori et al., 2002) (Figure S3C).

CagA Translocation and ALPK1-TIFA Activation Constitute Independent Pathways

In line with several previous studies (reviewed in Backert and Naumann (2010)) p65 translocation, TAK1 activation and IL-8 mRNA upregulation in our system were dependent on the T4SS, but not on CagA (Figures S4A–S4C). To test whether activation of the ALPK1-TIFA axis itself also depends solely on T4SS function, we transfected AGS SIB02 cells with TIFA-dtTomato for monitoring TIFAsome formation after infection (Figure 4A). As anticipated, TIFAsomes quickly formed after infection with WT and CagA-deficient *H. pylori* but not the T4SS-deficient cagPAI mutant (Figure 4A). To test whether ALPK1 or TIFA in turn affect CagA translocation, we analyzed CagA phosphorylation. It occurred readily in TIFA- or ALPK1-deficient cells (Figure 4B), suggesting that neither is required for CagA translocation.

T4SS Required for Release of HBP and ALPK1-TIFA-Dependent NF- κ B Activation

We speculated that TIFAsome formation and NF- κ B activation could be triggered by a bacterial component secreted in a T4SS-dependent fashion. The pathogen-associated molecular pattern (PAMP) heptose-1,7-bisphosphate (HBP), which has recently been shown to activate TIFA (Gaudet et al., 2015; Milivojevic et al., 2017), appeared a likely candidate. HBP is a mono-saccharide produced in the synthesis pathway of Gram-negative lipopolysaccharides (Valvano et al., 2002). We thus prepared bacterial lysates as previously described (Gaudet et al., 2015) from WT *H. pylori* and the corresponding Δ cagA, Δ cagL, and Δ cagPAI mutant strains and used them to transfect WT, as well as TIFA- and ALPK1-deficient AGS SIB02 cells. Real-time qPCR analysis showed that within 3 hr IL-8 expression was upregulated in control cells transfected with lysates from T4SS-competent and T4SS-deficient bacteria, as well as with lysate prepared from Gram-negative *N. gonorrhoeae* but not from Gram-positive *L. monocytogenes* (Figure 5A). By contrast, in ALPK1- or TIFA-deficient cells no increase in IL-8 expression was observed with any lysates. Moreover, we observed

