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Vorstand: Prof. Dr. Sebastian Suerbaum

Integrin-independent *Helicobacter pylori* CagA translocation via type IV secretion

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Abbreviations

aa	Amino acids
BB	Brucella broth
BER	Base excision repair
BLA	Beta-lactamase
BSA	Bovine serum albumin
CagA	cytotoxin-associated gene A
Cas	CRISPR associate
CEACAM	carcinoembryonic antigen-related cell adhesion molecules
CG	cholesteryl glucosides
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
DSB	Double strand break
ECM	Extracellular matrix
EMT	epithelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FAK	focal adhesion kinase
FCS	Fetal calf serum
FN	Fibronectin
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescence protein
HDR	homology directed repair
Hpy	*Helicobacter pylori*
IL	interleukin
ILK	Integrin-linked kinase
ITG	integrin
KO	knockout
MALT	mucosa-associated lymphoid tissue

MAPK	mitogen-activated protein kinases
MβCD	methyl-β-cyclodextrin
NHEJ	non-homologous end joining
OMP	outer membrane protein
PAI	pathogenicity island
PAM	protospacer adjacent motif
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog
sgRNA	short guide RNA
SSM	slipped strand mispairing
SP	*Streptococcus thermophiles*
ssODN	single strand DNA oligonucleotides
T4SS	type IV secretion system
TALEN	transcription activator-like effector
trancrRNA	trans-activating crRNA
TrkB	tropomycin-related kinase B
UPEC	Uropathogenic *Escherichia coli*
VacA	Vacuolating cytotoxin A
YTH	Yeast Two Hybrid
ZFN	Zinc finger nuclease

Summary

Helicobacter pylori is a Gram-negative gastric pathogen that infects approximately half of the world population. *H.pylori* infection is associated with many gastric diseases like gastritis, peptic ulcers and gastric cancer. Especially, translocation of the cytotoxin-associated gene A (CagA) protein via the *cag*-type IV secretion system (*cag*-T4SS) into host gastric epithelial cells is a major risk factor for developing gastric cancer. However, the exact molecular mechanism of CagA translocation into host cells and host factors involved in this process are not well characterized. Previously, host cell integrin β1 was reported to be exploited by the *H.pylori cag*-T4SS for CagA translocation. In addition, host carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) were recently identified to interact with the *H.pylori* adhesin HopQ to contribute to CagA translocation.

In order to study the role of host cell integrin in CagA translocation and to identify other integrins (besides integrin β1) which might be exploited by *H.pylori*, the CRISPR-Cas system was applied to generate single to multiple integrin depletion AGS and KatoIII gastric epithelial cell lines. By targeting the integrin β1, αv and β4 genes first individually, then simultaneously, all 17 potential non-leukocyte and non-platelet integrins, including 9 epithelial-specific integrins were systematically depleted on the cell surface. CagA translocation efficiency was subsequently evaluated in each integrin-depletion cell line by a quantitative β-lactamase dependent reporter assay. Unexpectedly, the CagA translocation efficiency was not found to be reduced in AGS cells in the context of single or multiple integrin depletion. However, CagA translocation efficiency was compromised in KatoIII cells, not because of the absence of host cell integrins, but because of decreased CEACAM5 and CEACAM6 expression which has been induced by integrin depletion.

Therefore this work describes for the first time a novel CagA translocation mechanism into gastric epithelial cell lines (AGS and KatoIII) *in vitro,* which is completely independent of host cell integrins. In addition, the data confirmed that the HopQ-CEACAM interaction is necessary for CagA translocation. Besides the CEACAMs, probably other unknown host receptors are exploited by *H.pylori* for CagA translocation.

Zusammenfassung

Etwa die Hälfte der Weltbevölkerung ist mit dem pathogenen Magenbakterium *Helicobacter pylori* infiziert. Die Infektion mit *H. pylori* ist mit verschiedenen Magenerkrankungen wie Gastritis, Magengeschwüren und Magenkrebs assoziiert. Insbesondere die Translokation des Zytotoxin-assoziierten Gen A (CagA) Proteins in Magenepithelzellen des Wirtes, was durch das *cag*-Typ IV-Sekretionssystem (*cag*-T4SS) ermöglicht wird, ist ein Risikofaktor für die Entstehung von Magenkrebs. Dennoch ist der exakte molekulare Mechanismus der CagA-Translokation in Wirtszellen sowie Wirtsfaktoren, die in diesen Prozess involviert sind, nicht genau charakterisiert. In früheren Studien wurde gezeigt, dass das Wirtszellintegrin β1 durch das *cag*-T4SS für die CagA-Translokation genutzt wird. Zusätzlich wurden Zelladhäsionsmoleküle der karzinoembryonalen Antigenfamilie (CEACAMs) identifiziert, welche mit dem *H. pylori* Adhesin HopQ interagieren und dadurch zur CagA-Translokation beitragen.

Um den Einfluss von Wirtszellintegrinen auf die CagA-Translokation zu untersuchen und neue von *H. pylori* ausgenutze Integrine (neben Integrin β1) zu identifizieren, wurde das CRISPR-Cas System angewandt, um Integrine in epithelialen Magenadenokarzinom-Zelllinien (AGS und KatoIII) zu depletieren. Indem die Integringene β1, αv und β4 sowohl einzeln als auch simultan inaktiviert wurden, konnten alle 17 nicht mit Leukozyten oder Thrombozyten assoziierten Integrine, einschließlich 9 mit Epithelien assoziierte Integrine, systematisch von der Zelloberfläche depletiert werden. Die CagA-Translokations-effizienz wurde in jeder Integrin-depletierten Zelllinie mittels eines quantitativen β-Lactamase Reporterassays bestimmt. Überraschenderweise war die CagA-Translokationseffizienz in keiner der Integrin-depletierten AGS Zelllinien reduziert. Hingegen war die CagA-Translokationseffizienz in Kato III Zellen reduziert, was jedoch nicht auf die Abwesenheit der Wirtszellintegrine, sondern auf eine durch Integrindepletion induzierte Expressionsreduktion von CEACAM5 und CEACAM6 zurückgeführt wurde.

Daher beschreibt diese Arbeit erstmals eine von Wirtszellintegrin unabhängige CagA-Translokation in epitheliale Magenadenokarzinom-Zelllinien (AGS and Kato III) *in vitro*.

Zusätzlich wurde die Relevanz der HopQ-CEACAM Interaktion für die CagA-Translokation bestätigt. Neben CEACAMs könnten weitere, unbekannte Wirtszellrezeporten für die CagA-Translokation von *H. pylori* ausgenutzt werden.

1 Introduction

1.1 The CRISPR-Cas system

During the past 20 years, different genome engineering technologies have raised considerable attention. For the first time, molecular biologists were able to manipulate DNA molecules to investigate and elucidate their role and function in the context of the whole genome. This holds promises for the development of novel drugs and therapeutic strategies. Early genome editing tools include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Both systems were designed as chimeric nucleases composed of sequence-specific DNA binding proteins fused to non-specific DNA cleavage modules. After introduction of site-specific double strand breaks (DSBs) by chimeric nucleases, DNA repair pathways are initiated to facilitate genome editing. However, these systems have their limitations, since they both exhibit context-dependent specificity due to the crosstalk between DNA binding modules and their target DNA sequences. In addition, the pre-design and post-screening processes are time-consuming, labor intensive and costly. Of the most thrilling invention is the recent discovery and application of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system as a powerful alternative for genome engineering in a variety of cell types and organisms with high efficiency, easy design and low cost.

The CRISPR-Cas system is naturally present in roughly 40% of sequenced bacteria and 90% of archaea. It represents a prokaryotic adaptive immune defense system against viral infection. Different from ZFNs and TALENs, the CRISPR-Cas system uses short RNAs to guide nucleases for recognizing target DNA through Watson-Crick base paring and introducing DSBs specifically. Three broad groups (type I-III) of CRISPR-Cas systems have been identified across different bacterial and archaeal hosts. Each system comprises a CRISPR array, a cluster of CRISPR associated (Cas) genes and noncoding RNAs. CRISPR array is a regulatory array containing multiple signature short direct repeats, which are interrupted by similarly sized variable sequences termed “spacers” that derived from phages and invaders. Most CRISPR arrays are transcribed

and processed as mature CRISPR RNAs (crRNAs). Whereas Cas genes are typically adjacent to CRISPR arrays and are translated into nucleases, which subsequently form complexes with and are guided by crRNAs to the target DNA to introduce DSBs. In addition, non-coding RNAs in the system are required as tans-activating RNA to facilitate and support the DNA targeting. Comparing to type I and type III CRISPR loci which encode redundant and multiple Cas genes, the type II loci contain significantly reduced numbers of Cas genes and are thus favored by scientists to harness for an genome editing tool.

1.1.1 History of the CRISPR-Cas system development

The CRISPR system was first reported in 1987 [1], Nakata and colleges were curious about the mysterious 29nt repeats downstream of the *iap* gene, when they studied alkaline phosphatase isozyme conversion in *E.coli*. After this, other components belonging to the system were identified one after the other. By 2005, more than one group found that the spacers derived from foreign genetic elements had phage-associated origins [2, 3]. This was the first time for scientists to surmise the biological function of the CRISPR system — it might be involved in microbial adaptive immunity against bacteriophage attack. As the CRISPR story became more interesting, researchers accelerated their paces. By 2011, the basic structure and function of this system became clear [4, 5]. However, the potential of the CRISPR system as a powerful genome editing tool had not been explored until 2013, when two studies simultaneously showed the first experimental evidences of applying CRISPR system in eukaryotic cell lines for the purpose of gene editing [6, 7]. This sparked the beginning of a new era for eukaryotic genome engineering. From then on, many labs applied this powerful technology and hence the whole molecular biology research had accelerated greatly.

1.1.2 Working mechanism of the natural prokaryotic CRISPR-Cas system

Bacteria and archaea are under constant attacks from viruses and other invaders. Not surprisingly, they evolved a prokaryotic adaptive immune system against foreign DNA. Taken the type II CRISPR-Cas system as an example, the defense mechanism involves

two phases. In the first so-called "immunization" phase, the Cas protein complex cleaves the injected DNA from invading phages into small fragments and inserts them in the CRISPR array between the direct repeats as "spacers". In the second "immunity" phase, the CRISPR array is transcribed as a long pre-crRNA, which recruits Cas9 nucleases to form a complex. At the same time, trancrRNA, an auxiliary trans-activating crRNA, is transcribed and binds to the repeat region of the pre-crRNA. Subsequently, RNase III recognizes the hybridized crRNA-trancrRNA and cleaves site-specifically, producing short crRNAs which form a mature complex with both Cas9 and trancrRNA. Eventually, mature crRNAs guide Cas9 nucleases to the target DNA to introduce precise cleavages and to produce DSBs. In this way, foreign DNA is degraded and the harmful invaders are defeated.

It is worth noting that recognition and cleavage of target DNA by the crRNA-trancrRNA-Cas9 complex not only involves the Watson-Crick pairing between the spacer and the complementary target sequence (also termed as "protospacer"), but also the presence of a specific short sequence immediately following the 3'-end of the target, which is termed as protospacer adjacent motif (PAM). PAM differs according to its Cas9 orthologue, for example, *Streptococcus pyogenes* requires NGG as the PAM, where N is any nucleotide [8]; *Neisseria meningitides* requires NNNNGATT [9, 10]. PAM is crucial for the system to distinguish between self and non-self to avoid autoimmunity since PAM doesn't exist in the repeat region of the CRISPR locus. In this way, Cas9 can only recognize and cleave target DNA, but not the spacer in the CRISPR locus [11].

Wildtype Cas9 in type II system was reported to make a blunt-ended, double-stranded break 3 bp upstream of the PAM sequence [12]. Double strand breaks are a crucial first step for genome editing, since the DNA repair system can be triggered when the DSBs are created [13]. DSBs can be repaired either by nonhomologous end-joining (NHEJ) or by homology directed repair (HDR) in nearly all different cells and organisms [14]. In the absence of the repair templates, DSBs are re-ligated by the error prone NHEJ pathway, which results in random indel (insertion/deletion) mutations. Indel mutations within coding exons can lead to frame shift and downstream stop codons. Therefore, DSB repair in the absence of homologous templates can mediate gene knockout [15]. In the presence of the homologous templates, dividing cells can undergo high fidelity HDR, generating gene knockin or precise point mutations specified

by the provided exogenous templates [16]. The repair template can be designed as single-stranded DNA oligonucleotides (ssODN) [17, 18] which appears simple and effective.

