The *Helicobacter pylori* type IV secretion system upregulates epithelial cortactin expression by a CagA- and JNK-dependent pathway

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**Abstract**

Cortactin represents an important actin-binding factor, which controls actin-cytoskeletal remodelling in host cells. In this way, cortactin has been shown to exhibit crucial functions both for cell movement and tumour cell invasion. In addition, the cortactin gene *cttn* is amplified in various cancer types of humans. *Helicobacter pylori* is the causative agent of multiple gastric diseases and represents a significant risk factor for the development of gastric adenocarcinoma. It has been repeatedly shown that *H. pylori* manipulates cancer-related signal transduction events in infected gastric epithelial cells such as the phosphorylation status of cortactin. In fact, *H. pylori* modifies the activity of cortactin's binding partners to stimulate changes in the actin-cytoskeleton, cell adhesion and motility. Here we show that *H. pylori* infection of cultured AGS and Caco-2 cells for 24–48 hr leads to the overexpression of cortactin by 2–3 fold at the protein level. We demonstrate that this activity requires the integrity of the type IV secretion system (T4SS) encoded by the *cag* pathogenicity island (*cag*PAI) as well as the translocated effector protein CagA. We further show that ectopic expression of CagA is sufficient to stimulate cortactin overexpression. Furthermore, phosphorylation of CagA at the EPIYA-repeat region is not required, suggesting that this CagA activity proceeds in a phosphorylation-independent fashion. Inhibitor studies further demonstrate that the involved signalling pathway comprises the mitogen-activated protein kinase JNK (c-Jun N-terminal kinase), but not ERK1/2 or p38. Taken together, using *H. pylori* as a model system, this study discovered a previously unrecognised cortactin activation cascade by a microbial pathogen. We suggest that *H. pylori* targets cortactin to manipulate the cellular architecture and epithelial barrier functions that can impact gastric cancer development.

**Take Aways**

- *Helicobacter pylori* infection induces overexpression of cortactin at the protein level
- Cortactin upregulation requires the T4SS and effector protein CagA

**Abbreviations:** cagA, cytotoxin-associated gene A; cagPAI, cag pathogenicity island; SD, standard deviation; wt, wild-type.


• Ectopic expression of CagA is sufficient to stimulate cortactin overexpression
• Overexpression of cortactin proceeds CagA phosphorylation-independent
• The involved host cell signalling pathway comprises the MAP kinase JNK

**KEYWORDS**
cancer, cortactin, Helicobacter, JNK, pathogenesis, pathogenicity island, signalling, virulence

## 1 | INTRODUCTION

*Helicobacter pylori* is a Gram-negative, spiral-shaped, microaerophilic pathogen colonising the human stomach of about 44%–60% of the world’s population (Hooi et al., 2017; Zamani et al., 2018). Naturally living in the gastric environment of humans for years without any symptoms, this bacterium can also trigger the development of gastric disorders such as gastritis and peptic ulcers, in the worst case scenario gastric cancer and, infrequently, MALT lymphoma in a small subset of individuals (Amieva & Peek, 2016; Cover & Blaser, 2009; Hatakeyama, 2019; Salama, Hartung, & Muller, 2013; Yamaoka & Graham, 2014). *Helicobacter pylori* eradication in patients with early gastric cancer resulted in lower rates of subsequent development of metachronous gastric cancer and more improvement from baseline in the grade of gastric corpus atrophy (Choi et al., 2018). Nevertheless, eradication of *H. pylori* seems to face certain difficulties, since the resistance of this microbe to antibiotics has reached alarming levels worldwide (Savoldi, Carrara, Graham, Conti, & Tacconelli, 2018). Contrary, there are numerous reports suggesting that *H. pylori* is an evolutionary “friend” of humans, rather than a “foe,” because it can protect the host against gastroesophageal and inflammatory bowel diseases as well as certain allergies such as asthma (Blaser, 2010; Jessurun, 2021).