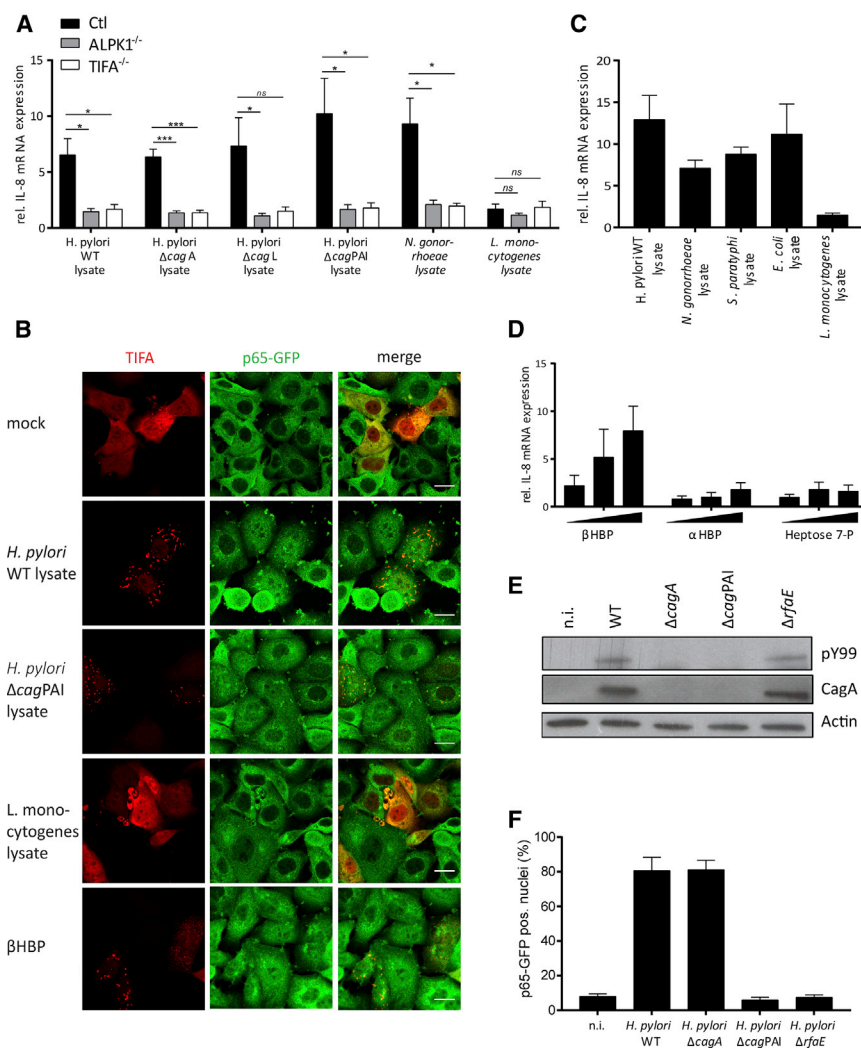


Figure 5. Bacterial Lysates (HBP) Trigger ALPK1-TIFA-Dependent NF-κB Activation in Permeabilized Cells

(A) Treatment of permeabilized AGS cells with bacterial lysates from T4SS-proficient (P12 WT and P12Δ*cagA*) or -deficient *H. pylori* induces IL-8 mRNA expression in normal, but not TIFA or ALPK1-depleted cells generated by CRISPR/Cas9. mRNA levels were measured by real-time qPCR 3 hr post-transfection and normalized to mock-transfected control cells. Data represent mean ± SEM of three independent experiments; **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001; ns, not significant, Student's *t* test.

(B) TIFAsome formation induced by bacterial lysates and βHBP. AGS SIB02 cells were transfected for 24 hr with TIFA-dtTomato (red) and then permeabilized for treatment with lysates from *H. pylori* WT, the Δ*cagPAI* mutant, *L. monocytogenes*, or synthetic βHBP for 3 hr. Scale bar, 20 μm.

(C) Lysates from Gram-negative bacteria but not from Gram-positive *Listeria* induce IL-8 mRNA expression in AGS cells treated as in (A). Data represent mean ± SEM of three independent experiments.

(D) D-Glycero-β-D-manno-heptose -1,7-bisphosphate (βHBP) but not D-glycero-α-D-manno-heptose-1,7-bisphosphate (αHBP) or D-glycero-D-manno-heptose-7-phosphate (heptose 7-P) induce IL-8 mRNA expression in permeabilized AGS cells 3 hr post-treatment as described in (A). Compounds were used at a final concentration of 0.5, 5 and 50 μM. Data are normalized to mock-transfected control cells.

(E) CagA is phosphorylated upon infection with a *rfaE* mutant. Wild-type cells and cells infected with *H. pylori* WT and Δ*rfaE* mutant at MOI 100 were analyzed by western blot 3 hr p.i. for tyrosine phosphorylation and total CagA. Blots are representative of two independent experiments.

(F) NF-κB activation by *H. pylori* depends on expression of RfaE. AGS SIB02 cells were infected

with wild-type P12, Δ*rfaE*, Δ*cagA*, and Δ*cagPAI* *H. pylori* (MOI 100, 30 min), and nuclear p65 translocation was analyzed by automated microscopy. Unless otherwise indicated, data represent mean ± SEM of at least two independent experiments. See also [Movie S2](#). See also [Figure S5](#).