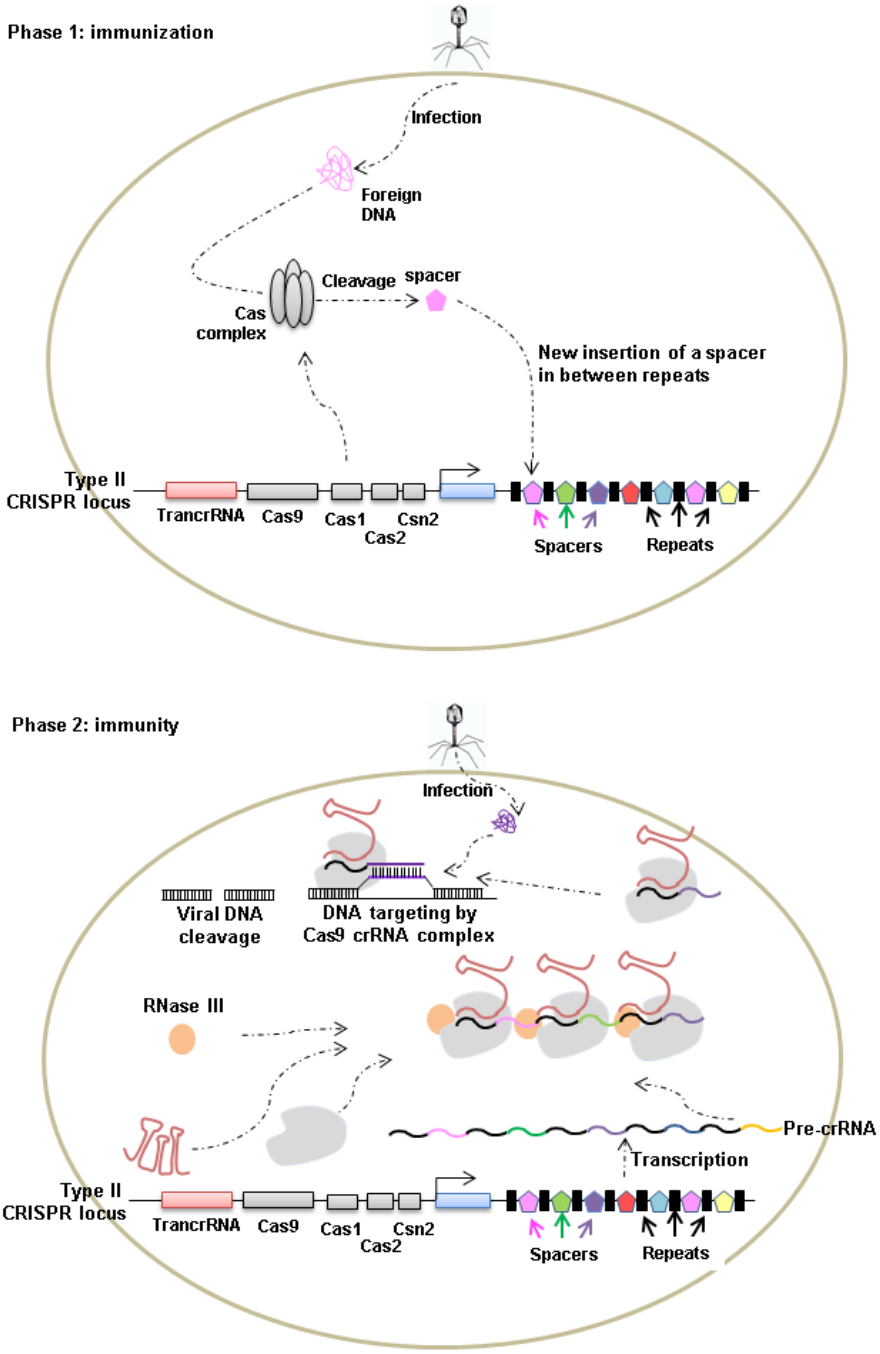

Figure 1.1 Working mecahnisms of type II CRISPR-Cas9 system (modified from Prashant Mali, 2013). In the first "immunization" phase, the Cas protein complex cleaves the injected DNA from invading phages into small fragments and inserts them in the CRISPR array between the direct repeats as "spacers". In the second "immunity" phase, the CRISPR array is transcribed as a long pre-crRNA, which recruits Cas9 nucleases to form a complex. At the same time, trancrRNA, an auxiliary trans-activating crRNA, is transcribed and binds to repeat region of the pre-crRNA. Subsequently, RNase III recognizes the hybridized crRNA-trancrRNA and cleaves site-specifically, producing short crRNAs which form a mature complex with both Cas9 and trancrRNA. Eventually, mature crRNAs guide Cas9 nucleases to the target DNA to introduce precise cleavages and produce DSBs.

1.1.3 Initial harnessing of the CRISPR-Cas system for biological research

How does a mysterious prokaryotic adaptive immune system become the most powerful and robust eukaryotic genetic engineering platform? The story began in 2011, when Siksnys and colleagues first found out that the type II CRISPR-Cas system from *Streptococcus thermophiles* could be functionally reconstituted in *E.coli* [19]. After the inspiring initial research, several studies made great progresses in the next two years in harnessing the type II system from *S. pyogenes* and *S. thermophiles* in eukaryotic cells. Progress was made through modification of the key components including expression of a human-codon-optimized Cas9 nuclease with a nuclear localization signal, and expression of crRNA-trancrRNA as a single chimera, also known as the short guide RNA (sgRNA) [6, 8, 20].

1.1.4 Specificity improvement of the CRISPR-Cas system

The specificity of the *S.pyogenes* Cas9 nuclease has raised considerable attention, since unexpected genome modifications could lead to unpredictable catastrophes especially for clinical gene therapy. Several studies have demonstrated that Cas9 could tolerate up to 5 mismatches between guide sequences and protospacers, which leads to off-target DNA cleavages [21-24]. The structure of Cas9 nuclease revealed, there are two signature catalytic domains responsible for cleavage of the double strand DNA: The HNH domain cleaves the strand which is complementary to the guide RNA, and the RuvC-like domain cleaves the non-complementary strand [8]. Several studies have converted SpCas9 into a nickase which produces single-stranded nicks by inactivating one of the catalytic domains [8, 19, 20]. To improve the nuclease targeting specificity, a double nicking strategy was applied in the CRISPR-Cas9 system with a pair of sgRNAs guiding Cas9 nickase targeting the opposite strands of the target site within an appropriate offset. Since nicks can be repaired without indel mutations by high fidelity base excision repair pathway, two nicks which are close enough and properly spaced can mimic the DSBs to induce indel formation. In this way, targeting specificity of a double nicking strategy is 1500 times higher than the wildtype Cas9 nuclease [24, 25].

1.2 Helicobacter pylori

In 1983, Warren and Marshall cultured slow growing microaerophilic bacteria and first reported an etiological correlation between these bacteria, later termed *Helicobater pylori* (*H.pylori*), and gastric diseases [26]. The outcome was remarkably surprising, since nobody expected microbes to survive in the highly acidic stomach. Because of this discovery, gastritis and peptic ulcers were no longer considered as chronic diseases caused by stress, but curable infections that can be treated with antibiotics and acid secretion inhibitors. Therefore, Warren and Marshall were awarded the Nobel Prize in Physiology or Medicine for 2005.

H.pylori, Gram-negative bacilli which colonize half of the world's population, are the main cause of gastritis, peptic ulcers, gastric lymphoma and even gastric adenocarcinoma. Of note, *H.pylori*-induced gastric cancer is one of the most common cancer and the second leading cause of cancer-related death worldwide. It is dominantly transmitted orally in childhood within a family and the infection mostly persists life-long in the infected individual [27]. *H.pylori* have colonized human stomachs for at least 60,000 years [28] and they have developed many strategies to adapt to the hostile niche and to successfully evade and manipulate human immune recognition to facilitate their survival and persistence [29].

1.2.1 Overview of the *H.pylori* infection process

The normal stomach is highly acidic with a pH of 1-2. Many pathogens conferring acidic tolerance or acidic resistance like *Vibrio cholerae* or *Escherichia coli*, can take up amino acids to produce amine by decarboxylation to neutralize the cytoplasm and facilitate bacterial survival, but not colonization [30, 31]. However, *H.pylori* can colonize in the acidic lumen of the stomach by using a unique strategy called acid acclimation [32, 33]. Unlike acid resistance, acid acclimation can buffer the periplasm of *H.pylori* to a near neutral pH by utilizing abundant surface urease to hydrolyse urea into ammonia. Periplasm neutral pH is important for *H.pylori* colonization because it assures synthesis and correct folding of membrane proteins and metabolite transport [34, 35].

Upon infection, *H.pylori* swims and escapes from the acidic lumen and quickly migrates to the epithelial surface where the pH is near neutral. *H.pylori* is highly motile, owning to their helical shape and flagella. Flagella motility is also required for the bacteria to penetrate through the physical barrier of the mucus layer [36]. Abundant surface ureases protects bacteria from local acid [34] and chemotaxis senses the pH gradient to guide bacteria swimming in the right direction [37, 38]. Furthermore, outer membrane proteins, such as BabA, SabA, or HopQ can help *H.pylori* to attach to the epithelial cells to avoid shedding from mucus turnover. Highly virulent *H. pylori* harbor the *cag* type IV secretion system (*cag*-T4SS), a needle-like apparatus protruding from the bacterial surface, which is able to inject the effector protein CagA (cytotoxin-associated gene A) and peptidoglycan into host cells. After translocation, CagA can be phosphorylated by host cell kinases and then further interact with a set of eukaryotic signaling molecules to disrupt different signal transduction pathways, resulting in induction of a proinflammatory response and precancerous changes [39-42]. Therefore, CagA is considered a bacterial-derived carcinogen and a broad-spectrum intracellular toxin [43]. In addition, vacuolating cytotoxin A (VacA) is secreted to target epithelial cells and immune cells to facilitate bacterial growth, colonization, immune evasion and persistent infection [44].

1.2.2 *H.pylori cag*-Type IV secretion system

The *cag*-T4SS has raised considerable attention during the past decades due to its role in the pathogenesis of *Helicobacter*-related diseases, including gastric inflammation and gastric cancer development [45]. The *cag*-T4SS is encoded by a 37 kb genomic region, the *cag*-pathogenicity island (*cag*-PAI), which encodes all the components of a T4SS, ranging from structural proteins, ATPases, coupling proteins to the only effector protein CagA and its secretion chaperone CagF [46]. The main feature of the Cag secretion system is to translocate CagA into host epithelial cells, which is associated with a high risk of developing gastric cancer [47]. The prototype T4SS, the VirB-T4SS in *Agrobacterium tumefaciens,* contains 11 structural components to form the apparatus (VirB1-VirB11) and a coupling protein VirD4 to recognize the substrate [48]. However, the *H.pylori cag*-T4SS encodes only some of the components sharing similarities with the VirB system and others are unique to the Cag system [46]. The

cag-T4SS apparatus is a long channel-like structure consisting of an inner membrane complex, a core complex spanning inner and outer membrane, as well as an external pilus protruding outside of the bacteria. The detailed information about the architecture of the secretion system and its components is reviewed in several articles [46, 49, 50].

The comprehensive molecular mechanisms of CagA translocation dynamics through the *cag*-T4SS apparatus are so far poorly understood. Before entering the translocation channel, CagF recognizes and binds specifically to CagA as a chaperone-like protein [51, 52], and the coupling protein Cagβ and its accessory protein CagZ form a complex to recognize the C-terminal secretion signal of CagA and to support CagA translocation [53-55]. After that, CagA is located at the pilus tip and further interacts with host factors to facilitate its uptake by host cells.

1.2.3 CagA and its cytotoxic and oncogenic effects in host cells

Translocation of CagA into leukocytes leads to the proteolytic cleavage of native CagA protein into an N-terminal fragment (1-867 amino acid) and a C-terminal part (868-1214 amino acid), indicating that there are 2 major domains of CagA [56]. The N-terminal domain can be further divided into D1, D2 and D3/D4 domains according to its crystal structure determination. The C-terminal domain contains the EPIYA region, the CagF binding region and the 20 amino acid secretion signal [40, 57]. The C-terminal secretion signal is essential for CagA translocation, however, presence of the N-terminal is critical as well, since the signal region alone cannot be translocated [52, 58]. Furthermore, N-domain mutation and CagA immunoprecipitation revealed that CagF binding to both N-terminal and C-terminal regions of CagA serves as a quality control mechanism to guarantee the translocation of the integral CagA [52, 59].

After translocation in gastric epithelial cells, CagA can bind to around 20 different host cell proteins in both phosphorylation-dependent and -independent manners to manipulate host cell signaling processes [43]. CagA can be phosphorylated in the EPIYA region by Src family kinases and C-Abl kinase at the host inner leaflet of the plasma membrane [60]. Phosphorylated CagA can bind to several host SH2 domain containing proteins. For example, phospho-CagA can bind and activate SHP2, a tyrosine phosphatase in the Ras-Erk mitogenic pathway [61]. SHP2 is considered as a proto-oncogenic phosphatase since mutations are detected in the gene encoding SHP2

in leukemia and other cancers [62]. Especially, CagA from East Asian *H.pylori* strains can bind to SHP2 stronger than western CagA, indicating that East Asian *H.pylori* represents a higher risk for gastric cancer development. Moreover, activated SHP2 can further dephosphorylate and inhibit focal adhesion kinase, which regulates cell shape and motility, resulting in actin cytoskeleton rearrangement and abnormal cell shape, like the hummingbird phenotype in AGS cells [63, 64]. In addition, CagA can also bind to Crk adaptor proteins in a phosphorylation-dependent way, which is associated with disruption of E-cadherin/catenin-containing adherence junctions of gastric epithelium [65]. Furthermore, CagA has also been reported to interact with SH2 domains of Grb2, promoting proto-oncogenic Ras-Erk signaling in a different way [66]. Altogether, phosphorylated CagA can bind several SH2 domain-containing proteins in host cells to manipulate and perturb multiple signaling pathways as well as host cell functions.

