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FORMAL COMMENT

Different roles of integrin-β_1 and integrin-α_v for type IV secretion of CagA versus cell elongation phenotype and cell lifting by *Helicobacter pylori*

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Formal comment for Zhao et al. 2018 (PLoS Pathog. 14: e1007359).

Using CRISPR/Cas9 gene knockout, Zhao *et al.* [1] unexpectedly reported that neither heterodimers of integrin-β_1 nor integrin-α_v are essential for translocation of CagA by a type IV secretion system (T4SS) and for induction of the cell elongation phenotype by *H. pylori*. We performed infection experiments using the reported AGS wild-type and isogenic knockout cells for integrin-β_1 (ΔITGB1) and integrin-α_v/integrin-β_4 (ΔITGAvB4) [1] with various worldwide T4SS-positive strains including TN2-GF4, G27 and P12 used by the authors. The absence or presence of integrin-β_1 and integrin-α_v in the respective cell lines was confirmed (Fig 1A–1C). Subsequently, the blots were probed with α-CagA and α-PY-99 antibodies to monitor CagA phosphorylation ($CagA^{PY}$), indicating successful translocation. Infection with all strains produced strong $CagA^{PY}$ signals in all cell lines. Densitometric quantification revealed some differences among band intensities between cell lines and strains, but did not reach statistical significance (Fig 1A–1C). Thus, we can confirm that the knockout of ITGB1 or ITGAvB4 in AGS cells did not significantly affect CagA delivery and phosphorylation. Surprisingly, however, we found that knockout of ITGB1 completely abolished the cell elongation phenotype (Fig 1D and 1E), while inactivation of ITGAvB4 even enhanced this phenotype significantly (Fig 1F). These data contradict above findings, suggesting that corresponding pictures have been differently interpreted [1]. Our data instead suggest a yet unrecognized and important role of ITGB1 and suppressive function for ITGAvB4. However, numerous pressing questions arose by the above new findings.

First, how can one explain the negative results concerning the role of integrins on T4SS-dependent CagA delivery? AGS cells express a series of integrin-β_1-based heterodimers ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$), integrin-α_v heterodimers ($\alpha_v\beta_5$ and $\alpha_v\beta_6$) and integrin-$\alpha_6\beta_4$ [1,2]. Knockout of the β_1 gene should therefore abrogate surface exposure of every possible β_1-comprising integrin heterodimers [1]. To avoid functional substitution of β_1 by other integrin combinations (such as $\alpha_v\beta_5$, $\alpha_v\beta_6$ or $\alpha_6\beta_4$), integrins α_v and β_4 were also inactivated, and did not significantly compromise CagA translocation either [1]. These data contradict earlier publications that CagA delivery was achieved in integrin-β_1 expressing GE11 or GD25 mouse cell lines, but not in their isogenic integrin-β_1 knockouts [2,3]. However, bacterial binding to these cells may differ, but was not tested. In addition, we know today that these cells do not express human CEACAMs, which are newly discovered receptors for CagA delivery [4,5]. It was proposed that CagA translocation in the integrin-β_1-expressing counterparts was only at low background levels in cells lacking human CEACAMs with integrins having only a small