Though *H. pylori* is considered as a key risk factor for gastric disorder development, not all strains exhibit the same virulence capabilities. In fact, *H. pylori* isolates have been divided into two major groups, reflecting their pathogenic capacities. Type-I strains can produce cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), which both contribute to disease progression. However, type-II strains still produce VacA, but lack CagA and are therefore less virulent (Covacci & Rappuoli, 2000). VacA represents a pore-forming toxin and can affect both epithelial and immune cells, promoting immune tolerance and subsequently persistent infection (Chauhan, Tay, Marshall, & Jain, 2019; McClain, Beckett, & Cover, 2017). Interestingly, VacA has also been proposed to downregulate CagA’s effects on epithelial cells, probably due to avoid excessive cellular damage in response to *H. pylori* (Oldani et al., 2009; Tegtmeyer et al., 2009). CagA represents a translocated effector protein of the type IV secretion system (T4SS) encoded by the cag pathogenicity island, cagPAI (Backert, Haas, Gerhard, & Naumann, 2018). *Helicobacter pylori* strains possessing the cagPAI are more prevalent in East Asian than in Western countries, showing the geographical diversity of the bacterium. CagA translocated into the host cytoplasm can lead to a variety of disturbances in cellular signalling resulting in immune responses, apoptosis, cytoskeletal rearrangements, altered cell polarity and disruption of the epithelial barrier (Naumann, Sokolova, Tegtmeyer, & Backert, 2017; Saadat et al., 2007; Tegtmeyer et al., 2017). For instance, transfected and translocated CagA from a subset of *H. pylori* strains was shown to induce IL-8 release through pro-inflammatory transcription factor NF-κB activation (Brandt, Kwok, Hartig, Konig, & Backert, 2005). During infection, *H. pylori* can stimulate the oncopgenic Src tyrosine kinase in a CagL/integrin-β1-dependent manner (Kwok et al., 2007; Tegtmeyer et al., 2010). In turn, activated Src can phosphorylate bacterial CagA at the EPIYA-repeats (Mueller et al., 2012). Phosphorylated CagA finally inactivates Src kinase via a negative feedback loop. CagA-mediated Src inactivation can dysregulate various proteins, vital in regulating cellular actin dynamics, including cortactin, ezrin and vinculin (Moese et al., 2007; Selbach, Moese, Backert, Jungblut, & Meyer, 2004; Tegtmeyer & Backert, 2011). Injected CagA can further disturb a variety of other host cell signalling pathways, including the mitogen-activated protein (MAP) kinases such as the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase (Keates et al., 1999).

One of the major host target proteins during *H. pylori* infection is cortactin, a factor involved in cellular cytoskeletal rearrangements (Sharafutdinov, Backert, & Tegtmeyer, 2020). Cortactin has multiple functional domains including an N-terminal acidic domain (NTA), a filamentous actin (F-actin) binding region, a proline-rich domain and a C-terminal Src homology 3 (SH3) domain (Ammer & Weed, 2008; Cosen-Binker & Kapus, 2006; Selbach & Backert, 2005). Cortactin can directly bind the Arp2/3 complex or via N-WASP, and promote actin polymerisation by enhancing Arp2/3 interaction with actin filaments (Urino et al., 2001). Interaction of cortactin with more than 15 known protein partners makes it an attractive target for multiple pathogens, including viruses, bacteria, fungi and protozoa (Bonfim-Melo et al., 2015; Jouvenet et al., 2006; Moreno-Ruiz et al., 2009; Nguyen et al., 2020; Selbach & Backert, 2005). Upon infection with *H. pylori*, cortactin undergoes tyrosine dephosphorylation at Y-421 and Y-486 in a CagA-dependent manner (Selbach et al., 2003; Tegtmeyer et al., 2011). However, the actual phosphatase involved in these dephosphorylation events is yet unknown (Knorr, Backert, & Tegtmeyer, 2020). However, tyrosine dephosphorylation of cortactin allows ERK1/2 kinases to phosphorylate cortactin at S-405 and S-418, resulting in its interaction with focal adhesion kinase (FAK) followed by its subsequent activation (Tegtmeyer et al., 2011). FAK activation finally prevents host cells from the excessive detachment (cell lifting) and therefore supports the chronic infection (Tegtmeyer & Backert, 2020). Moreover, the p21-associated kinase (PAK) can...
phalloidin (visualising actin filaments) and DAPI (4,6-diamidino-2-phenylindole, visualising DNA). In denaturing polyacrylamide gel electrophoresis, cortactin appears as two bands of 80 and 85 kDa (p80 and p85), as has been shown in various cell lines (Kelley, Hayes, Ammer, Martin, & Weed, 2010). Helicobacter pylori-dependent phosphorylation of cortactin at S-405 generates p85, which may trigger downstream signal transduction contributing to the process of gastric carcinogenesis (Tegtmeyer et al., 2011). High expression of the p85 cortactin was also observed contributing to the process of gastric carcinogenesis (Tegtmeyer et al., 2011). Cortactin 2 (cttn) (Schuring, Verhoeven, Litvinov, & Michalides, 1993; Weaver, 2008; Yamaguchi & Condeelis, 2007; Yin, Ma, & An, 2017). For example, immunohistochemical analysis of 109 patients with precancerous laryngeal lesions revealed a strong correlation of cortactin and FAK overexpression with a significantly higher cancer incidence (Villaronga et al., 2018). Similarly, cortactin overexpression might contribute to gastric cancer development as shown in vitro and in vivo (Wang et al., 2010; Wei, Zhao, Li, Zhou, & You, 2014). Besides, overexpression of cortactin increased the cellular invasion and adhesion to endothelial cells, implying a substantial role in tumour metastasis (Y. S. Li et al., 2001). Since H. pylori is considered (a) as an established factor for gastric cancer development and (b) to target cortactin, we were interested to study whether H. pylori might induce the overexpression of cortactin. Here we report that AGS and Caco-2 epithelial cells overexpress cortactin 2–3 fold upon infection with H. pylori, and identified the bacterial factor CagA and JNK MAP kinase pathway to be involved in this particular host response.