TIFAsome formation and nuclear translocation of p65 in NF-κB reporter cells upon transfection of all *H. pylori*- (WT, Δ*cagA*, Δ*cagL*, and Δ*cagPAI*) and *N. gonorrhoeae*- but not *L. monocytogenes*-derived lysates ([Figure 5B](#); data not shown). Translocation of p65 in NF-κB reporter cells transfected with *H. pylori* WT lysate was also monitored by time-lapse video microscopy and, in contrast to the activation by live *H. pylori*, occurred in a non-synchronized fashion ([Movie S2](#)).

IL-8 was also upregulated upon transfection with lysates from the Gram-negative *S. paratyphi* and *E. coli* ([Figure 5C](#)), further supporting the idea that the NF-κB-inducing factor is a molecule expressed specifically by Gram-negative bacteria such as HBP. Thus, we chemically synthesized D-glycero-β-D-manno-heptose -1,7-bisphosphate (βHBP), as well as its anomer D-glycero-α-D-manno-heptose -1,7-bisphosphate (αHBP), and the monophosphate D-glycero-D-manno-heptose-7-phosphate (heptose 7-P) ([Figures S5A–S5C](#)). Transfecting AGS cells with

increasing amounts of βHBP, as compared to αHBP or heptose 7-P, induced IL-8 expression ([Figure 5D](#)) and formation of TIFAsomes ([Figure 5B](#), lower panel), an observation further confirmed by time-lapse video microscopy of p65 translocation, similar to the data presented in [Movie S2](#) (data not shown). This suggests that NF-κB activation can be induced by translocation of the HBP β-anomer via the ALPK1-TIFA axis. Generation of a *H. pylori* *rfaE* knockout mutant corroborated the action of HBP as the specific inducer of NF-κB. RfaE catalyzes the phosphorylation of D-glycero-D-manno-heptose-7-phosphate at the C-1 position to generate HBP. Infection with this mutant still allowed for CagA translocation but not NF-κB activation ([Figures 5E](#) and [5F](#)). The NOD1 agonist Tri-DAP only induced very minor IL-8 mRNA upregulation upon transfection of AGS cells; however, in contrast to *H. pylori* lysates this activation was independent of ALPK1 ([Figure S6](#)). Thus, although the NOD1 receptor is clearly stimulated, it does not act via the ALPK1-TIFA pathway,