In addition, CagA can also subvert host cell functions in a phosphorylation-independent manner. CagA is reported to disrupt tight junctions of gastric epithelium by targeting PAR1 kinase, which leads to disorganization of epithelial apical-basal polarity and gastric mucosal architecture [67]. In addition, PAR1 also regulates microtubules, and inhibition of PAR1 by CagA perturbs segregation of sister chromatids in mitosis. As a result, CagA injected cells show chromosome instability and high risk of neoplastic transformation [68]. Furthermore, translocated CagA can hijack many other host signaling cascades important for cell proliferation, apoptosis and pro-inflammation [69-73]. In conclusion, the CagA toxin represents a sophisticated and versatile oncoprotein promoting host gastric carcinogenesis and tumor progression in many different ways.

1.2.4 Other virulence factors of *H.pylori*

The *cag*-T4SS and CagA described above is one of the major virulence factors among *H.pylori*, other virulence factors include urease, chemotaxis, flagella, VacA, HtrA, different outer membrane proteins and others. VacA and some well-characterized outer membrane proteins are discussed in the following.

The vacuolating cytotoxin VacA

VacA is present in virtually all *H.pylori* strains. As a multifunctional secreted toxin, not only can VacA produce large intracellular vesicles after internalization (also known as vacuoles), but also contributes to bacterial pathogenesis in diverse ways. VacA can bind to T cells to inhibit T cell activation and proliferation for the sake of immune suppression and persistent colonization [74, 75]. Moreover, it can target mitochondria in epithelial cells to induce apoptosis, resulting in accelerated turnover to prevent cancer formation and tissue damage, and thus avoid immune cell recruitment [76]. Furthermore, VacA can increase permeability of epithelial monolayers to get access to nutrients like iron, sugars and amino acids, although the mechanisms are not yet clear [77]. Most importantly, CagA and VacA can work together in a coordinate fashion, sometimes against each other and sometimes cooperate, to facilitate colonization, bacterial growth, and virulence [76, 78–80].

Outer membrane proteins (OMPs)

H.pylori contains five major outer membrane protein (OMP) families, which include more than 30 paralogous genes. However, only a few of them are well-characterized, most of the members remain uncharacterized [81]. OMPs mainly function as adhesins to facilitate initial colonization by attaching to epithelial cell surface to prevent mechanical clearance, especially when the gastric environment is dynamic and under conditions of peristaltic movement and secretion of gastric juices [82]. In addition, *H.pylori* adherence plays an important role in persistent infection and chronic inflammation, since it is highly unlikely that serious diseases like gastritis, gastric or duodenal ulcers, and MALT lymphoma can develop without adhesin-host interactions. Importantly, outer membrane proteins are regulated by several mechanisms such as point mutations in the coding region, phase variation, gene conversion, slipped strand mispairing (SSM), or gene duplication [81]. These regulations are beneficial for *H.pylori* survival by facilitating adaptation to the changing host environment and escaping host immune systems.

Outer membrane protein BabA

BabA (**B**lood group **A**ntigen **B**inding adhesin) was the first identified outer membrane protein adhesin [83, 84]. BabA can bind specifically to fucosylated ABO blood group antigens and Lewis b antigens which are abundantly expressed in gastrointestinal epithelium [85, 86]. The interaction of the adhesin BabA and host Lewis b was shown

related to T4SS function and T4SS-induced effects in host cells. Ishijima et al. showed that the *H.pylori* wildtype strain ATCC 43504, but not its isogenic *babA* deletion mutant induced significantly increased IL-8 and a stronger CagA phosphorylation in Lewis b stably transfected Martin Dearby kidney cells (MDCK) cells. These in vitro data were confirmed in vivo in the Mongolian gerbil animal model by demonstrating that BabA was important for inducing enhanced IL-8 and chemokine (C-X-C motif) ligand 1 (CXCL1) [87]. In addition, BabA was described to co-localize with gastric mucin MUC5AC to promote damage to the gastric mucous gel [88]. Moreover, *H.pylori* can induce host DNA double strand breaks (DSBs) upon close contact with host cells, and this effect was shown to be BabA-dependent [89], suggesting BabA has carcinogenic potential, since constant repair of DSBs could highly enhance the probability of mutagenic events and genetic instability which in turn increase the cancer rate [13].

Outer membrane protein SabA

SabA expression is related to development of gastric atrophy, intestinal metaplasia and gastric cancer [90]. Healthy gastric mucosa expresses nonsialylated antigens, however, *H.pylori* infection induces gastric inflammation and gastric adenocarcinoma and presents a low level of nonsialylated Lewis b antigens, but upregulated sialyl-dimeric-Lewis x antigens and sialyl-dimeric-Lewis a antigens (sLe^x and sLe^a, respectively). *H.pylori* outer membrane protein SabA was shown to bind sLe^x and sLe^a and mediates bacterial adherence to inflamed tissue by mimicking selectin [91]. Marcos *et al.* demonstrated that highly virulent *H.pylori* strains containing *cag* pathogenicity island could manipulate expression of sLe^x by modulating host glycan biosynthesis, especially by inducing overexpression of a GlcNAc transferase (β3GnT5), to upregulate sLe^x expression and by enhancing SabA-mediated adherence [92]. Moreover, β3GnT5 induction is CagA- and CagE-dependent [92]. In this way, *H.pylori* expand their adherence ability by binding to both healthy tissue (BabA mediated) in the initial stage of infection and inflamed gastric mucosa (SabA mediated) in late stage. Therefore SabA contributes to *H.pylori* virulence and chronic inflammation [93].

Outer membrane protein HopQ

HopQ belongs to the *hop* family of outer membrane proteins and the *hopQ* locus exhibits genetic diversity. There are two alleles in the locus: allele type I and allele type II. Type I and type II alleles show around 80% identity in nucleotide sequences and

around 70% identity in amino acid sequences [94]. Moreover, Type I *hopQ* is usually found in the strains harboring the *cag*-PAI, but in some strains, both types are present at the same time [94]. Falkow and colleagues also reported that HopQ shows "co-inheritance" with the *cag* island, which suggests that HopQ might contribute to the virulence of *H. pylori* [95]. Indeed, HopQ was recently identified as a virulence factor by Meyer and colleagues using a large transposon-based mutant library of *H. pylori* strain G27 to determine essential factors affecting nuclear translocation of the NFkB subunit P65 in AGS cells [96, 97]. These data were confirmed in strain P12. An isogenic *hopQ* deletion mutant exhibited significantly reduced NFkB translocation, IL-8 secretion, CagA phosphorylation and "hummingbird" cell shape formation in AGS cells. Taken together, these data suggest that HopQ is an essential accessory non-*cag*-PAI factor for T4SS function [96].

Other outer membrane proteins

Other outer membrane proteins are found to contribute to the *H.pylori* virulence as well. For example, AlpA and AlpB, two proteins that shared homology in their gene sequences, are involved in adherence. Deletion of these two genes resulted in reduced induction of IL-8 upon east Asian *H.pylori* strains infecting gastric epithelial cell lines [98]. Moreover, OipA, an OMP encoded by the *hopH* gene, also showed an assistant role in T4SS dependent IL-8 induction besides the adherence ability [99], and the presence of OipA is associated with enhanced neutrophil infiltration, gastric inflammation and cancer [100]. However, the host receptor for OipA has not been discovered yet. As we gain insight into the sophisticated outer membrane proteins, some questions are waiting to be answered. For examples, do these outer membrane proteins work sequentially or coordinately? Which adhesins are associated with or even essential to *cag*-T4SS function? How do adhesins and the *cag*-T4SS work together? Are adhesins and *cag*-T4SS components co-regulated?

1.3 Integrins as receptors exploited by pathogens

integrins are transmembrane receptors with long extracellular domains and short cytoplasmic tails, and they can bind to different extracellular matrix (ECM) ligands like collagens, laminins or RGD motif-containing proteins. Integrins are typically heterodimers with an α and a β subunit. In mammals, there are 18 α subunits and 8 β subunits which assemble into 24 distinct integrins (**Figure 1.2**). Among them, integrin α6β4, αvβ5, αvβ6 and several β1 integrins (α1β1, α2β1, α3β1, α5β1, α6β1 and α9β1) are expressed on epithelial cells [101, 102]. Ligation of integrins to their ligands can trigger divergent signal transduction events known as "outside-in" signaling [103, 104]. However, integrins are also capable of "inside-out" signaling in ways that intracellular talin or kindlin binds to the cytoplasmic domain of integrins to further promote extracellular ligand binding [105, 106]. Through bidirectional signaling, integrins are involved in various cellular processes like cell survival and proliferation, homeostasis, cell motility, leukocyte trafficing and cancer progression [107-109]. In addition, integrins have either extended or bent conformations, indicating activated and inactivated status, respectively [103]. Of note, integrins are not constitutively active, and they are often kept in the inactive state which is important for their biological function [103, 110].

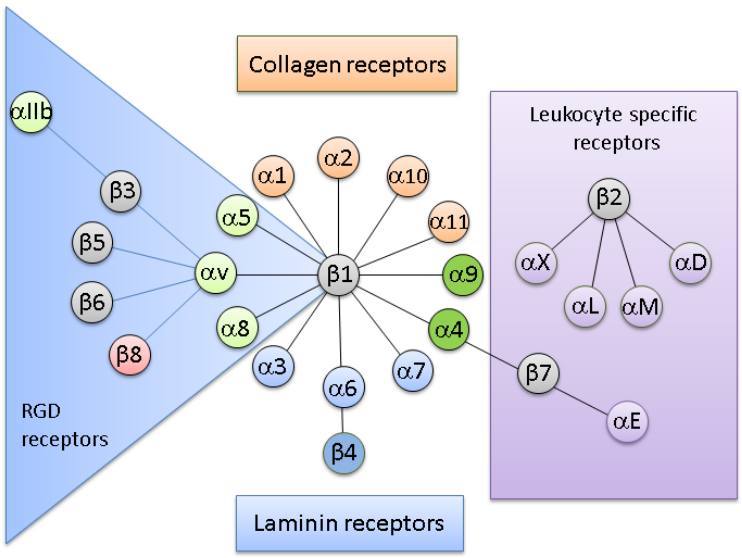

Figure 1.2 The mammalian integrin receptor family and their ligands (modified from Hynes, 2002). The figure illustrates integrin subunits αβ association and their ligands.

Meanwhile, integrins are recognized as the favored receptors of different viruses and bacteria for their adhesion, invasion or cellular entry [111-116]. Many pathogens can engage integrins directly or indirectly, not only to get access to their favored cells and tissues, but also to interfere with integrin linked signaling pathways and cellular functions to facilitate their surviving and virulence. For instance, many pathogens can directly bind to integrin with high affinity or bind to ECM ligands to facilitate adherence and/or invasion like *Yersinia pseudotuberculosis* [117], *Yersina enterocolitica* [118], *Staphylococcus aureus* [119], *Streptococcus pyogenes* [120] , *Leptospira interrogans* [121], *Campylobacter jejuni [122] , etc.* Some pathogens can utilize integrin to deliver their virulence factor into host cells and further alter host cell functions like *H.pylori*. Although host epithelium have developed exfoliation as a host defense mechanism to restraint bacteria colonization, some bacteria establish strategies to slow down the turn over rate by increasing the integrin mediated cell adhesion to extracellular matrix to counteract the detachment of infected cells. For example, *Neisseria gonorrhoeeae* and *Haemophilus influenzae* can bind to CEACAMs and thus trigger endoglin (CD105) upregulation [123, 124]. Upregulated CD105 can change focal adhesion conformation and stimulate integrin β1 inside out signaling and therefore promote enhanced binding of integrin β1 and extracellular matrix [125]. On the contrary, some pathogens accelerate shedding of epithelial cells to get access to deeper tissues. For example, the uropathogenic *E.coli* (UPEC) produces a pore forming toxin for insertion into epithelial cell membranes. The toxin can degrade focal adhesion components paxillin and therefore disrupt cell-matrix adhesion [126]. Taken together, it is a common strategy for many pathogens to target integrins in order to break epithelial barriers, disrupt host first line of defense and alter integrin mediated cellular functions for their own advantages.

1.4 *Helicobacter pylori* host-pathogen interactions

1.4.1 Interaction of the T4SS and host cell receptors in CagA translocation

For many years the interaction of the *cag*-T4SS with the host cell membrane was not fully understood. From 2007 to 2010, researchers proposed different interaction models of CagA and/or T4SS with three diverse host cell receptors, including host cell integrin β1, host membrane phosphatidylserine and host membrane cholesterol.