2 | RESULTS

2.1 | Helicobacter pylori infection triggers cortactin overexpression in Caco-2 and AGS cells

Numerous previous studies have reported cortactin overexpression in tumour biopsies of patients with either breast cancer, pancreatic cancer, gastric cancer or other tumour types (MacGrath & Koleske, 2012; Mazloomi et al., 2021; Ramos-Garcia et al., 2019; Wang et al., 2010; Yang et al., 2020). To analyse if cortactin expression can be regulated by H. pylori infection, we utilised two standard epithelial cell lines, AGS gastric cells and Caco-2 intestinal cells, which allow prominent bacterial binding and translocation of T4SS substrates (Javaheri et al., 2017; Tegtmeyer, Harrer, Schmitt, Singer, & Backert, 2019). First, we infected Caco-2 cells with wild-type (wt) H. pylori for 24 hr or left them untreated. Cells were then fixed with paraformaldehyde (PFA), stained with α-cortactin antibodies and counterstained using phalloidin (visualising actin filaments) and DAPI (4',6-diamidino-2-phenylindole, visualising DNA). Infection with H. pylori wt led to a significant increase in cortactin staining compared to the F-actin patterns (Figure 1a). Afterwards, we quantified cortactin and F-actin fluorescence levels in the cells, showing that the overexpression of cortactin in H. pylori-infected Caco-2 cells is significant (Figure 1b). Similar observations were obtained using infected AGS cells (see below). These results suggest that cortactin can be overexpressed both in gastric and intestinal epithelial cells upon infection by H. pylori.

2.2 | Cortactin overexpression is independent of H. pylori factors secreted into the medium

In order to study these interesting findings in more detail by another approach, we applied Western blotting and infected the cells with H. pylori wt in a time-course of 1–48 hr. As a control, we co-incubated the cells with bacteria-free culture supernatants of H. pylori, grown in liquid broth. As shown in the probed blots, H. pylori infection over time leads to phosphorylation of CagA in a time-dependent fashion (Figure 2a), while this is not the case during treatment with the bacterial supernatant, as expected (Figure 2c). Probing the blots with the α-cortactin antibodies and quantification of cortactin band intensity showed that cortactin overexpression is significantly visible between 24 hr and 48 hr only due to infection with H. pylori, but not supernatant (Figure 2b,d). Similar results were obtained in infected AGS and Caco-2 cells (Figure 2 and data not shown). These results suggest that direct cell contact of the bacterium and not a secreted factor in the supernatant is responsible for overexpression of cortactin in infected epithelial cells.

2.3 | Cortactin overexpression is MOI-dependent and triggered by a proteinaceous factor

Next, we treated AGS cells with H. pylori wt using different multiplicities of infection (MOIs from 1 to 100) of bacteria for 48 hr, and then collected cell pellets for Western blotting. Co-incubation of cells with growing numbers of H. pylori bacteria increased the expression of cortactin (Figure 3a). The quantification of the band intensities showed that cortactin overexpression is MOI-dependent (Figure 3b). As next, we wanted to know, which bacterial factor is mediating cortactin overexpression. For this purpose, we performed different pretreatments of the bacteria, for example, by addition of certain antibiotics, proteinase K, PFA fixation or heat treatment (Figure 3c,d). We found that infection with H. pylori wt is susceptible to pretreatment with proteinase K, suggesting that the bacterial factor responsible for cortactin overexpression is a protein. Either pretreatment of bacteria at 95°C or PFA fixation also abolished the overexpression, further demonstrating the factor’s proposed protein nature. Besides, we pretreated the cells with penicillin/streptomycin showing an inhibitory effect on cortactin overexpression, suggesting that live bacteria are essential. Furthermore, pretreatment with chloramphenicol also suppressed cortactin overexpression, suggesting that bacterial protein biosynthesis is also required.
2.4 | **The *H. pylori* cag T4SS and injected CagA are required for cortactin overexpression**