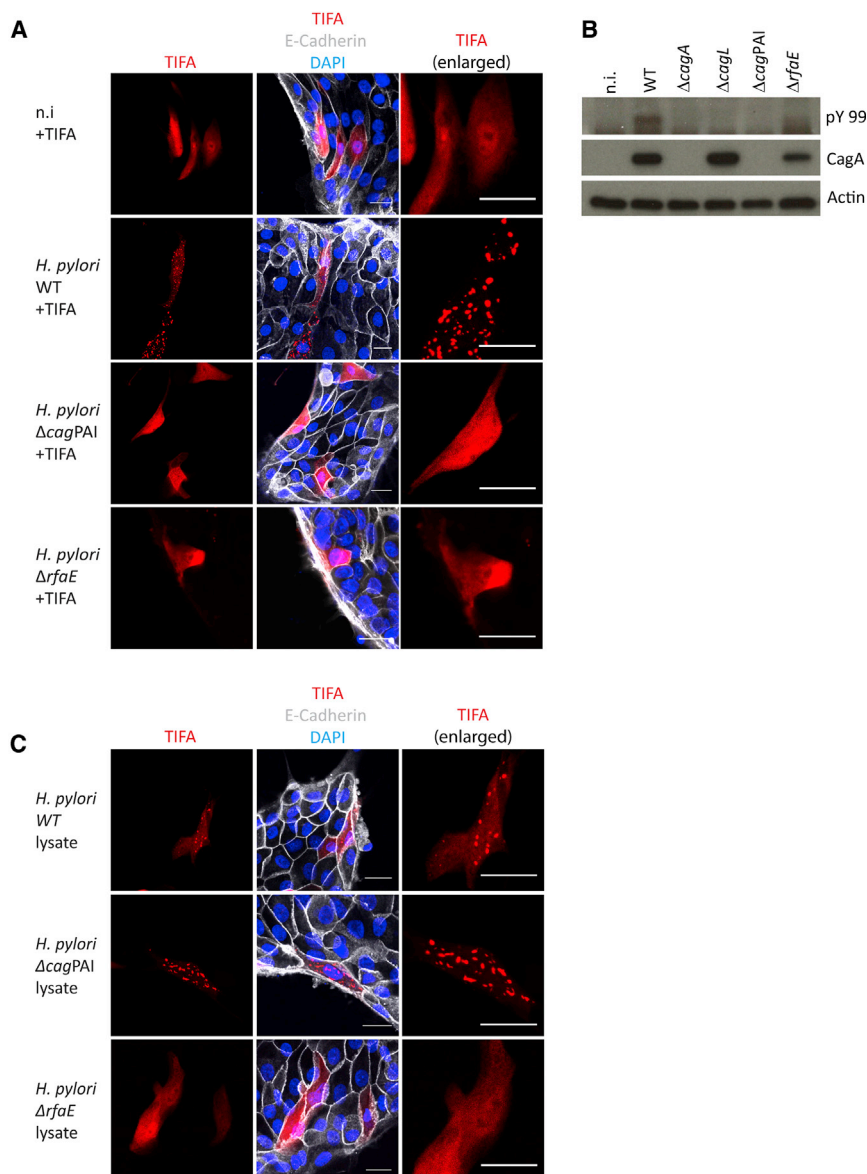


Figure 6. TIFAsome Formation in Primary Gastric Epithelial Cells

(A) Human gastric primary cells grown as mono-layers and transfected with TIFA-dtTomato (red) for 24 hr using FuGENE6 were left uninfected or infected with *H. pylori* P12 WT, Δ cagPAI, or Δ rfaE (MOI 100, 3 hr), fixed, and counterstained for DNA (DAPI, blue).

(B) *RfaE* mutant bacteria show CagA translocation and phosphorylation in primary cells infected with WT or mutant *H. pylori* (MOI 100, 3 hr) and analyzed by western blot for tyrosine phosphorylation and total CagA.

(C) Cells as in (A) transfected with respective bacterial lysates (3 hr) as indicated. Scale bars, 20 μ m.

DISCUSSION

Discriminating pathogenic from non-pathogenic microbial traits is one of the prime functions of the innate immune system, and the epithelial barrier is equipped with exquisite sensory means to accomplish this task. We wanted to understand the basis of innate recognition of highly pathogenic strains of *H. pylori*, known to elicit particularly strong NF- κ B-based immune reactions in the gastric mucosa due to their cagPAI-encoded T4SS (Backert and Naumann, 2010; Koch et al., 2016). Our extensive screening program provides a list of host factors involved in nuclear translocation of p65 selectively stimulated by *H. pylori*. Of the main hits, ALPK1 identified in the initial kinome screen (Bartfeld, 2009) appeared to be furthest upstream in the pathway, triggered by the T4SS-dependent infusion of HBP to the host cell cytosol. ALPK1, either directly or indirectly, then causes TIFA phosphorylation and complex formation with an array of

host factors, leading to activation of classical NF- κ B signaling. This uncovers the HBP-ALPK1-TIFA axis as the key regulon of *H. pylori* T4SS-dependent NF- κ B activation.