The first model was proposed in 2007, when Backert and colleagues first identified that host cell integrin β1 is exploited by *H.pylori* as host receptor for CagA translocation [127]. The first line of surmise came from the observation of co-localization of phosphorylated CagA (CagA-PY) and focal adhesion kinase (FAK) or vinculin at the focal adhesions, which led to the hypothesis that integrin β1 was involved in CagA translocation as the receptor. Indeed, more experiments supported this hypothesis: CagA phosphorylation was blocked *in vitro* by integrin β1 specific antibodies and a bacterial integrin β1 binding protein, *Yersinia* invasin InvA [127]. Furthermore, CagA phosphorylation was not observed in the integrin β1 knockout mouse fibroblast cell line GD25 upon *H.pylori* infection, but was restored in integrin β1 stable transfected GD25 cells [127]. In addition, the RGD motif-containing CagL, a structural protein located in the pili of the T4SS, was verified as the binding partner of integrin β1. The RGD motif is the classical domain present in many extracellular matrix proteins, which can be recognized and bound by integrin β1. However, mutated CagL (with RGD changed into RAG motif) did not support CagA phosphorylation [127]. Therefore it was strongly suggested that the T4SS pili-associated protein CagL directly interacts with the host cell surface protein integrin β1 to trigger CagA translocation in an RGD dependent manner. However, an independent study by our group confirmed that *H.pylori* utilize integrin β1 as the receptor, but in an RGD-independent fashion [128]. Firstly, interaction of CagL and integrin β1 could not be detected in either a Yeast Two Hybrid (YTH) screen,or in pull down assays. Secondly, either CagA phosphorylation efficiency or Interleukin-8 (IL-8) induction was not impaired when RGD motif was completely removed from CagL protein upon *H. pylori* infection. Instead, T4SS components CagY, CagI and CagA itself were identified to interact with host cell integrin β1 *in vitro, as* proven by YTH and pull-

down assays. However, integrin β1 ligands fibronectin, invasin and RGD peptide failed to block or reduce CagA phosphorylation. Moreover, eight out of nine different monoclonal antibodies which target different epitopes of integrin β1 could not abolish CagA phosphorylation with the exception of 9EG7, an antibody binding to EGF domain of integrin β1 and therefore locking integrin β1 in the extended conformation, accomplished to block. These results have led to the conclusion that the interaction of the *cag*-T4SS and host cell integrin β1 was not like the known binding mode of classic ligand/integrin interaction. Instead, the T4SS was proposed to bind to the extended conformation of integrin β1 to further force integrin to the bent conformation by unknown mechanisms. As a result, the T4SS pilus was pulled physically closer to the host cell to facilitate CagA translocation. However, the authors claimed that this outcome did not exclude the possibility that other co-receptors necessary for CagA phosphorylation were blocked by 9EG7.

In 2008, Lai and colleagues showed that host cell membrane cholesterol is exploited by the *H. pylori* T4SS for CagA translocation [129], an observation which was inspired by the previous finding that host cholesterol is important for *H. pylori* virulence. Interestingly, *H.pylori* cholesterol-α-glucosyltransferase (CapJ) is responsible to exploit host cell cholesterol for subsequent glucosylation in order to promote immune evasion by escaping phagocytosis and inhibiting T-cell effects [130]. This discovery led them to propose that host cholesterol is utilized by *H. pylori* as the receptor for CagA translocation, which was supported by following evidences. First, host cell cholesterol depletion by methyl-β-cyclodextrin (MβCD) could significantly reduce CagA phosphorylation and IL-8 induction, as well as CagA-induced hummingbird phenotype [129]. In addition, *H. pylori* 26695 isogenic *capJ* deletion mutant led to drastically reduced CagA phosphorylation and CagA associated signaling pathways. Therefore, the authors proposed that *H. pylori* CapJ could convert host cholesterol into its cell wall components cholesteryl glucosides (CG). CG could further promote lipid rafts associated components clustering at the infection site, which might influence host membrane mobility and dynamics. Altered membrane architecture could be recognized by CG and thus trigger T4SS assembly and secretion function.

In 2010, Hatakeyama and colleagues proposed a different CagA translocation mechanism. They showed that T4SS tip located CagA can directly interact with host

phosphatidylserine (PS) to mediate CagA internalization [131]. PS is an important phospholipid membrane component which constitutes about 10% of the total cell lipid content [132, 133]. PS is usually located in the inner leaflet of the cell membrane facing the cytosol [134]. If a cell undergoes apoptosis, the PS is flipped to the outside of the cell and is recognized by macrophages for phagocytosis of the cell [135]. However, in some non-apoptosis cases, PS can be aberrantly externalized by some pathogens like Chlamydia [136], Herpes simplex virus 1 (HSV-1) [137], vaccinia virus [138], hepatitis C virus [139] , pichinde virus [140] and others, to induce host cell apoptosis or to facilitate microbe internalization. *H. pylori* seems to trigger PS externalization upon infection with the similar motivation like other pathogens. Indeed, several facts supported their perspective: (i) CagA translocation was partially blocked by annexin V and PS specific antibodies. (ii) Recombinant CagA could bind PS *in vitro* and a classical PS binding motif KXnRXR was found to be present in the central region of CagA and is conserved throughout different *H.pylori* strains [131]. (iii) Mutated CagA-R619/621A within the motif could not be translocated upon infection, suggesting CagA-PS interaction is essential for CagA translocation. Although the exact mechanism of PS-bound CagA internalization into host cells was unclear, the author proposed that *H. pylori* infection could trigger temporary externalization of PS without actually inducing apoptosis to enable bacterial surface exposed CagA binding. Subsequently, CagA-bound PS could flip back to the inside of cells with an unknown mechanism, and inner membrane PS-tethering CagA could further interact with PAR1 in polarized MDCK cells to disrupt tight junctions and cell polarity [67, 131].

The aforementioned studies and data suggested there highlight at least three host cell factors involved in *H.pylori* type IV secretion. These researches have inspired us with comprehensive and insightful perspectives to the molecular mechanisms of type IV secretion and CagA translocation in the context of host-pathogen interaction. However, a lot of questions did also arise accordingly. For example, how does the CagL-integrin β1 interaction possibly contribute to the CagA transport across the host membrane? If CagY, CagI and CagA all bind to integrin β1, why did none of the tested integrin antibodies or integrin ligands block CagA translocation except one antibody, 9EG7? What is the molecular mechanism underlying the internalization of CagA-bound PS? Since cholesterol depletion can lead to many side effects, what is the real reason for the CagA translocation blocking outcome by cholesterol depletion? Is there

cooperation between three reported host factors during CagA translocation? Taken together, the *H. pylori* CagA translocation mechanism is more complicated than originally proposed and *H. pylori* may use more host factors as receptors and might as well use backup receptors. Therefore, future studies need to investigate thoroughly to reveal the molecular mechanisms of *H.pylori* Type IV secretion.

1.4.2 Interaction of *H.pylori* outer membrane protein HopQ and host cell receptor CEACAMs -- Contribution of non-T4SS factors in CagA translocation

It is worth noting that our group has recently proven that host epithelial cell surface carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are exploited by HopQ as receptors[141, 142]. CEACAMs belong to the IgCAMs (immunoglobulin superfamily of cell adhesion molecules) and are cell surface glycoproteins expressed in normal and malignant tissues mediating cell-cell adhesion and other important cellular processes including T cell proliferation, insulin homeostasis, neovascularization, apoptosis, cancer progression and metastasis. [143-145]. A lot of pathogens are reported to use CEACAMs as receptors for adhesion or invasion [111, 112, 123, 146-149]. Our group has identified that host CEACAMs are exploited by HopQ for adhesion and CagA translocation, as indicated by the following observations. First, *H.pylori* can specifically bind to soluble GFP fusion proteins of the N-terminal domain of CEACAM1 and CEACAM5, as shown by pull-down assays. Second, HopQ was identified as a binding partner of CEACAMs since an isogenic *hopQ* deletion mutant of strain P12 lost the binding ability in the pull-down assays. Third, HopQ-CEACAM interaction correlated with CagA translocation, as illustrated by the facts that those cell lines which allow CagA translocation did express CEACAMs on the surface (including AGS, KatoIII and MKN45), whereas cell lines non-permissive for CagA translocation only produced little or no CEACAMs (including MKN28, HeLa and HEK293). Significantly, HEK293 cells could become permissive for CagA translocation when CEACAM1 or CEACAM5 was stably transfected. Fourth, co-localization of *H.pylori* and CEACAM5 was detected in gastric biopsy isolates from *H.pylori* infected patients via microscopy studies[141, 142]. These various findings showed conclusively that the HopQ-CEACAM interaction is essential for *cag*-T4SS function and reveal a distinct CagA translocation mechanism.

1.5 Aim of my study

H.pylori host-pathogen interaction has raised considerable attention because of its role in pathogenesis. Particularly, understanding the molecular mechanism of CagA translocation holds promise in providing new insights and therapeutic target candidates for treating *H.pylori* induced gastric diseases, especially gastric cancer.

Previous studies have confirmed that *H.pylori* exploits host cell integrin β1 for CagA translocation, which has been summarized in detail in the introduction section 1.4. However, several outcomes were difficult to understand or interpret. For example, it was shown that CagL can bind host cell integrin β1 to facilitate CagA translocation in a RGD-dependent manner. Whereas conflicting results were obtained by our group suggesting that the RGD motif is not necessary for CagA translocation. Moreover, our group has proven that several T4SS components can bind to integrin β1 to facilitate CagA translocation, but when the authors were trying to block CagA translocation with many different integrin β1 ligands, binding proteins or specific antibodies targeting different epitopes, none of them could abolish CagA translocation except for one of the antibodies (9EG7).

These discrepancies led us to ask more questions. For example, is integrin β1 alone sufficient for CagA translocation? Since integrin αvβ3 showed high affinity for CagA binding, as revealed by surface plasmon resonance, is it possible that integrin αvβ3 can function as the receptor for CagA translocation as well? Are there other host cell integrins involved in this process? To answer these questions, my project mainly focused on the systematic generation of single to multiple integrin depletion gastric epithelial cell lines, and the subsequent evaluation of the CagA translocation efficiency in these integrin-depletion cell lines upon *H.pylori* infection. In this way, the study attempted to identify potential other host cell integrins which are exploited by *H.pylori* for CagA translocation.

2 Results

2.1 Gene targeting of different integrins with the CRISPR-Cas system

In order to study the role of different host cell integrins in *H.pylori* CagA translocation, generation of different integrin-depletion cell lines is the first step. AGS and KatoIII cell lines are generally used for the evaluation of CagA translocation efficiency, since both cell lines were derived from gastric epithelial cells and are standard cell lines to study *H.pylori* pathogenesis *in vitro.* Therefore, integrin-depletion AGS and KatoIII cell lines were planed to establish.

The integrin receptor family is composed of 24 distinct integrins which are heterodimers consisting of different α and β subunits distributed in different cell types and tissues. Figure 2.1 A shows the αβ association of integrins which are expressed on non-leukocyte and non-platelet cell surfaces. Among them, six β1 integrins (α1β1, α2β1, α3β1, α5β1, α6β1 and α9β1), two αv integrins (αvβ5 and αvβ6) and the integrin α6β4 are shown to be epithelial-specific [102, 150-153].

Gene targeting with the CRISPR-Cas9 system in the absence of the repair template can lead to gene knockout. Furthermore, gene targeting in either subunit of the integrin heterodimer can eventually result in depletion of surface expression of targeted integrin, since it was shown that a defect in either subunit prevented intracellular αβ association and therefore abolished a functional integrin heterodimer surface expression [154, 155]. Thus, we sought to finally target three important genes in parallel, the integrin β1 gene, the integrin αv gene and the integrin β4 gene. This should generate the integrin β1 depletion cell lines (AGS and KatoIII cell lines) without surface expression of all 12 potential β1 containing integrins, including 6 epithelial-specific β1 integrins, the integrin αv knockout cell lines without surface expression of all 4 potential αv containing integrins, including 2 epithelial-specific ones, as well as the integrin β4 knockout cell lines without expression of the integrin α6β4. Furthermore, by targeting different combinations of two of the aforementioned genes at the same time, cell lines deprived

of even more integrins should be generated. Ultimately, by targeting three genes at the same time, cell lines without any integrin surface expression should be generated.

2.1.1 Integrin profiling in AGS and KatoIII cells

To first have an overview of integrin expression on the surface of AGS and Kato III, the cells were stained with different integrin antibodies and the integrin expression was profiled by flow cytometry. Therefore, integrin β1 antibody, integrin αv antibody, integrin β4 antibody and a few others were used for the profiling. Moreover, goat-anti mouse and goat-anti rat secondary antibodies were used for the negative controls, to exclude false positive results.

Indeed, AGS and KatoIII cells were found to express β1 integrins (including α2β1, α3β1 and possibly others), αv integrin(s) and the β4 integrin (α6β4) on their surfaces with varying expression levels (Figure2.1 B and C).