The experiments above suggested that the bacterial factor responsible for cortactin overexpression is of protein nature, and we subsequently aimed to identify the factor(s). *Helicobacter pylori* type-I strains possess the cag pathogenicity island, while type-II strains do not (Covacci & Rappuoli, 2000). Therefore, we infected the cells with various clinical type-I strains and type-II strains for 24 hr to specify their possible importance in cortactin overexpression. As a control, we performed phospho-tyrosine stainings of the samples showing that CagA is injected into the host cells. As expected, phosphorylated CagA appeared when infected with the type-I strains, but not type-II isolates (Figure 4a). Re-probing of the samples with the α-cortactin

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**FIGURE 1** *Helicobacter pylori* infection for 24 hr leads to an increase of cellular cortactin in cultured Caco-2 cells. (a) Confocal laser scanning microscopy with the upper part of images showing an X:Y projection (top view) of Caco-2 cell monolayers, and lower – Their Z-stacks (side view). Caco-2 monolayers without infection (mock) or after infection with *H. pylori* N6 wt were immunostained with α-cortactin (green), and counterstained for actin filaments and nuclei with rhodamine phalloidin and DAPI, respectively. (b) Relative fluorescence intensity from cortactin and actin filaments was further assessed and quantified as mean per cell ±SD (n = 50 for each dataset) by segmentation of cells into regions of interest (ROIs). Scale bars are 20 μm

**FIGURE 2** *Helicobacter pylori* induces cortactin overexpression in a time-dependent manner upon bacterial-host contact analysed by Western blotting. AGS cells were co-incubated with *H. pylori* NCTC11637 wt cells (a) or bacterial supernatants (c) in a time-course manner for 1 hr, 2 hr, 3 hr, 6 hr, 12 hr, 24 hr and 48 hr. After infection, the samples were probed against α-PY99, α-CagA and α-cortactin. Cortactin bands were quantified by measuring band intensities densitometrically either after infection with *H. pylori* (b) or after co-incubation with bacterial supernatants (d). The asterisks in panels a and c denote an unknown phosphorylated host cell protein of about 125 kDa

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(a)  
H. pylori wt

(b)  
Supernatant

(c)  
CagA α-PY99

(d)  
CagA α-GAPDH

(e)  
CagA α-Cortactin

(f)  
CagA α-GAPDH

(g)  
CagA α-Cortactin
antibodies suggested that the overexpression of cortactin is a hallmark of type-I, but not type-II strains, implying that the T4SS is involved (Figure 4a,b). As next, we infected the cells with isogenic mutants encoding structural components of the T4SS apparatus (ΔcagY and ΔcagL), effector protein CagA (ΔcagA) as well as translocated bacterial peptidoglycan and ADP-heptose (Backert
et al., 2018). In fact, mutations in the soluble lytic murein trans-glycosylase (Δslt) and sedoheptulose 7-phosphate isomerase (ΔgmhA) genes are known to diminish bacterial muropeptide production and ADP-heptose synthesis, respectively, while the ΔvacA mutant served as control. The Western blot results show that cortactin over-expression is independent of the H. pylori genes vacA, slt and gmhA (Figure 4c,d). However, deletion of any T4SS genes, including cagY, cagL or cagA abolished cortactin overexpression, suggesting that injection of CagA is essential for this process, but not the delivery of peptidoglycan or ADP-heptose. To confirm this, we performed immunofluorescence microscopy of AGS cells infected with either H. pylori wt or ΔcagA mutant. After 24 hr, infected cells were fixed with PFA, immunostained with α-cortactin and α-H. pylori antibodies and counterstained using phalloidin and DAPI. Cortactin staining in AGS cells infected with H. pylori wt was higher than in non-infected mock cells, while this was not the case when infected with isogenic ΔcagA mutant (Figure 5). Altogether, these results confirm the critical role of CagA delivery in cortactin overexpression upon H. pylori infection.

2.5 Cortactin overexpression is induced by ectopic expression of CagA independent of EPIYA phosphorylation

To study the role of CagA in more detail, we performed a series of transfection experiments using established CagA expression constructs (Higashi et al., 2002). For this purpose, AGS cells were

![FIGURE 5](image-url)
transfected for 12 hr, 24 hr and 48 hr and then probed with PY99 and CagA antibodies, showing that CagA is expressed and phosphorylated over time (Figure 6a). Cortactin staining revealed that cortactin was overexpressed at 24 hr and 48 hr, similar to the above infection experiments, suggesting that ectopic expression of CagA in AGS cells alone is sufficient to induce cortactin overexpression (Figure 6a,b). A hallmark of CagA translocation is its tyrosine phosphorylation (Backert et al., 2018). To investigate if tyrosine phosphorylation is involved, we transfected the cells with CagA wt and phosphorylation-deficient mutant CagA (CagA YF) for 48 hr. Only CagA wt, but not a phosphorylation-deficient YF mutant, underwent phosphorylation as expected (Figure 6c). However, both constructs significantly overexpressed cortactin, suggesting that CagA phosphorylation is not required for cortactin overexpression (Figure 6c,d).