ALPK1 is a member of the atypical kinase family alpha kinases that recognize phosphorylation sites within α helices (Ryazanov et al., 1999). Until recently, the only substrates suggested for ALPK1 were myosin IA and IIA (Heine et al., 2005; Lee et al., 2016). Indeed, we found myosin IIA to be part of the TIFAsomes, but whether it is phosphorylated by ALPK1 or plays a role in the activation of innate immunity during *H. pylori* infection still has to be elucidated. A recent report also identified ALPK1 as necessary for TIFA oligomerization after infection with *S. flexneri* (Milivojevic et al., 2017), connecting it to the innate immune response. A role for ALPK1 and TIFA in inflammation was first predicted from an analysis of major genetic susceptibility loci

in congruence with Gaudet et al. (2017). To demonstrate that the observed ALPK1-TIFA axis-dependent induction of NF- κ B is not a cell-line-specific phenomenon, we tested gastric primary cells isolated from patient samples, expanded as organoids, and seeded in 2D (Schlaermann et al., 2016) prior to transfection with recombinant TIFA-dtTomato. Consistent with our previous observations, we found that *H. pylori* WT and Δ cagA infection, but not the cagPAI or *rfaE* mutant, caused formation of TIFAsomes in these authentic gastric cells (Figure 6A; data not shown) with both the WT *H. pylori* and the *rfaE* mutant being capable of translocating CagA (Figure 6B). However, while transfecting lysates of the PAI mutant induced formation of TIFAsomes, lysates of the *rfaE* mutant did not (Figure 6C). This indicates that T4SS-dependent induction of the ALPK1-TIFA signaling route via HBP is active in human primary cells.

in the experimental IL10^{-/-} mouse model of inflammatory bowel disease (Bleich et al., 2010). ALPK1 was later found to be upregulated in patients with gout and to modulate expression of inflammatory cytokines in an experimental gout model (Wang et al., 2011). Its role in these inflammatory scenarios is still unknown, but the importance in innate immune sensing of *S. flexneri* (Milivojevic et al., 2017) together with the data presented here point to a critical role of ALPK1 in HBP sensing, making it a key regulator of inflammation.

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

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

As the *H. pylori* component responsible for NF- κ B activation, we here identify the small molecule metabolite HBP, a precursor of LPS previously recognized as a PAMP of several Gram-negative bacteria, including *N. gonorrhoeae* (Gaudet et al., 2015) and *S. flexneri* (Milivojevic et al., 2017). We demonstrate strict dependency of NF- κ B activation on *H. pylori*'s functional T4SS, suggesting that it delivers HBP to the inside of host cells. Infection with mutants of either the T4SS or the responsible LPS biosynthetic pathway *rfaE* fails to induce NF- κ B or IL-8. No evidence was found to support the previously proposed functions of either CagA (Lamb et al., 2009) or the small peptidoglycans supposedly translocated by *H. pylori* in NF- κ B activation (Viala et al., 2004). Instead, our transfection experiments clearly show that induction of NF- κ B depends strictly on the *rfaE* gene product. Moreover, no significant hits in our screens pointed to a role of NOD1, NOD2, or respective downstream factors (Lipinski et al., 2012; Warner et al., 2013; Yeretssian et al., 2011). Although NF- κ B acti-

vation in epithelial cells occurs only marginally and robust activation requires NOD1 overexpression (Bielig et al., 2014), we show here, using high levels of Tri-DAP as agonist, that NF- κ B activation via NOD1 is independent of ALPK1. We thus conclude that the HBP-mediated pathway constitutes the initial NF- κ B activating event immediately after contact of T4SS-proficient *H. pylori* with epithelial cells. We do not rule out that other reported NF- κ B activating pathways may come into play at later stages of the infection once inflammatory cytokines or remnants of bacterial degradation are released in the infected tissue (Backert et al., 2016; Velin et al., 2016).

Interestingly, while the T4SS is capable of activating the ALPK1-TIFA-NF- κ B axis irrespective of CagA, both the bacterial *rfaE* or the host ALPK1 mutants still support CagA translocation. These findings mechanistically separate CagA translocation from HBP delivery, inspiring future investigations into other substrate-specific pathogen or host translocation functions. It will further be interesting to see whether and to what extent HBP influences host cell pathways apart from NF- κ B that branch-off from activated ALPK1 and TIFAsomes and what the contribution of this signaling route is to gastric pathogenesis.