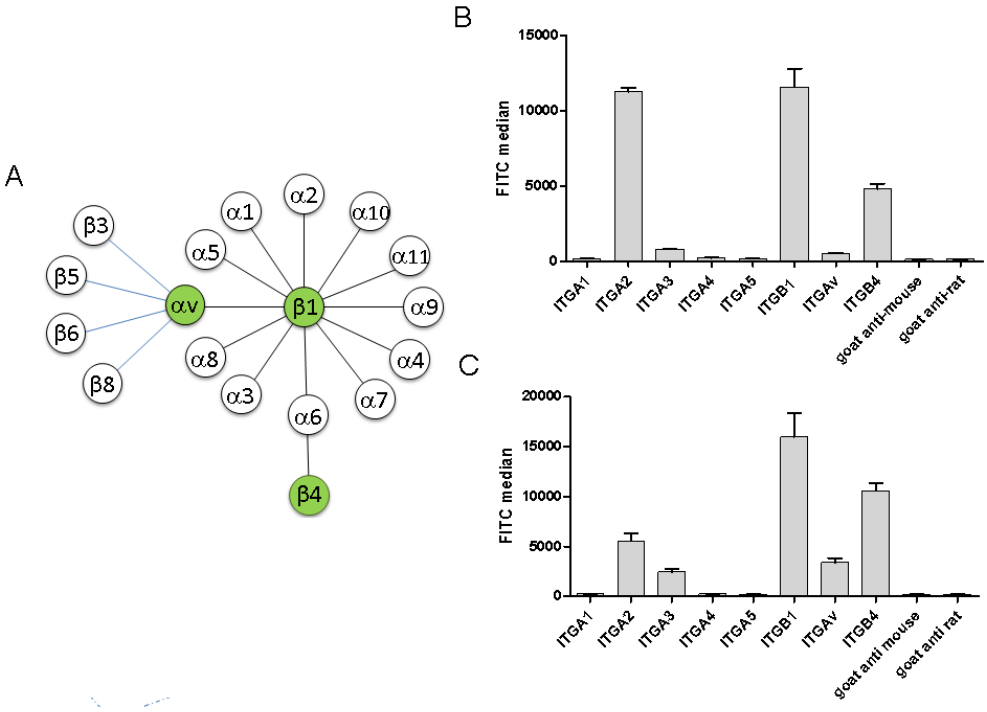

Figure2.1 The integrin expression profiling in AGS and KatoIII cells. A, The αβ association of non-leukocyte and non-platelet integrins. B, Integrin profiling in wildtype AGS cells using different integrin antibodies (n=3). C, Integrin profiling in wildtype KatoIII cells using different integrin antibodies (n=3). All values in B and C were indicated as standard errors of the mean (±SEM) from three independent experiments. ITGA, integrin α; ITGB, integrin β. Integrin antibodies were used as following: integrin α1 antibody (clone FB12), integrin α2 antibody (clone P1E6), integrin α3 antibody (clone P1B5), integrin α4 antibody (clone P1H4), integrin α5 antibody (clone P1D6), integrinβ1 antibody (AIIB2), integrin αv antibody (clone P2W7), integrinβ4 antibody (clone 439-9B).

2.1.2 Integrin β1 gene targeting with the CRISPR-Cas9 system

2.1.2.1 Design of paired 20-nt guide sequences for targeting the integrin β1 gene with the double-nicking strategy

In order to obtain integrin β1 depletion cell lines without undesired off-target mutagenesis, the double nicking strategy was applied. It was reported that the Cas9 nuclease can tolerate certain mismatches in the base-pairing between guide RNAs and their target locus, and therefore exhibits off-target potential [6]. Cas9 nucleases contain two catalytic domains: HNH and RuvC; each of them cut one strand of the target DNA. It was shown that an aspartate to alanine (D10A) mutation in the RuvC domain renders the Cas9 nuclease to a Cas9 nickase [156]. Paired sgRNAs can lead Cas9 nickase making single-stranded nicks on both strands of the target DNA simultaneously. As a result, the double strand breaks (DSBs) are generated from double nicking and therefore facilitate the target gene knockout, as illustrated in Figure 2.2. Of note, as nicks can be repaired scarlessly by the high-fidelity BER pathway [157], double strand breaks only occur if a pair of sgRNAs target opposite strands of a target locus within a defined space (0-100 bp offset). In this way, the double nicking strategy requires 2 targets to introduce DSBs, which doubles the base-pairing length between sgRNAs and their target locus. As a result, it effectively minimizes off-target activity and greatly enhances the genome editing specificity.

For design of paired short guide RNAs (sgRNAs) targeting integrin β1 (ITGB1) gene, an online CRISPR design tool (http://tools.genome-engineering.org) was used for optimal sgRNA analysis and identification. To begin with, the genomic DNA sequence of the ITGB1 gene was downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/nuccore/NG_029012.1?from=5001&to=63048&report=genbank). Subsequently, the genomic fragments of 23-250 bp from *exon 1* to *exon 5* of the integrin β1 gene were loaded into the design tool one after another for screening optimum targets.

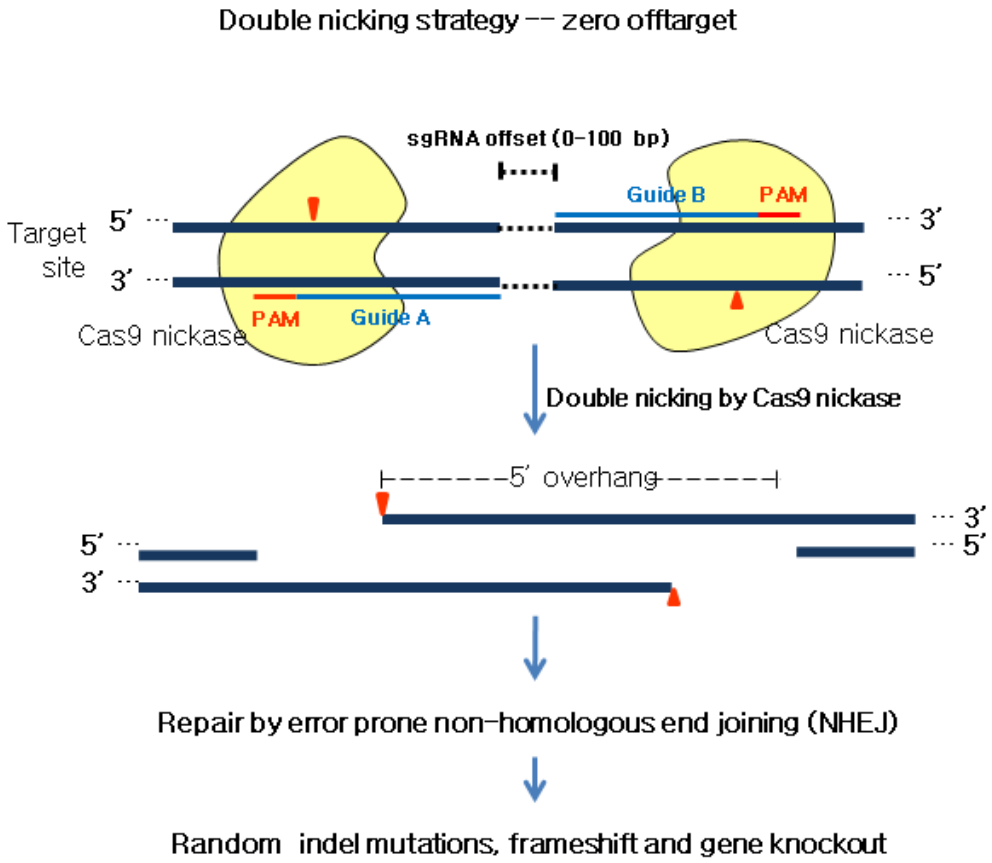

Figure 2.2 Gene targeting by the double nicking strategy. Double nicking strategy requires paired sgRNAs at the same time guiding Cas9 D10A nickase. Cas9 nickases mediates nicks 3 bp upstream the PAMs on the opposite strand of the targets (see illustration: target DNA loci: highlighted in blue; PAMs: highlighted in orange; nickase cleavage sites on both DNA strand: orange triangles). To promote efficient double nicking, the pair of sgRNAs should be designed that 5' overhangs are generated rather than 3' overhangs. The DSBs from double nicking are repaired by non-homologous end joining (NHEJ) producing indel formations, frame shifts and premature stop codons, which result in gene knockout.

After screening and analysis *in silico*, all the possible sgRNA target sites were identified, ranked and scored by the design tool according to the predicted efficiency, number of off-target sites and secondary structures. There were 5 pairs of sgRNAs available in the integrin β1 gene *exon 5*, and the detailed information of each pair was shown in the design tool when the interested pair was clicked. As illustrated in Figure 2.3, the scores, sequences, as well as the number of offtargets and genic offtargets (offtargets located in gene-coding region) were shown for the selected top sgRNA pair on the list. The sgRNA A (guide A) and sgRNA B (guide B) were appropriately spaced and oriented in integrin β1 gene *exon 5* as illustrated in Figure 2.4 A and named as ZQ80 and ZQ82, respectively.

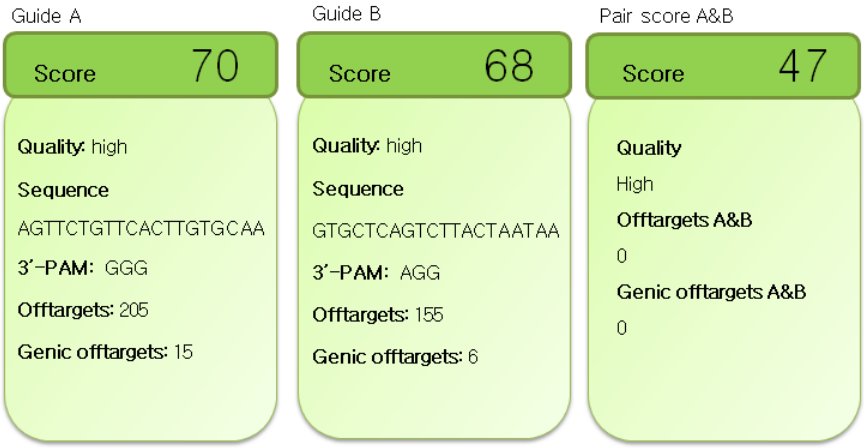

Figure 2.3 Recommended paired sgRNAs targeting the ITGB1 gene by the online CRISPR design tool. When the top pair with the highest score was chosen, the design tool showed the sequences, scores, the 3'-PAM sequences, number of offtargets and genic offtargets of each sgRNA. Guide A alone exhibited 205 off-targets including 15 genic offtargets and guide B 155 off-targets with 6 genic offtargets. Whereas guide A and B pair presented no offtarget.

2.1.2.2 CRISPR plasmids construction

In order to clone designed sgRNAs into CRISPR vectors to obtain functional CRISPR constructs, designed paired guides by the online tool were optimized. An additional guanine (G highlighted in yellow rectangle) was added 5' immediately before the guide sequences (in blue) for the preference of the U6 transcription. And an overhang (CACC in black) was added for the ligation into the BbsI sites in the CRISPR vector pSpCas9 nickase (BB)-2A-Puro (PX462, Addgene ID: 48141). Subsequently, the complementary oligos of guide A and guide B were designed accordingly and all the oligos needed for the ITGB1 gene targeting were ordered commercially as listed in Table 2.1.

Table 2.1 sequences of paired sgRNAs designed for targeting ITGB1 gene

sgRNA targeting ITGB1 gene	Guide A	top oligo (ZQ80)	5'- CACCGAGTTCTGTTCACTTGTGCAA - 3'
		bottom oligo (ZQ81)	5'- AAACTTGCACAAGTGAACAGAACTC - 3'
	Guide B	top oligo (ZQ82)	5'- CACCGGTGCTCAGTCTTACTAATAA - 3'
		bottom oligo (ZQ83)	5'- AAACTTATTAGTAAGACTGAGCACC - 3'

The complementary oligo pairs were annealed before cloning into the vector. The cloning scheme for PX462-8081 (sgRNA guide A expressing CRISPR construct) and

PX462-8283 (sgRNA guide B expressing CRISPR construct) is illustrated as in Figure 2.4 B.