2.6 | The MAP kinase pathway JNK is involved in cortactin overexpression by H. pylori

Finally, we wanted to identify the downstream signalling pathway that could be involved in CagA-mediated cortactin overexpression. It was previously reported that translocation and transfection of CagA can induce several MAP kinases, including ERK and p38 (Brandt et al., 2005; Z. F. Liu et al., 2012). To study if one of the MAP kinase pathways is involved in CagA-induced cortactin overexpression, we transfected CagA wt in the cells for 48 hr in the presence of PD98059, SB203580 and SP600125 inhibiting ERK, p38 and JNK, respectively. Then we probed the samples with corresponding antibodies and found that inhibition of ERK1/2 or p38 had no impact on cortactin overexpression, but inhibition of JNK abolished this effect suggesting that the JNK pathway is involved in this response (Figure 7a,b). As next, we infected AGS cells for 24 hr with H. pylori wt in the presence or absence of the above inhibitors. Western blotting of resulting cell lysates using phospho-specific antibodies revealed that H. pylori activated ERK, p38 and JNK, which was blocked in the presence of PD98059, SB203580 and SP600125, respectively (Figure 7c,d). Re-probing of the samples with α-cortactin antibodies confirmed that inhibition of JNK, but not ERK1/2 or p38, abrogated cortactin overexpression not only by CagA transfection as shown above, but also by H. pylori during infection (Figure 7c,d).

3 | DISCUSSION

Cortactin is an important actin-binding protein, which plays an intrinsic role in actin-cytoskeletal rearrangements and cellular motility by interacting with numerous regulatory proteins (Schnoor, Stradal, & Rottner, 2018). Thus, it is not surprising that various microbial pathogens can target cortactin to manipulate bacterial attachment, host cell invasion and intracellular motility as well as cell scattering (Selbach & Backert, 2005; Sharafutdinov et al., 2020). Here we show for the first time that cortactin is overexpressed in AGS and Caco-2 epithelial cells upon infection with highly pathogenic H. pylori strains. Our infection experiments in a time-course revealed that cortactin overexpression increases up to 2–3 fold at 24 hr and 48 hr after exposure to the bacteria. Using a set of clinical H. pylori type-I and type-II strains as well as isogenic mutants, we identified that the bacterial factor responsible for cortactin overexpression is encoded by a bacterial T4SS (Grohmann, Christie, Waksman, & Backert, 2018). We found that cortactin overexpression is dependent on the structural T4SS components CagY and CagL as well as the effector protein CagA, suggesting that injection of CagA is required. Translocated CagA is well-known to disturb various host cell signalling pathways, including a network of different kinases (Backert & Blaser, 2016; Hatakeyama, 2019; Tegtmeier, Neddermann, Asche, & Backert, 2017). The MAP kinases play an important role in cell differentiation, proliferation and cytokine production and the major members are presented by ERK, JNK and p38 (Arthur & Ley, 2013). Multiple previous studies have confirmed the critical role of CagA in the regulation of these pathways. For example, infection of AGS cells with type-I strains expressing a functional T4SS affected all three major MAP kinases, ERK, JNK and p38 (Keates et al., 1999). Transfection of CagA in human gastric epithelialGES-1 cells activated both ERK and p38 MAP kinase pathways (Z. F. Liu et al., 2012). In our experiments, we identified JNK as an important MAP kinase involved in cortactin overexpression in...
response to CagA. JNK is a major kinase regulating migration of various human cell types (C. Huang, Rajfur, Borchers, Schaller, & Jacobson, 2003; Z. Huang, Yan, & Ge, 2008; E. H. Lee et al., 2020; Xu & Hu, 2020). Our studies are in agreement with other studies showing that transgenic CagA can induce JNK activation in Drosophila (Wandler & Guillemin, 2012). Previous studies also showed that H. pylori infection could activate JNK in AGS cells (Krueger et al., 2006) and stimulate cell motility through a T4SS-dependent mechanism (Snider, Allison, Bellaire, Ferrero, & Cardelli, 2008). However, in the latter study JNK activation alone was CagA-independent and instead depended on the integrin β1 pathway since an integrin β1-blocking antibody significantly inhibited JNK activation (Snider et al., 2008). In addition, other work showed that JNK activation by H. pylori was independent of CagA, but was rather dependent on the T4SS itself (Glowinski, Holland, Thiede, Jungblut, & Meyer, 2014). The authors suggested that CagA primarily induced ERK1 activation, while the T4SS components activated JNK and p38 (Glowinski et al., 2014). However, to analyse H. pylori-induced JNK activation in the latter studies, the 1.5 hr and 7 hr (Glowinski et al., 2014) or 18 hr (Snider et al., 2008) time points of infection were used. Indeed, in our infection and transfection experiments, the upregulation of cortactin was observed only after 24 hr of infection. It might be assumed that CagA at the early stage of infection activates primarily the ERK kinase, while JNK activation can be observed later. Time-dependent variation in the activation of host kinases by H. pylori has been already shown for Src, Abl and ERK (Tegtmeyer et al., 2011), suggesting that a similar scenario might also be applied for JNK activation over time.