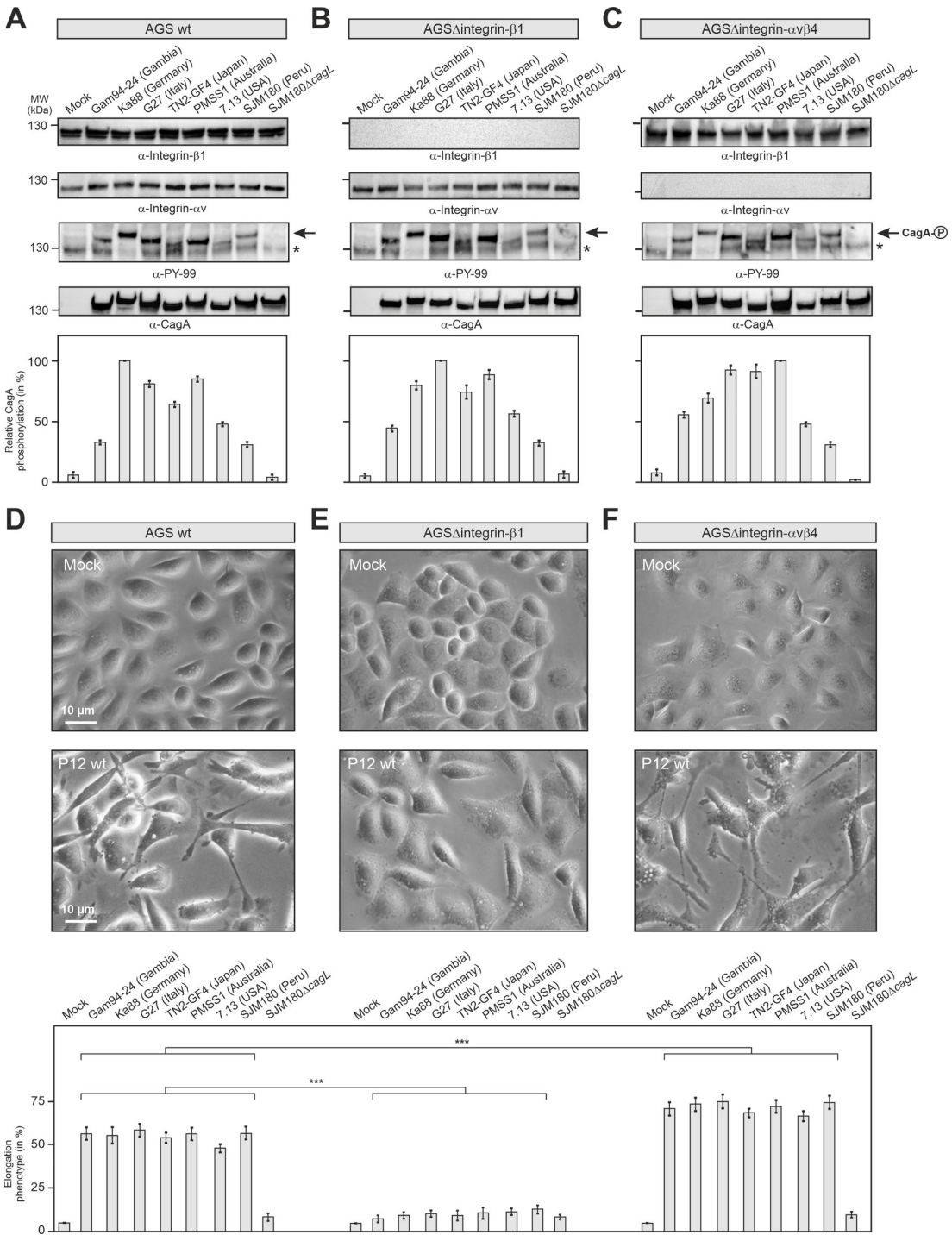

Fig 1. Quantification of CagA phosphorylation and elongation phenotype upon *H. pylori* infection of AGS gastric epithelial cell types. (A) AGS wild-type, (B) AGSΔITGB1 and (C) AGSΔITGAvB4 knockout cells [1] were infected with the indicated *H. pylori* strains for 6 hours using a multiplicity of infection of 50. Western blotting using specific antibodies confirmed the integrin-β_1 and integrin-α_v expression status (top panels). Probing with α-CagA and α-PY-99 antibodies (middle panels) was applied to quantitate the degrees of CagA phosphorylation in triplicates by densitometry (bottom panels). The asterisks indicate the position of a phosphorylated ~125 kDa host cell protein on the gels. (D-F) Phase contrast microscopy of non-infected vs. infected AGS cell lines (top and middle panels). The elongation phenotype was quantitated in triplicate experiments showing inhibition in infected ΔITGB1 cells and enhancement in ΔITGAvB4 cells compared to the AGS wild-type control (bottom panels).

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supportive effect on CagA delivery [1]. Other earlier data showed that two function-interfering integrin-β_1 antibodies or recombinant CagA fragments, comprising the integrin-β_1 binding site, downregulated CagA translocation [2,3,6]. In the light of the new data, these previous observations can be interpreted as indirect impact of integrin-β_1 on CagA translocation, such as steric hindrance of CEACAM receptors, together implying that integrin-β_1 is not essential.