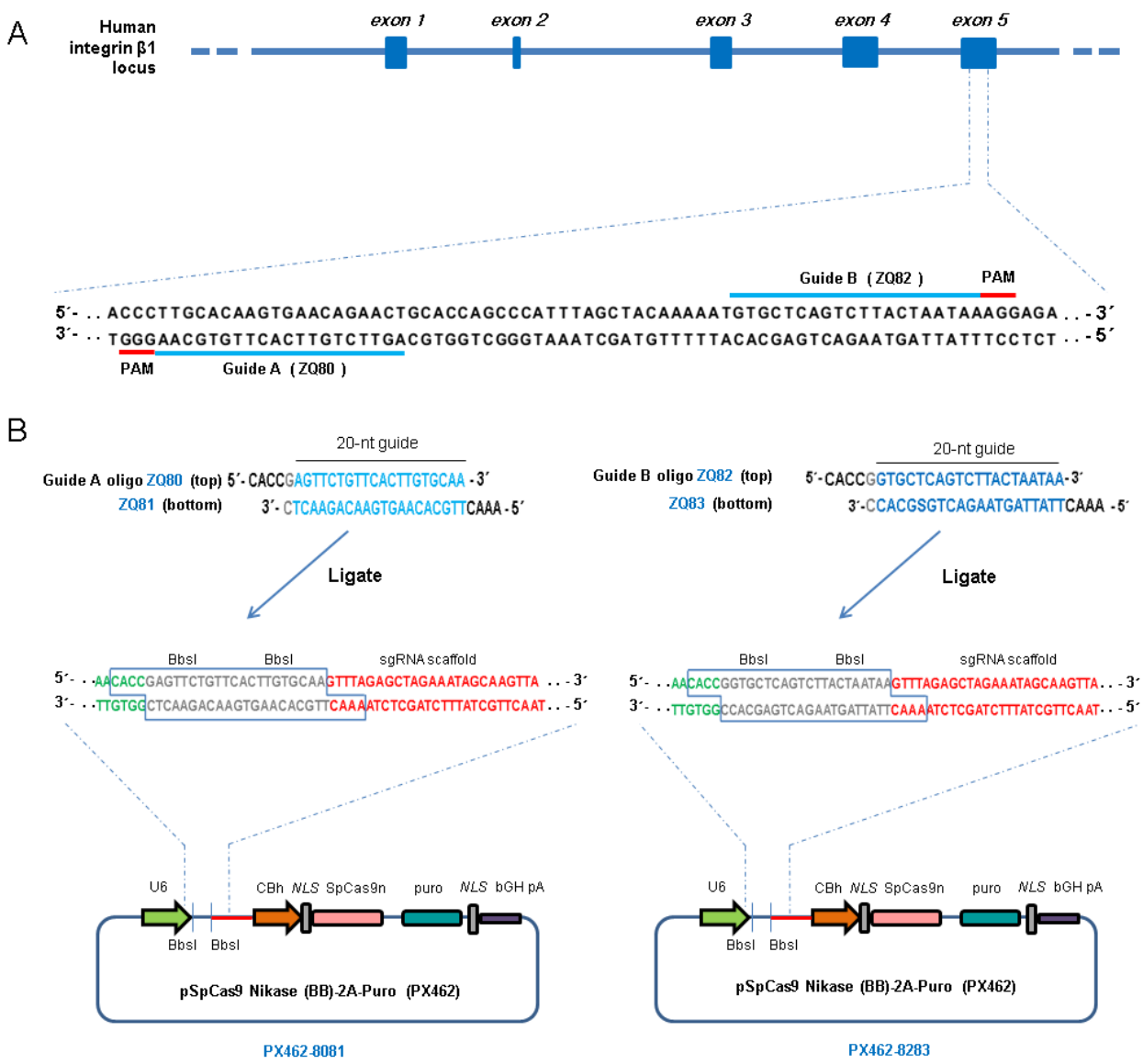

Figure 2.4 The sgRNA locations in integrin β1 gene and the generation of corresponding plasmids. (A) For *Streptococcus pyogenes* Cas9 nickase, 20bp targets (highlighted in blue) were immediately followed at their 3' ends by the 5'-NGG PAM (protospacer adjacent motif). The sgRNA pairs located on both strands of target DNA and were spaced with a 25 bp gap. Two appropriate targets in *exon 5* of integrin β1 gene were selected *in silico* by using the online CRISPR Design Tool (http://tools.genome-engineering.org). (B) Cloning scheme of the CRISPR plasmids. Top and bottom oligos were commercially ordered and anealed before ligation. Restrictive digestion of vector pSpCas9nickase(BB) with BbsI allowed direct insertion of anealed oligos. Importantly, an extra G-C base pair (in gray) was appended at the 5' end of the guide sequence for the transcription preference of the U6 RNA polymerase III promoter.

Sequencing of each construct was started from the U6 promoter in both vectors by using the U6-Fwd primer (ZQ66, section 4.1.3). Sequencing results showed that both

constructs, PX462-8081 (clone 1) and PX462-8283 (clone1), had the correct insertion of guide sequences sgRNA 8081 and 8283 in the vector PX462 (Figure 2.5).

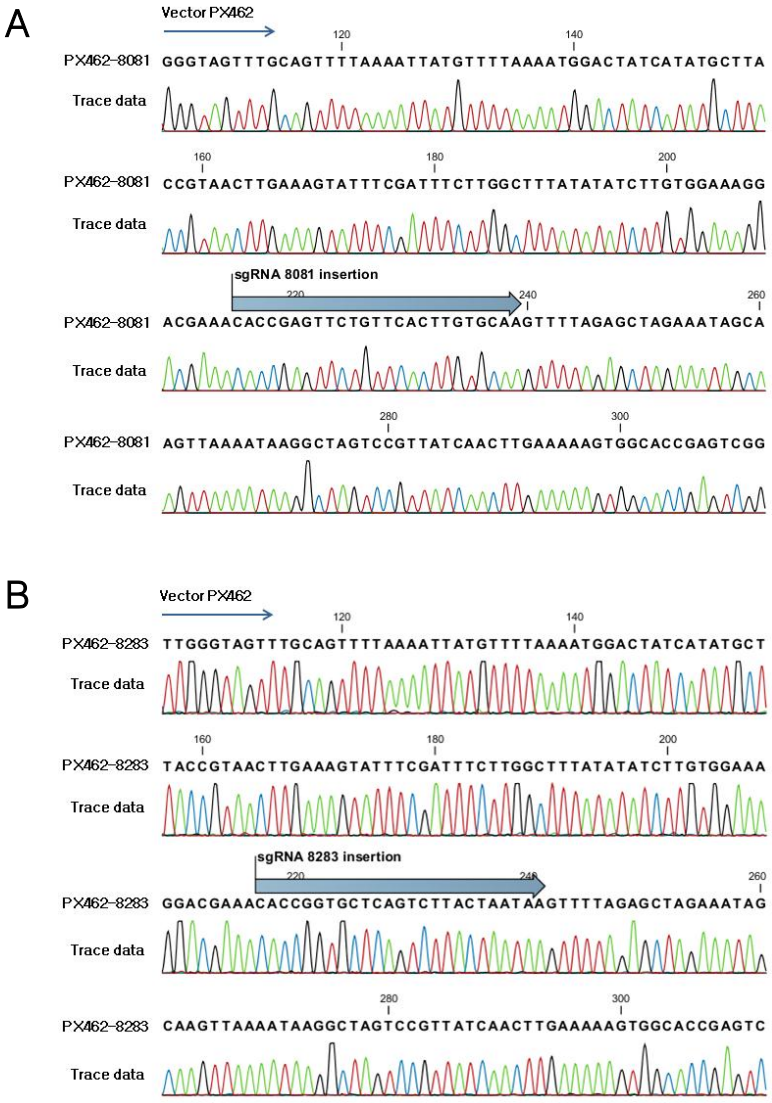

Figure 2.5 sequence verification of CRISPR construct targeting ITGB1 gene. A, Sequencing verification of PX462-8081 (clone1). Designed sgRNA ZQ8081 was correctly inserted in the vector PX462. **B**, Sequencing verification of PX462-8283 (clone1). Designed sgRNA ZQ8283 was correctly inserted in the vector PX462.

2.1.3 Integrin αv targeting with the CRISPR-Cas9 system

Design of sgRNAs for targeting human integrin αv (ITGAv) gene and the CRISPR plasmids construction were similar as in section 2.1.2, except that integrin αv genomic DNA sequence were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NG_029012.1?from=5001&to=63048&report=genbank). A pair of sgRNA guides which located in the *exon 4* of the integrin αv gene was chosen among the recommendations from the online CRISPR design tool according to the ranking and scoring, and the sequences and orientation of the guides are illustrated in Figure 2.6.

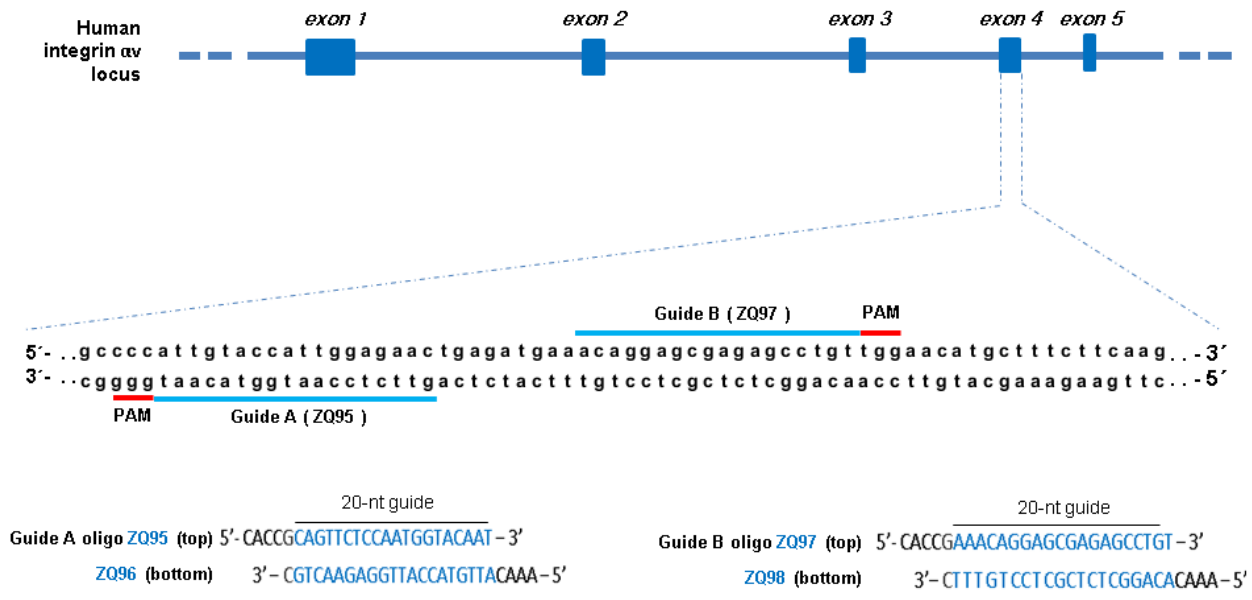

Figure 2.6 The oriantation and location of the sgRNA pair selected for Integrin αv gene targeting. For *S.pyogenes* Cas9 nickase, 20bp sgRNA pairs (hignlighted in blue) were immediately followed at their 3' ends by the 5'-NGG PAM (protospacer adjacent motif). The sgRNA pairs located on both strands of target DNA and were spaced with a 9 bp gap. Two appropriate targets in *exon 4* of integrin αv gene were recommended *in silico* by using the online CRISPR Design Tool (http://tools.genome-engineering.org).

All the oligos designed for targeting the ITGAv gene are listed in **Table 2.2**. Furthermore, the construction of the CRISPR plasmid PX462-9596 and PX462-9798 targeting integrin αv gene was similar as in section 2.1.2.2 and will not be repeated here. The sequencing results of the two constructs revealed correct insertion of designed sgRNAs in the CRISPR vector PX462 (data not shown).

Table 2.2 Sequences of paired sgRNAs designed for targeting integrin αv gene

sgRNAs targeting ITGAv gene	Guide A	top oligo (ZQ95)	5'- CACCGCAGTTCTCCAATGGTACAAT - 3'
		bottom oligo (ZQ96)	5'- AAACATTGTACCATTGGAGAACTGC - 3'
	Guide B	top oligo (ZQ97)	5'- CACCGAAACAGGAGCGAGAGCCTGT - 3'
		bottom oligo (ZQ98)	5'- AAACACAGGCTCTCGCTCCTGTTTC - 3'

2.1.4 Integrin β4 targeting with the CRISPR-Cas9 system

Procedures for designing the paired sgRNAs targeting integrin β4 (ITGB4) gene and the preparation of sgRNA constructs were similar as described in section 2.1.2 and 2.1.3.

However, the integrin β4 genomic DNA sequence was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NG_007372.1?from=5001&to=41384&report=genebank) and the selected paired sgRNAs were located in *exon 6* (Figure 2.7).

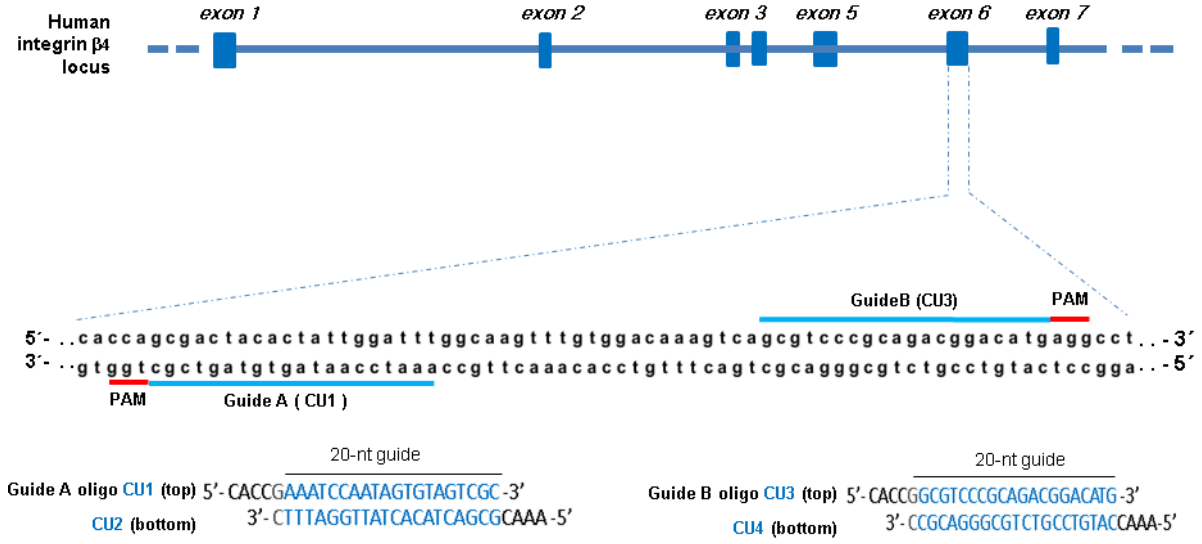

Figure 2.7 The oriantation and location of the sgRNA pair selected for Integrin β4 gene targeting. For *S.pyogenes* Cas9 nickase, 20bp sgRNA pairs (hignlighted in blue) were immediately followed at their 3' ends by the 5'-NGG PAM (protospacer adjacent motif). The sgRNA pairs located on both strands of target DNA and were spaced with a 21 bp gap. Two appropriate targets in *exon 6* of the integrin β4 gene were recommended *in silico* by using the online CRISPR Design Tool (http://tools.genome-engineering.org).