We propose that overexpression of cortactin by translocated CagA is based both on enhanced mRNA expression and protein stability (model in Figure 8). In this model, injected CagA induces downstream signalling leading to cortactin overexpression in a phosphorylation-independent manner. The first signalling cascade triggers the Ras/ERK axis activation through CagA interaction with the adaptor protein Grb2 (Mimuro et al., 2002). Subsequently, ERK can phosphorylate cortactin at S-405 and S-418, followed by interaction with and activation of FAK through its autophosphorylation at Y-397, Y-407 and Y-576 (Tegtmeyer et al., 2011). Once activated, FAK can either signal to JNK via cortactin (Eke et al., 2012) or activate the focal adhesion-associated adapter protein paxillin by phosphorylation at Y-31 and Y-118 (Z. Huang et al., 2008). Prior to tyrosine phosphorylation at Y-31 and Y-118, paxillin requires its serine phosphorylation at S-178 by JNK (C. Huang et al., 2003). The second proposed signalling cascade, leading to JNK activation, involves the partitioning-defective kinase Par1b forming a complex with CagA through the CRPIA motif (Saadat et al., 2007). This complex mediates Par1b inactivation, so that Par1b can no longer phosphorylate guanine exchange factor GEF-H1 at S-885 and S-959, resulting in its activation and stimulation of the small GTPase RhoA (Yamahashi, Saito, Murata-
Kamiya, & Hatakeyama, 2011). Activated RhoA finally triggers JNK signalling leading either to cell apoptosis or tumour progression, as shown by transgenic expression of CagA in Drosophila melanogaster (Wandler & Guillemin, 2012). Furthermore, CagA inhibited the neoplastic tumour suppressors (nTSGs), driving JNK-mediated tumour development generated by oncogenic Ras expression (Wandler & Guillemin, 2012). In addition, JNK inactivation was shown to activate the transcription factor Snail1 via Smad2/4, repressing cortactin gene expression (M. S. Lee et al., 2014). Interestingly, infection of AGS cells with H. pylori led to a slight increase in cttn mRNA level as was shown by RNA-seq (Koeppel, Garcia-Alcalde, Glowinski, Schlaermann, & Meyer, 2015); however, more research in this regard is required. Therefore, cortactin overexpression at the transcription level, at least partially, is dependent on JNK activity. On the other hand, we propose that cortactin overexpression is related to the enhanced protein stability, for instance, through interaction with FAK and/or paxillin (Bowden, Barth, Thomas, Glazer, & Mueller, 1999; Eke et al., 2012). Indeed, serine-phosphorylated cortactin is prone to degradation, for instance, through degradation by calpain (Perrin, Amann, & Huttenlocher, 2006) or ubiquitin-proteasome complex (Zhao et al., 2012). Therefore, association with other proteins might preserve cortactin from the interaction with proteases and subsequent apoptosis.
expression has also been shown to enhance EGFR protein levels, expression (H. S. Liu et al., 2009). Interestingly, cortactin overexpression (Figure 8). Therefore, paxillin and FAK might represent factors, indirectly enabling JNK-mediated cortactin overexpression (Figure 8).

Cortactin has been clearly implicated in various human diseases, including tumour growth and metastasis (MacGrath & Koleske, 2012; Villaronga et al., 2018; Wang et al., 2010; Wei et al., 2014). Interestingly, the cortactin gene actn1 is highly expressed in various human cancers and is associated with increased cell motility and tumor invasion (Sanchez, 2019). Similar to cortactin, paxillin and FAK are associated with increased cell motility and tumor invasion and both are targeted by H. pylori for cytoskeletal rearrangements (Badowski et al., 2008; Bowden et al., 1999; Tabassam, Graham, & Yamaoka, 2011; Tegtmeyer et al., 2011). Therefore, paxillin and FAK might represent factors, indirectly enabling JNK-mediated cortactin overexpression.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell lines and culture conditions

The human gastric adenocarcinoma cell line AGS (ATCC CRL-1739™) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS; Gibco, Paisley, UK) and 2 mM L-glutamine (Invitrogen, Karlsruhe, Germany) (Hartung et al., 2015). The Caco-2 cell line (ATCC HTB-37™) derived from a human colon adenocarcinoma was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and 4 mM L-glutamine. Both cell lines were cultivated at 37°C in incubators with 5% (v/v) CO2 followed by subculturing at a ratio of 1:3−1:5 every 2−3 days at a confluence of 70%–90%. All cells were routinely grown in 75 cm² tissue culture flasks and seeded into 6- or 12-well plates (Greiner-Bio-One, Frickenhausen, Germany) before infection.