EXPERIMENTAL PROCEDURES

Ethical Permissions

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

Bacterial Strains and Cultivation

The following *H. pylori* strains were used: P1 and P12 WT (strain collection no. P213 and P511) and the mutant strains P12 Δ cagPAI, P12 Δ cagA, P12 Δ cagL, and P12 Δ rfaE (strain collection no. P387, P378, P454, and P588). *H. pylori* was cultivated as described before (Backert et al., 2000). If not otherwise indicated MOI was 100.

Cell Culture

AGS cells stably transfected with a p65-GFP construct (AGS SIB02) (Bartfeld et al., 2010), AGS SIB02 CRISPR/Cas9 control and knockout cells (AGS STZ001, AGS STZ003, and AGS STZ004), and the parental AGS cells (ATCC CRL 1739) were cultivated in RPMI medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamate. A549 cells stably transfected with a p65-GFP construct (A549 SIB01) (Bartfeld et al., 2010), A549 SIB01 CRISPR/Cas9 control and knockout cells (A549 STZ001, A549 STZ002, and A549 STZ003) were cultivated in DMEM medium (GIBCO) supplemented with 10% heat inactivated FCS, 1 mM Na-pyruvate, and 2 mM L-glutamine and kept at 37°C in a humidified atmosphere with 5% CO₂. AGS cell infections were carried out after serum starvation in serum-free medium.

Primary Cell Culture

Gastric primary glands were cultured as described previously (Schlaermann et al., 2016). Briefly, cells from freshly isolated glands were seeded in Matrigel to form organoids and expanded. Organoids were sheared every 10 days and for experimental use seeded on collagen-coated coverslips and grown for 1–2 days in 2D medium.

p65 Translocation Assay

AGS SIB02 cells were seeded in 12-well plates and infected with *H. pylori* (MOI 100) or stimulated by IL-1 β (10 ng/mL, PeproTech) or TNF- α (20 ng/mL, PeproTech) for 30 min under serum-free conditions and fixed with ice-cold

methanol, and nuclei were counterstained with Hoechst 33342 (2 μ g/mL, Molecular Probes). Images were acquired using automated microscopy (Olympus). NF- κ B translocation was analyzed using an automated cell-based assay.

Screening Procedure

For the kinome screen we used the QIAGEN "Human Kinase siRNA Set" containing 1,292 siRNAs targeting 646 kinase and kinase-associated genes. To reduce sample size, two siRNAs for each gene were pooled and tested in six conditions: *H. pylori* (45 and 90 min), TNF- α (30 and 75 min), and IL-1 β (45 and 90 min) using four independent biological replicates. AGS SIB02 cells were seeded in 96-well plates and transfected using the RNAiFect Transfection Kit (QIAGEN). One day later, cells were split into new wells, and experiments were conducted after at least 60 hr to allow reduction of target protein levels.

For the primary screen, the QIAGEN Human Druggable Genome siRNA Set v.2.0 Library containing four siRNAs per gene and the QIAGEN Human Genome 1.0 Library containing two siRNAs per gene were used. Altogether 24,000 genes were targeted by 62,000 individual siRNAs. For the validation screen, four individual siRNAs were purchased from QIAGEN. AGS SIB02 cells were seeded in 384-well plates, siRNAs were transfected with HiperFect transfection reagent (QIAGEN), and 72 hr post-transfection cells were stimulated for 45 min by infecting with *H. pylori* (MOI 100) or in the validation screen cells were additionally induced by IL-1 β (10 ng/mL, Miltenyi Biotec) or TNF- α (10 ng/mL, Becton Dickinson) for 45 min under serum-free conditions and fixed with ice-cold methanol, and nuclei were counterstained with Hoechst. Images were acquired using automated microscopy (Olympus), and NF- κ B translocation was analyzed using an automated cell-based assay as described (Bartfeld et al., 2010). In short, nuclei were detected, and the surrounding cytoplasmic area was set using image analysis software (Scan R, Olympus) following quantification of translocation of p65-GFP. Experiments were performed in triplicates. The hit validation screen was performed for 300 selected primary hits (200 positive and 100 negative regulators) using four siRNAs per gene. Cells were transfected for 72 hr, and NF- κ B was activated with either *H. pylori* infection, TNF- α , or IL-1 β . Cells were fixed after 45 min, and p65-GFP translocation was quantified automatically. As a positive control for all screening experiments, the combinatorial knockdown of IKK α and β was used.