The paired sgRNAs designed for targeting integrin β4 (ITGB4) gene are listed in Table 2.3, and the sequencing results of CRISPR constructs PX462-1&2 and PX462-3&4 showed that sgRNAs were correctly inserted into the vector PX462 (data not shown).

Table 2.3 sequences of paired sgRNAs designed for targeting ITGB4 gene

sgRNAs targeting ITGB4 gene	Guide A	top oligo (CU1)	5' -CACCGAAATCCAATAGTGTAGTCGC-3'
		bottom oligo (CU2)	5' -AAACGCGACTACACTATTGGATTTC-3'
	Guide B	top oligo (CU3)	5'- CACCGGCGTCCCGCAGACGGACATG-3'
		bottom oligo (CU4)	5'- AAACCATGTCCGTCTGCGGGACGCC-3'

2.1.5 Generation of single- and multiple-integrin depletion cell lines

For generation of integrin-depletion AGS and KatoIII cell lines, verified CRISPR constructs targeting different integrin genes were transfected or cotransfected into AGS and KatoIII cells. Transfected cells went through several selection procedures in order to obtain the clonal deületion cell lines. Firstly, the transfected population was treated with puromycin so that the untransfected cells were killed since CRISPR constructs contain the puromycin resistance gene. Secondly, the surviving cells were stained with corresponding integrin antibodies for the negative selection by FACS sorting. Thridly, the sorted negative population was diluted by serial dilution to generate clonal cells. In the end, twelve clonal cell lines were obtained, including five integrin-depletion AGS cell lines and seven integrin-depletion KatoIII cell lines. All the depletion cell lines were established with the double nicking strategy by introducing paired CRISPR constructs targeting the respective integrin gene in target cell lines. All the constructs used for the cell line generation are listed in Table 2.4. In addition, all cell lines were generated by targeting wildtype AGS or KatoIII directly. However, ITGB1B4 KO AGS and ITGAvB1B4 KO KatoIII cell lines were generated by targeting previously generated integrin-depletion cell lines (Table 2.4).

All obtained integrin-depletion cell lines were verified by surface detection of different integrins using flow cytometry with corresponding antibodies. Compared to wildtype cell lines, each knockout cell line showed a complete depletion of the corresponding integrin(s) surface expression (Figure 2.8 – 2.9).

Table 2.4 CRISPR constructs and targeted cell lines for the generation of integrin-depletion AGS and KatoIII cell lines

Integrin knockout cell lines	Constructs used for transfection	Target cell line
ITGB1 KO AGS	PX462-5859 PX462-6061	Wildtype AGS
ITGB1 KO KatoIII		Wildtype KatoIII
ITGAv KO AGS	PX462-9596 PX462-9798	Wildtype AGS
ITGAv KO KatoIII		Wildtype KatoIII
ITGB4 KO AGS	PX462-1&2 PX462-3&4	Wildtype AGS
ITGB4 KO KatoIII		Wildtype KatoIII
ITGB1B4 KO AGS	PX462-1&2 PX462-3&4	ITGB1 KO AGS
ITGB1B4 KO KatoIII	PX462-5859 PX462-6061 PX462-1&2 PX462-3&4	Wildtype KatoIII
ITGAvB4 KO AGS	PX462-9596 PX462-9798	Wildtype AGS
ITGAvB4 KO KatoIII	PX462-1&2 PX462-3&4	Wildtype KatoIII
ITGAvB1 KO KatoIII	PX462-5859 PX462-6061 PX462-9596 PX462-9798	Wildtype KatoIII
ITGAvB1B4 KO KatoIII	PX462-5859 PX462-6061	ITGAvB4 KO KatoIII

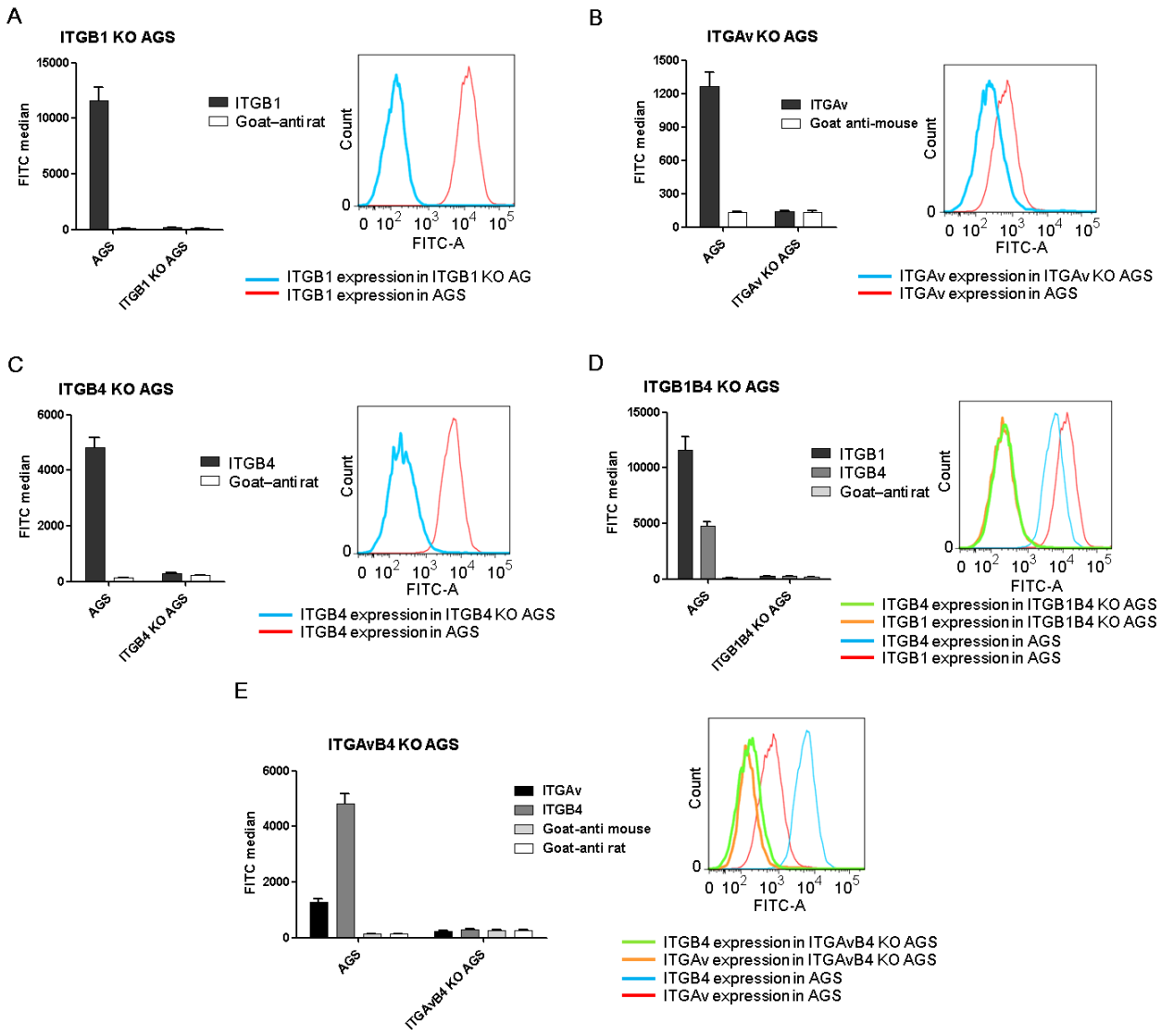

Figure 2.8 Verification of integrin expression on wildtype AGS and its five integrin-depletion cell lines (n=3). Integrin expression was determined showing FITC median from three independent flow cytometry experiments. FITC-A histograms are representatives of three experiments. As negative controls, cells were stained only with secondary antibody (Goat-anti mouse, Goat-anti rat). Comparing to wildtype AGS cells, all of the integrin-depletion cell lines showed the complete loss of the corresponding integrin expressions on their surfaces. A, ITGB1 surface expression on wildtype AGS cells and ITGB1 KO AGS cells. B, ITGAv surface expression on wildtype AGS cells and ITGAv KO AGS cells. C, ITGB4 surface expression on wildtype AGS cells and ITGB4 KO AGS cells. D, ITGB1 and ITGB4 surface expression in wildtype AGS cells and ITGB1B4 KO AGS cells. E, ITGAv and ITGB4 surface expression in wildtype AGS cells and ITGAvB4 KO AGS cells. All values were indicated as average values including standard errors of the mean (±SEM).

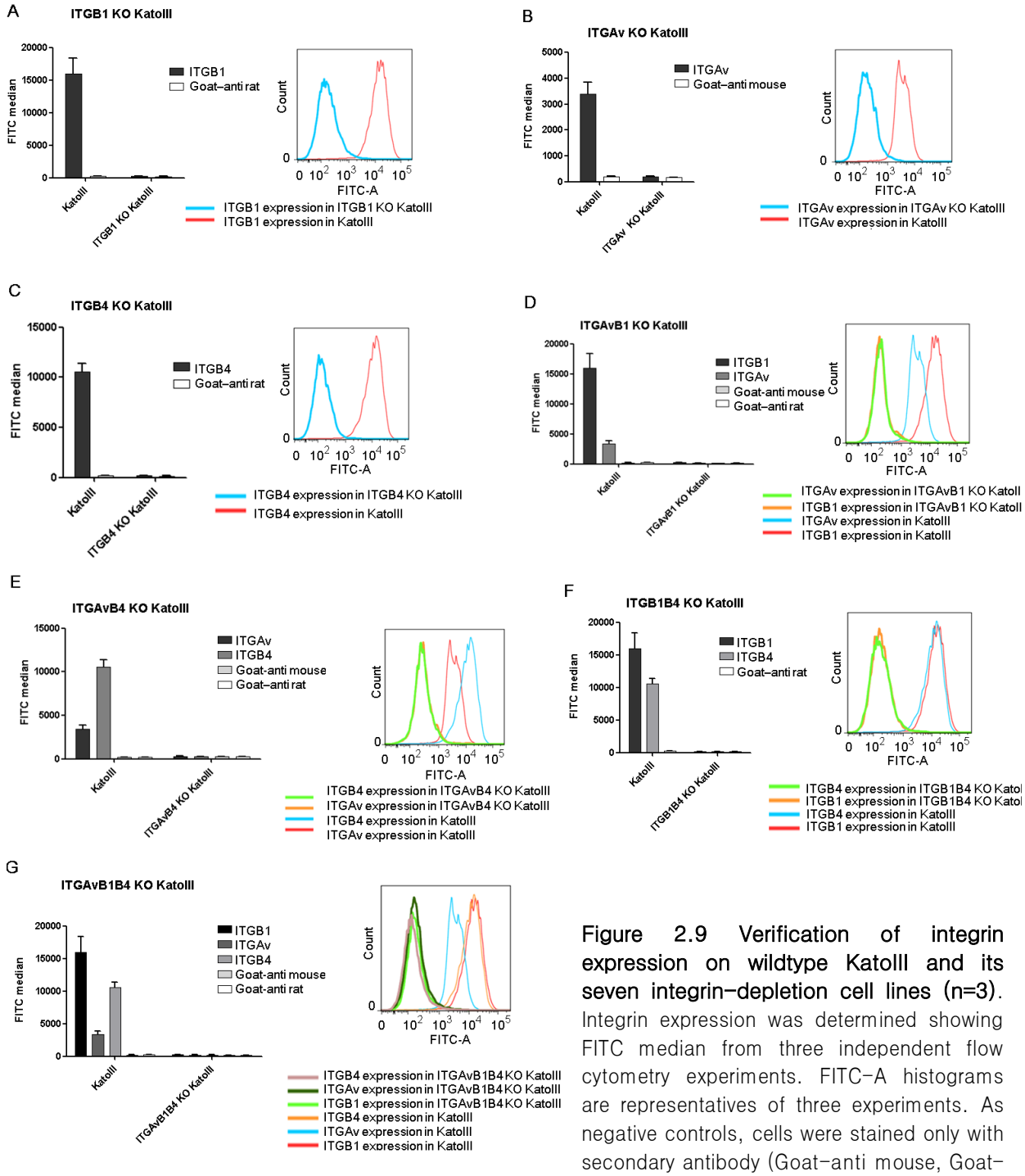

Figure 2.9 Verification of integrin expression on wildtype KatoIII and its seven integrin-depletion cell lines (n=3). Integrin expression was determined showing FITC median from three independent flow cytometry experiments. FITC-A histograms are representatives of three experiments. As negative controls, cells were stained only with secondary antibody (Goat-anti mouse, Goat-anti rat). Comparing to wildtype KatoIII cells, all the integrin-depleted cell lines showed the complete depletion of the corresponding integrin expression on their surface. **A**, ITGB1 surface expression in wildtype KatoIII cells and ITGB1 KO KatoIII cells. **B**, ITGAv surface expression in wildtype KatoIII cells and ITGAv KO KatoIII cells. **C**, ITGB4 surface expression in wildtype KatoIII cells and ITGB4 KO KatoIII cells. **D**, ITGAv and ITGB1 surface expression in wildtype KatoIII cells and ITGAvB1 KO KatoIII cells. **E**, ITGAv and ITGB4 surface expression in wildtype KatoIII cells and ITGAvB4 KO KatoIII cells. **F**, ITGB1 and ITGB4 surface expression in wildtype KatoIII cells and ITGB1B4 KO KatoIII cells. **G**, ITGAv, ITGB1 and ITGB4 surface expression in wildtype KatoIII cells and ITGAvB1B4 KO KatoIII cells. All values were indicated as average values including standard errors of the mean (±SEM).