4.2 | Helicobacter pylori strains and isogenic mutants

For the infection experiments, H. pylori cagPAI-positive wt strains N6, NCTC11637, P12, 26-695 and G27 and cagPAI-negative wt strains UH4, Ka125, Safr7 and 1061 were used (Backert et al., 2004; Conradi et al., 2016; Tegtmeyer et al., 2020). In addition, a set of isogenic H. pylori mutants was applied, including P12ΔvacA, P12Δstl, P12ΔgmiA, P12ΔcagA, P12ΔcagY and P12ΔcagI. (Pachathundikandi et al., 2019; Tegtmeyer et al., 2020). The H. pylori strains were stored as glycerol stocks at −80°C. Before each use, bacterial cells were cultivated as thin layers on GC agar plates supplemented with 5% horse serum, 1% vitamin mix, 5 μg/ml trimethoprim, 10 μg/ml vancomycin and 1 μg/ml nystatin (Patel et al., 2013). For the growth of isogenic H. pylori mutant strains, appropriate antibiotics were added in the culture medium. The antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Subsequently, bacteria were grown at 37°C for 2 days in anaerobic jars containing a CampyGen™ gas mix of 85% N2, 10% CO2 and 5% O2 (Oxoid, Wesel, Germany).

Before infection, bacterial cells were resuspended in phosphate-buffered saline (PBS, pH 7.4), and the bacterial number was determined by measuring an optical density at 600 nm. For infection, bacterial cells were added in cell culture medium (without antibiotics) at 10% CO2 and 5% O2 (Oxoid, Wesel, Germany).
an MOI of 50 for the times indicated in the figure legends. Uninfected control cells were incubated with a corresponding volume of PBS without bacteria. For inhibitory experiments, H. pylori were pretreated for 10 min with either 10 μg/ml proteinase K, 4% PFA, 10 μg/ml streptomycin/penicillin, 20 μg/ml chloramphenicol or heat-treated at 95°C before co-incubation with AGS cells.

4.3 | Immunofluorescence microscopy and fluorescence intensity quantification

For the immunofluorescence microscopy, Caco-2 and AGS cells were grown in 12-well plates as described above. The cells were co-incubated with H. pylori for 24 hr, then washed twice with PBS and fixed in 4% PFA. For cell permeabilization, 0.1% saponin was routinely used. Cells were blocked for 1 hr in 1% BSA and immunostained with primary mouse α-cortactin (#05–180, Merck-Millipore, Darmstadt, Germany), rabbit α-H. pylori (Dako, Glostrup, Denmark) and secondary FITC (fluorescein isothiocyanate)-conjugated α-mouse or Alexa Fluor 633-conjugated α-rabbit (Thermo Fisher Scientific, Darmstadt, Germany) antibodies. DAPI and rhodamine phalloidin conjugated with TRITC (tetramethylrhodamine isothiocyanate) were used for counterstaining cell nuclei and F-actin, respectively. Samples were investigated by confocal laser scanning microscopy using a Leica SP5 (Leica Microsystems, Wetzlar, Germany) at the Optical Imaging Centre Erlangen (OICE, Erlangen, Germany) (Pachathundikandi et al., 2019). Excitation/emission of the fluorescence from DAPI, FITC, TRITC and Alexa Fluor 633 was processed at 390/413–460 nm, 488/496–550 nm, 561/571–630 nm and 633/643–700 nm wavelengths, respectively. The obtained data were visualised using LAS AF computer software (Leica Microsystems). For the quantification of the fluorescence intensities of cortactin and F-actin, the images after pretreatment of the membranes with 20°C for 1 hr. Non-phosphorylated and phosphorylated CagA protein species were detected using the rabbit polyclonal α-CagA antibody (# HPP-5003-9, Austral Biologicals, San Ramon, CA, USA) and mouse monoclonal α-pan-phosphorytrosine antibody PY-99 (#sc-7020, Santa Cruz, Heidelberg, Germany), respectively (Moese et al., 2001). Monoclonal mouse antibodies recognising GAPDH expression (#sc-20357, Santa Cruz) were used as loading controls. Horseradish peroxidase-conjugated α-rabbit (#31462) or α-mouse (#31446) polyclonal goat immunoglobulins, respectively, were used as secondary antibodies (Thermo Fisher Scientific, MA, USA) (Tegtmeier et al., 2016). To detect the antibodies, ECL Prime chemiluminescence Western blot kit (GE Healthcare) was used (Blumenthal, Hoffmann, Aktories, Backert, & Schmidt, 2007; Krause-Grusczynska et al., 2011).