Second, how can we explain the different results concerning the role of integrins on the elongation phenotype? CagA translocation by *H*. *pylori* and subsequent phosphorylation were reported to be essential for the elongation phenotype [7,8]. The essentiality of the protein for phenotypical outcome was later confirmed by ectopic expression of CagA in AGS cells [9]. $CagA^{PY}$ induces this phenotype through a cell retraction defect during cell movement (Fig 2A and 2B). Time-lapse video microscopy has shown that infected cells undergo elongation because they failed to release their "back ends" upon cell locomotion [10]. These "back ends" represent enlarged focal adhesions (FAs) and their disassembly is inhibited resulting in elongated cell projections [8]. Thus, it was proposed that the function of $CagA^{PY}$ is to strengthen the FAs in epithelial cells, preventing excessive cell lifting during infection [11]. To achieve this goal, $CagA^{PY}$ manipulates the activities of tyrosine phosphatase SHP2 [9] and actin-binding protein cortactin [11,12], resulting in the deregulated action of focal adhesion kinase FAK [11–13]. In turn, FAK activity controls cell adhesion to the extracellular matrix (ECM) through integrin. Besides FAK, the FA complex comprises many other signaling factors including kinase Src and cytoskeletal proteins talin, paxillin, vinculin, p130Cas and α-actinin, connecting to the actin-cytoskeleton [14–16]. The cytoplasmic domain of integrin-β_1 was exemplary shown to interact directly with α-actinin, paxillin, talin and FAK [14–16], and is sufficient for its recruitment to preformed FAs and signal transduction to FAK [16–19]. This can explain why integrin-β_1 is absolutely required for the *H*. *pylori*-induced elongation phenotype in AGS cells. In the absence of integrin-β_1, α_v-containing FAs can be formed [20], however, we postulate that cortactin and SHP2 only have a low or no impact on the turnover of FAs upon infection of ΔITGB1 cells or the composition of the FAs and/or phosphorylation status of FA proteins might have changed (Fig 2C). On the other hand, knockout of ITGAvB4 in AGS cells enhances the elongation phenotype by a yet unknown mechanism, which needs to be elucidated in future studies.

Third, what is the role of the high affinity T4SS-integrin-β_1 interaction if not required for CagA delivery? It seems clear that *H*. *pylori* evolved at least four known T4SS proteins (CagA, CagI, CagL and CagY) that bind to the extracellular domain of integrin-β_1 [1–3,6]. Zhao et al. [1] proposed that binding of these *cag* proteins to integrin-β_1 heterodimers may allow tethering of the T4SS-pilus to the host cell resulting in a low level CagA translocation, but full CagA translocation requires the CEACAMs. In addition, the onset of intracellular signaling through integrins can be triggered. While there are no reports on the potential role of CagA, CagI and CagY for integrin signaling, a role of CagL has been described in detail. This includes the activation of transcription factor NF-κB leading to suppression of H,K-ATPase-α and gastric acid secretion [21], induction of IL-8 [22] and introduction of DNA double-strand breaks via endonucleases XPF and XPG leading to genome instability [23] as well as phosphorylation of cellular tyrosine kinases comprising Src, FAK, EGF receptor and its family member Her3/ErbB3 [24]. Remarkably, the ECM protein fibronectin can activate the same repertoire of kinases, except Her3/ErbB3 [20,25,26]. In addition, cultured host cells can robustly attach to immobilized CagL, which triggers FA formation and host cell spreading, a characteristic that was previously only known for ECM proteins like fibronectin [24]. Purified CagL could even complement the spreading defect of fibronectin knockout cells *in vitro*. These findings suggest that CagL displays functional mimicry with fibronectin [24]. In addition, CagL was previously shown to bind other integrins (AvB3, AvB5 and AvB6) with role in gastrin secretion and other