Immunoblotting

Cells were lysed in 2 \times Laemmli buffer, separated by 10% SDS-PAGE, transferred to PDVF membranes, blocked in TBS buffer supplemented with 0.1% Tween 20 and 5% milk, and probed against primary antibodies at 4°C overnight. Membranes were probed with matching secondary horseradish-peroxidase-conjugated antibodies (Amersham, 1:3,000) and detected with ECL reagent (PerkinElmer). Primary antibodies were as follows: anti-Ik β (44D4) #4812, p-TAK1 (Thr 184/187) #4508, p-Ik β (Ser32) (14D4) #2859, and pNF- κ B p65 (Ser536) #3033 from Cell Signaling Technology anti phospho-tyrosine 99 (pY99) sc-7020, anti CagA (b300) sc-25766, and β -actin A5441 from Sigma.

Real-Time qPCR

Real-time qPCRs were performed using Power SYBR Green RNA-to-CT 1-Step Kit according to manufacturer's instructions (Applied Biosystems) and analyzed using the delta-Cq method (Livak and Schmittgen, 2001). See Supplemental Experimental Procedures for further details and Table S4 for MIQE data.

Plasmids

For overexpression of N-terminal WT TIFA c-terminally fused to Myc-Flag we used full-length human TIFA cDNA (Origene #RC204357) and introduced a silent TGG to TCG point mutation encoding L103 to destroy the PAM site preventing CAS9 cutting of TIFA when overexpressed in TIFA-deficient cells (strain collection no. pMW930). Mutation of the PAM site and TIFA T9A mutation (strain collection no. pMW931) were introduced using PfuTurbo DNA Polymerase (Agilent Technologies) complying with the protocol of Stratagene's single-site QuikChange Site-Directed Mutagenesis Kit. For generating N-terminal TIFA c-terminally fused to dtTomato, TIFA from pMW930 was amplified and cloned into ptdTomato N1 (modified from Clontech #6085-1) (strain collection no. pSTZ006). Human ALPK1 was amplified and cloned into pLenti-c-Myc-DKK (strain collection no. pMW909).

The following primers were used: ALPK1-Myc-Flag 5'-CTGCCGCCGCGATC GCCATGAATAATCAAAAAGTGGTAGC-3' and 5'-TTGCGGCCGCGTGCATGG TTTCTCCATTGAAG-3', TIFA-dtTomato 5'-CGGCTAGCCGCCATGACCAGT TTTGAAG-3' and 5'-CGGAATCCGGTTGACTCATTTCATCCATTCT-3'.