4.4 | Transfection of AGS cells

AGS cells (0.8 × 10⁶) were cultivated in six-well plates and transfected with 2.5 μg of described CagA wt or phosphorylation-negative CagA YF (tyrosine to phenylalanine mutant) constructs and corresponding empty vector control (Higashi et al., 2002) with Lipofectamine™ 3000 reagent for 12 hr, 24 hr or 48 hr according to the supplier’s protocol (Thermo Fisher Scientific, Waltham, MA, USA).

4.5 | Specific MAP kinase inhibitors

For inhibition of the MAP kinases ERK, p38 and JNK, AGS cells were treated 30 min prior to infection with the specific inhibitors PD98059 (25 μM), SB203580 (25 μM) and SP600125 (25 μM), respectively. SB203580 is a selective inhibitor of p38 MAP kinase, which inhibits the activation of MAPKAPK-2 and subsequent phosphorylation of HSP27 (Cuenda et al., 1995). PD98059 is a highly selective in vitro inhibitor of MEK1 activation, an upstream activator of ERK1/2 (Rosen, Ginty, Weber, & Greenberg, 1994). SP600125 is an antrapyrazolone inhibitor of JNK that competes with ATP to inhibit the phosphorylation of c-Jun (Bennett et al., 2001). All inhibitors were purchased from Sigma-Aldrich. AGS cells were incubated with the corresponding inhibitors or with the same amount of DMSO without inhibitor (Mock) as control following Western blotting and probing with the α-CagA and α-cortactin antibodies.

4.6 | SDS-PAGE, antibodies and immunoblot analysis

For protein analysis, samples were harvested, boiled for 5 min, and then subjected to standard dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, protein lysates were loaded onto 6–12% polyacrylamide gels, with following blotting on polyvinylidene difluoride (Immobilon-P PVDF) membranes (Merck-Millipore), TBS-T buffer (25 mM Tris–HCl pH 7.4, 140 mM NaCl, 0.1% Tween-20) supplemented with 5% skim milk or 3% bovine serum albumin was used for blocking of the membranes at 20°C for 1 hr. Non-phosphorylated and phosphorylated CagA protein species were detected using the rabbit polyclonal α-CagA antibody (# HPP-5003-9, Austral Biologicals, San Ramon, CA, USA) and mouse monoclonal α-pan-phosphotyrosine antibody PY-99 (#sc-7020, Santa Cruz, Heidelberg, Germany), respectively (Moese et al., 2001). Monoclonal mouse antibodies recognising GAPDH expression (#sc-20357, Santa Cruz) were used as loading controls. Horseradish peroxidase-conjugated α-rabbit (#31462) or α-mouse (#31446) polyclonal goat immunoglobulins, respectively, were used as secondary antibodies (Thermo Fisher Scientific, MA, USA) (Tegtmeier et al., 2016). To detect the antibodies, ECL Prime chemiluminescence Western blot kit (GE Healthcare) was used (Blumenthal, Hoffmann, Aktories, Backert, & Schmidt, 2007; Krause-Grusczynska et al., 2011).

4.7 | Quantification of protein bands by Western blotting

For the quantification of band signals on immunoblots, the densitometric analysis was applied using the Image Lab software (BioRad, Munich, Germany). The fold change of cortactin expression per sample is shown and was normalised to the GAPDH expression blot, presented in the corresponding figures. The control band in the first lane of each blot was set at 1.

4.8 | Statistical data analyses

Each experiment was performed independently at least three times with similar results. Statistics was calculated by Student’s t-test or
one-way ANOVA followed by Tukey’s test. The obtained p values $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) were defined as significant.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Nicole Tegtmeyer; Methodology, Nicole Tegtmeyer and Steffen Backert; Investigation, Nicole Tegtmeyer, Irshad Sharafutdinov; Formal Analysis, Nicole Tegtmeyer, Irshad Sharafutdinov and Steffen Backert; Writing-Original Draft, Irshad Sharafutdinov and Nicole Tegtmeyer; Writing-Review & Editing, Irshad Sharafutdinov, Steffen Backert, Nicole Tegtmeyer; Supervision, Nicole Tegtmeyer.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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