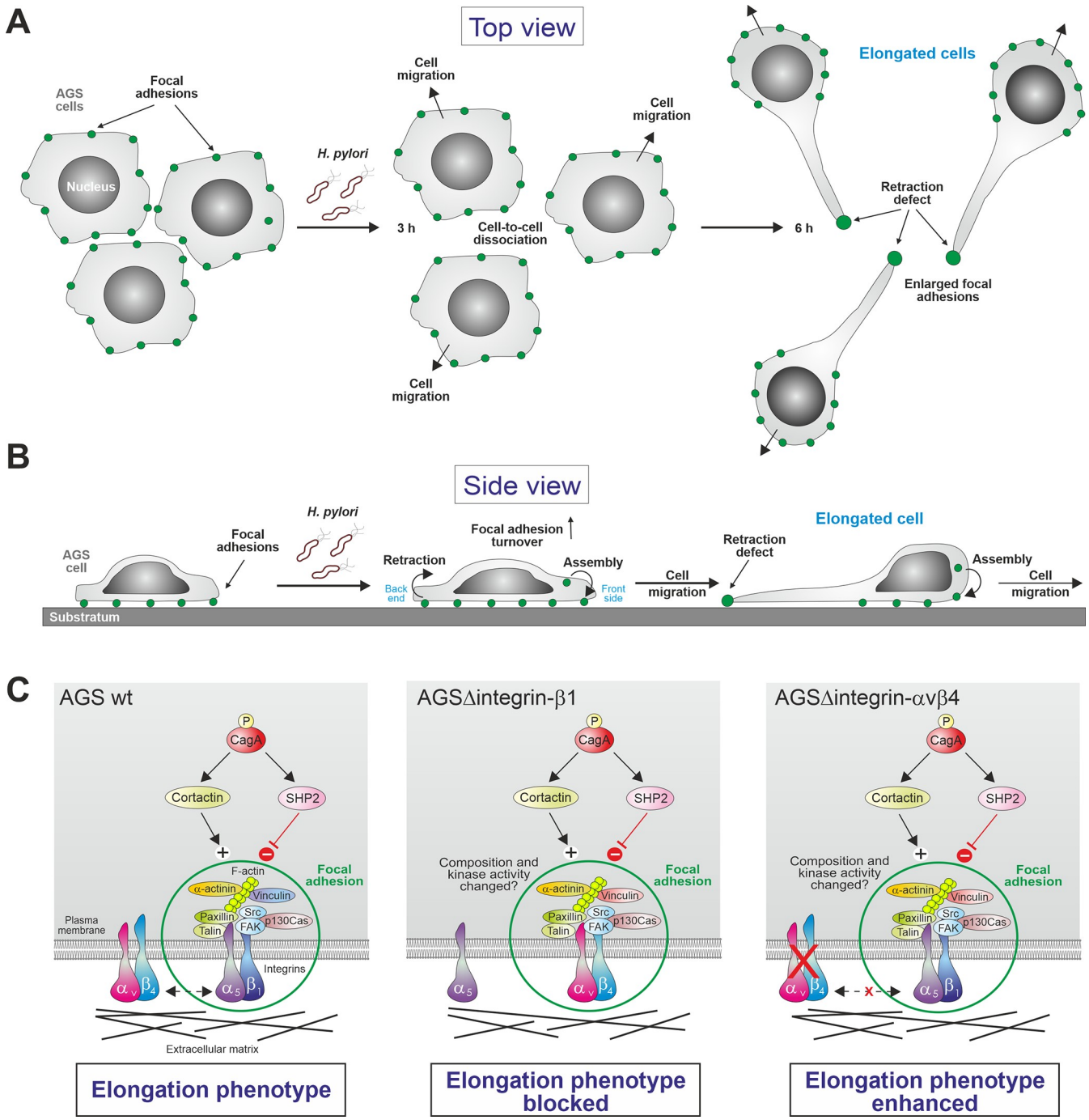

Fig 2. Schematic model for the elongation phenotype in *H. pylori* infected AGS cells and molecular events controlling phenotypic outcome. (A) Top view and (B) side view of infected AGS wild-type cells. Infection with T4SS-positive *H. pylori* induces host cell motility visible after 3–6 hours. The elongation phenotype depends on intracellular $CagA^{PY}$ facilitating a cell retraction defect at the focal adhesions (FAs, green) during cell movement as indicated by arrows. Cell migration is characterized by the controlled FA assembly at the front side and their disassembly (retraction) at the back end of the cells as shown. The back ends become enlarged through the activities of $CagA^{PY}$ and its disassembly is inhibited, resulting in elongated cell protrusions. (C) Proposed model for phenotypical outcome. The FA complexes comprise the indicated transmembrane integrin receptor heterodimers and a series of intracellular signaling factors including the tyrosine kinases Src and FAK and cytoskeletal proteins. Injected $CagA^{PY}$ manipulates the activities of phosphatase SHP2 and the actin-binding protein cortactin, leading to the deregulation of FAK kinase activity. In this fashion, FAK controls cell adhesion to the extracellular matrix (bottom). A positive regulatory role is played by the integrin-$\alpha_5\beta_1$ heterodimer to which some of the indicated factors directly interact, while ITGAvB4 knockout exhibits opposing effects. As a result, $CagA^{PY}$ triggers the adhesion of wild-type AGS cells (left) resulting in the elongation phenotype, while this phenotype is blocked in ΔITGB1 cells (middle) and enhanced in ΔITGAvB4 cells (right). For more details, see text.

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functions [27–29]. Together, we propose that a direct binding of components of the T4SS such as CagL (and probably also CagA, CagI, and CagY) to integrin-β_1 is to trigger intracellular signaling for bacterial advantage, but also enhance cell attachment and FA formation, thereby supporting the above discussed intracellular activities of $CagA^{PY}$ towards FAK, with the overall goal to prevent excessive cell lifting during the course of infection. Future experiments should address these interactions of *H. pylori* with integrins in more detail and study the exact function of CEACAM receptors for CagA delivery.

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