Confocal Microscopy

Cells were seeded on poly-L-lysine or collagen coated glass coverslips and transfected for 24 hr with recombinant ALPK1, recombinant TIFA, or its functional mutants using FuGENE6 (Promega). Cells were left uninfected or infected with *H. pylori* (MOI 100), treated with TNF- α (20 ng/mL), or transfected for 3 hr with bacterial lysates using Lipofectamine 2000 (Thermo Fisher Scientific) under serum-free conditions. For visualization of Myc-tagged protein, cells were fixed with 4% paraformaldehyde (PFA), blocked with 3% BSA, 5% FCS, and 0.3% Triton X-100 for 1 hr at room temperature (RT) and incubated with primary antibodies (anti-MYC [CST, cat. no. 9B11, 1:6,000], anti-E-Cadherin [BD Pharmingen, cat. no. 610181, 1:100]) overnight at 4°C, followed by secondary antibodies (donkey anti-mouse Cy3 [1:100, cat. no. 715-166-020], goat anti-rat Dylight 649 [1:100 cat. no. 112-486-062], DAPI [1:300, Roche, cat. no. H1840-10]), mounted with Mowiol 40-88 (Sigma, cat. no. 324590) and analyzed by laser scanning microscopy using a Leica SP8. Images were processed using ImageJ.

Bacterial Lysate Preparation and Delivery

Bacterial lysates were prepared as described in Gaudet et al. (2015). *H. pylori* and *N. gonorrhoeae* were grown on GC agar plates, *E. coli* on Luria broth (LB) agar plates, *L. monocytogenes* in brain heart infusion medium, and *S. paratyphi* in LB media, all overnight. Bacteria were then collected in PBS and resuspended in water at OD₆₀₀ L and boiled for 15 min at 400 rpm shaking. Debris was removed by centrifuging at 4,000 \times g for 3 min, and the supernatant was treated with 10 μ g/mL RNase A (New England Biolabs), 1 U/mL DNase 1 (QIAGEN), and 100 μ g/mL ProteinaseK (New England Biolabs) for 5 min at RT. Samples were boiled for 5 min and passed through a 0.22- μ m filter. Permeabilization of cellular membranes for lysate delivery to the cytoplasm was performed by treating cells in a 24-well format with a preparation of 5 μ L lysate mixed with 5 μ L Lipofectamine 2000 (Thermo Fisher Scientific) and 30 μ L OptiMEM (GIBCO), pre-incubated for 30 min at RT, and added to AGS cells at 70% confluence.

Statistical Analysis

For the kinome screen, data were normalized (using plate-median or, in comparison, Z score), and significance was assessed with Welch's t test. Primary hits were identified combining statistical significance (p value \leq 0.05). For the validation screen, the 5% top candidates were taken into account for further analysis and an additional four siRNAs were tested and hits confirmed with at least two siRNAs each with a Z score of \leq -1 or \geq +1. For the primary screen, plate normalization was performed using CellHTS2, and then Redundant siRNA Analysis (RSA) was used for defining the hit candidates. Positive and negative regulators were defined by an RSA score of \leq -2 and \geq +2, respectively. For the hit validation screen, data were analyzed using CellHTS2. Hit genes were defined according to their Z score: \leq -1 for positive and \geq +1 for negative regulators.

All graphs were prepared using GraphPad Prism 7 software (GraphPad). Data are presented as mean \pm SEM, and p values were calculated with a two-sided unpaired Student's t test. Data were considered significant if p < 0.05. Unless stated otherwise, figures represent three independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, four tables, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.08.039>.

AUTHOR CONTRIBUTIONS

S.B. and N.M. performed kinome screen and revealed ALPK1; J.L. and N.M. carried out the genome-wide screen; A.P.M., K.-P.P., and M.R. performed

bioinformatics analyses; M.K., F.G., and M.R. recognized factors including TIFA; C.R., M.R., S.B., O.S., and M.N. performed NF- κ B pathway analysis; S.Z. validated factors using CRISPR/Cas9; S.Z., M.A.A.-Z., and L.P. characterized HBP-ALPK1-TIFA interactions and performed cell assays; M.S. and A.K. generated genetic constructs; A.Z. and P.K. synthesized HBP; M.S. performed mass spectrometry; V.B., M.A.A.-Z., and L.P. performed confocal microscopy; T.F.M. conceived and supervised the project; S.Z., M.A.A.-Z., L.P., S.B., and T.F.M. wrote the manuscript.

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