2.2 Quantification of CagA translocation in integrin-depletion cell lines by measuring TEM-1 β-lactamase activity

In order to understand the role and the molecular basis of the interaction of T4SS and host cell integrins, the CagA translocation efficiency was evaluated in the generated integrin-depletion cell lines. The result should help to answer the question whether Integrin β1 alone is sufficient for CagA translocation, and whether it is possible that other integrins are involved in this process.

Traditionally, by using the fact that CagA can be phosphorylated by host cell kinases when translocated into the host cells, CagA translocation efficiency is assessed by detecting a phosphorylated CagA band in *H.pylori* infected cells via western blot. However, the use of western blot as a quantification method is questioned [158], especially when the conclusions are dependent on subtle differences between the samples.

Fortunately, a sensitive β-lactamase reporter system has recently been established in our lab to quantitatively examine *H.pylori* CagA translocation into host cells [52]. One of the major advantages about this system is that it can monitor and assess CagA translocation independently of its tyrosine phosphorylation and host cell kinase activity. β-lactamases (BLA) are enzymes produced by bacteria to facilitate resistance to β-lactam antibiotics by breaking the β-lactam ring open through hydrolysis. The ability to use BLA as a fluorescence-based reporter gene was not realized until the development and synthesis of the fluorescent substrates CCF2-AM [159] and CCF4-AM, which are composed of two fluorescent dyes, 7-hydroxycoumarin-3-carboxamide and fluorescein, bridged by a cephalosporin moiety (**Figure 2.10**). CCF4 is relatively more soluble than CCF2 and thus has been preferred for some experiments. Neither BLA nor its intact or cleaved substrate is toxic to eukaryotic cells, therefore, it is possible to introduce the system in live cells or live animals.

To apply the system for *H.pylori in vitro* infection experiments to measure CagA translocation, a TEM-1 β-lactamase was previously fused to the N-terminus of *CagA* from *H.pylori* strain P12 [52]. When host cells were infected by this modified *H. pylori*, the in frame protein fusion of TEM-1 β-lactamase and the CagA (TEM-CagA) was injected by the T4SS into host cells. Upon arrival of TEM-CagA, The fluorescent

substrate loaded into the host cells will be cleaved by TEM-1, thus resulting in disruption of Fluorescence Resonance Energy Transfer (FRET) of the substrate to convert the substrate fluorescence from green to blue (**Figure 2.10**). By measuring and calculating the ratio of blue to green fluorescence with either a plate reader or by flow cytometry, translocation of TEM-CagA can be evaluated quantitatively with high sensitivity.

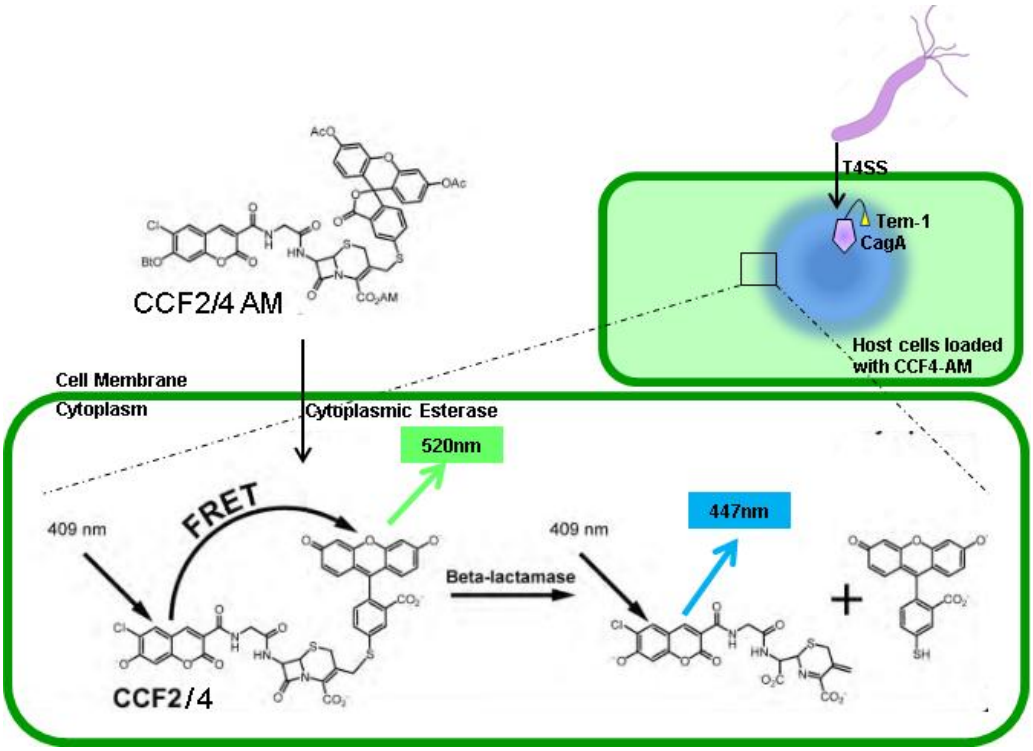

Figure 2.10 Illustration of the β-lactamase reporter system. The lipophilic and esterified (acetoxymethylated; AM) form of the substrate CCF2 or CCF4 (CCF2/4-AM) can enter eukaryotic cells with ease and readiness. Once CCF2/4-AM enters a cell, host esterases convert it into its negatively charged form (CCF2/4) which is maintained in the cytoplasm. In the absence of TEM-1, excitation of hydroxycoumarin at 409 nm exhibits FRET to fluorescein which generates green fluorescence (520 nm). In the presence of TEM-1, in this case when TEM-CagA is injected into host cells by the T4SS upon infection, CCF2/4 is hydrolyzed and therefore FRET is disrupted, coumarin emits at 447nm and produces the green fluorescence.

2.2.1 Host cell integrin-independent CagA translocation in AGS cells

To investigate CagA translocation in different integrin-depletion AGS cell lines independent of its tyrosine phosphorylation and host kinase activity, the β-lactamase reporter system was applied by using *H.pylori* strains to infect different integrin-depletion cell lines *in vitro*. The bacterial strains included P12[TEM-CagA], and its isogenic *hopQ* deletion mutant (P12Δ*hopQ*[TEM-CagA]). As mentioned in section

1.4.2, outer membrane protein HopQ can bind to host CEACAMs and the binding is essential for CagA translocation[141, 142]. Therefore the isogenic *hopQ* deletion mutant was used to disrupt the HopQ-CEACAM interaction, in order to evaluate CagA translocation efficiency when both host cell CEACAM and integrin receptors do not function properly. As negative controls, the isogenic *cagI* deletion mutant (P12Δ *cagI*[TEM-CagA]) was used, since deletion of *cagI* results in a defect in the Type IV secretion system [160].

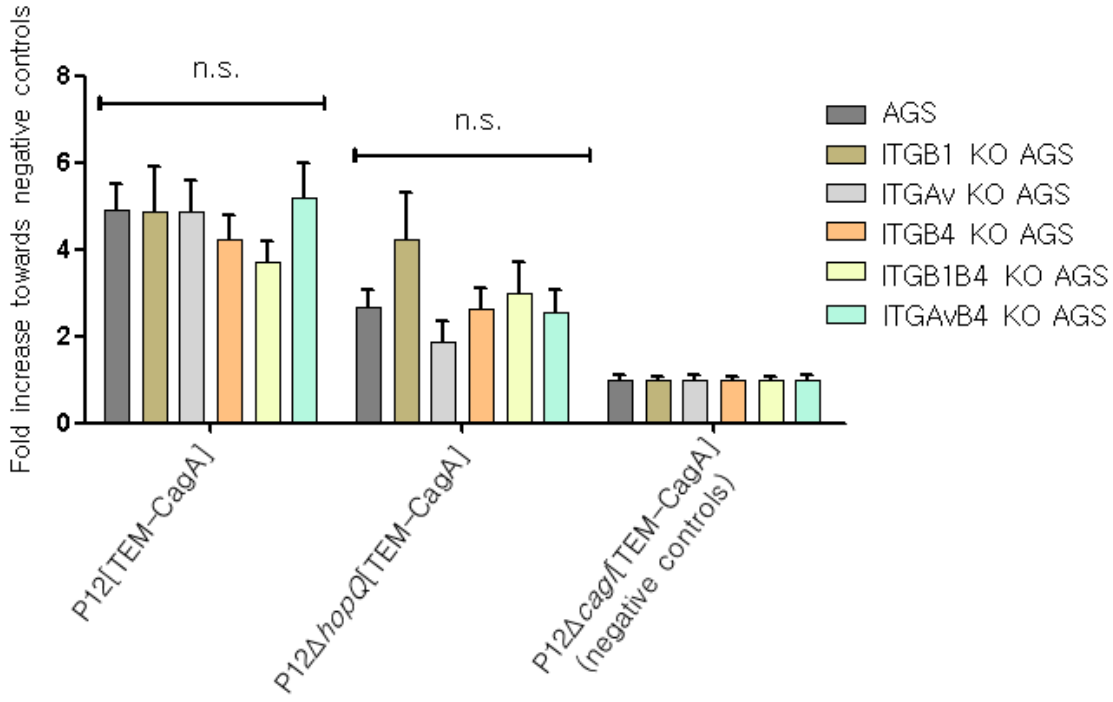

Figure 2.11 Quantitative evaluation of TEM-CagA translocation into AGS and its five integrin-depletion cell lines (n=5). AGS and its five integrin-depletion cell lines were seeded in 96-well plates and infected with P12[TEM-CagA], its *hopQ* deletion mutant and its *cagI* deletion mutant at an MOI of 60. After 2.5h infection, substrate CCF4-AM was loaded in each cell line for 2 h. Green and blue fluorescence of each well were measured by a plate reader. Ratios of blue to green fluorescence of each sample were calculated and normalized to the mean of blue to green ratio of its negative controls. All values were indicated as standard errors of the mean (±SEM) from five independent experiments. The significance of differences was analyzed using One way ANOVA (n.s., non-significant).

The blue to green fluorescence of each infection was measured by a plate reader and the ratio was calculated as an indicator for translocation efficiency. Surprisingly, as illustrated in **Figure 2.11**, all five different integrin-depletion cell lines showed comparable translocation efficiencies to the AGS wildtype cell line when infected with P12[TEM-CagA]. Moreover, all five integrin-depletion cell lines exhibited a similar level of translocation as AGS cells when infected with the P12Δ*hopQ*[TEM-CagA] strain,

which is strongly reduced in its capacity to translocate CagA. As expected, all cell lines showed a very low (background level) blue to green fluorescence ratio when infected with T4SS defective mutant P12Δ*cagI*[TEM-CagA]. The data presented here confirmed that the HopQ-CEACAM interaction is necessary for the CagA tanslocation. Moreover, it showed that single to multiple integrin depletion did not affect CagA tranlocation efficiency, suggesting a so-far unrecognizedl host cell integrin-independent CagA translocation into AGS cells.

2.2.2 Host cell integrin-independent CagA translocation in KatoIII cells

CagA translocation was quantified in seven different integrin-depletion KatoIII cell lines with the β-lactamase reporter system as well, and the blue and green fluorescence in each cell was measured by flow cytometry. After calculating of the blue-to-green ratio of each infection and the statistical analyzing from four independent experiments, the ITGAvB1 KO KatoIII cell line showed a significant reduction in CagA translocation when infected with P12[TEM-CagA], compared to wildtype KatoIII cells. However, when infected with P12Δh*opQ*[TEM-CagA], all cell lines including wildtype KatoIII showed a slight shift from green to blue, indicating arrival of a small amount of TEM-CagA in the cells, which did not make a statistical difference. The type IV secretion defective negative controls (P12Δ*cagI*[TEM-CagA]) showed no shift or only a background shift (**Figure 2.12**). The data suggested that the HopQ-CEACAM interaction was the major contributor in the CagA translocation process in KatoIII cells. Except for the ITGAvB1 KO KatoIII cell line, other integrin-depletion cell lines exhibited host cell integrin-independent CagA translocation. In addition, the ITGAvB1 KO KatoIII cell line showed a compromised CagA translocation efficiency, which was not observed in the ITGAvB1B4 KO KatoIII cell line. The reasons for this discrepancy will be further investigated in section 2.4.

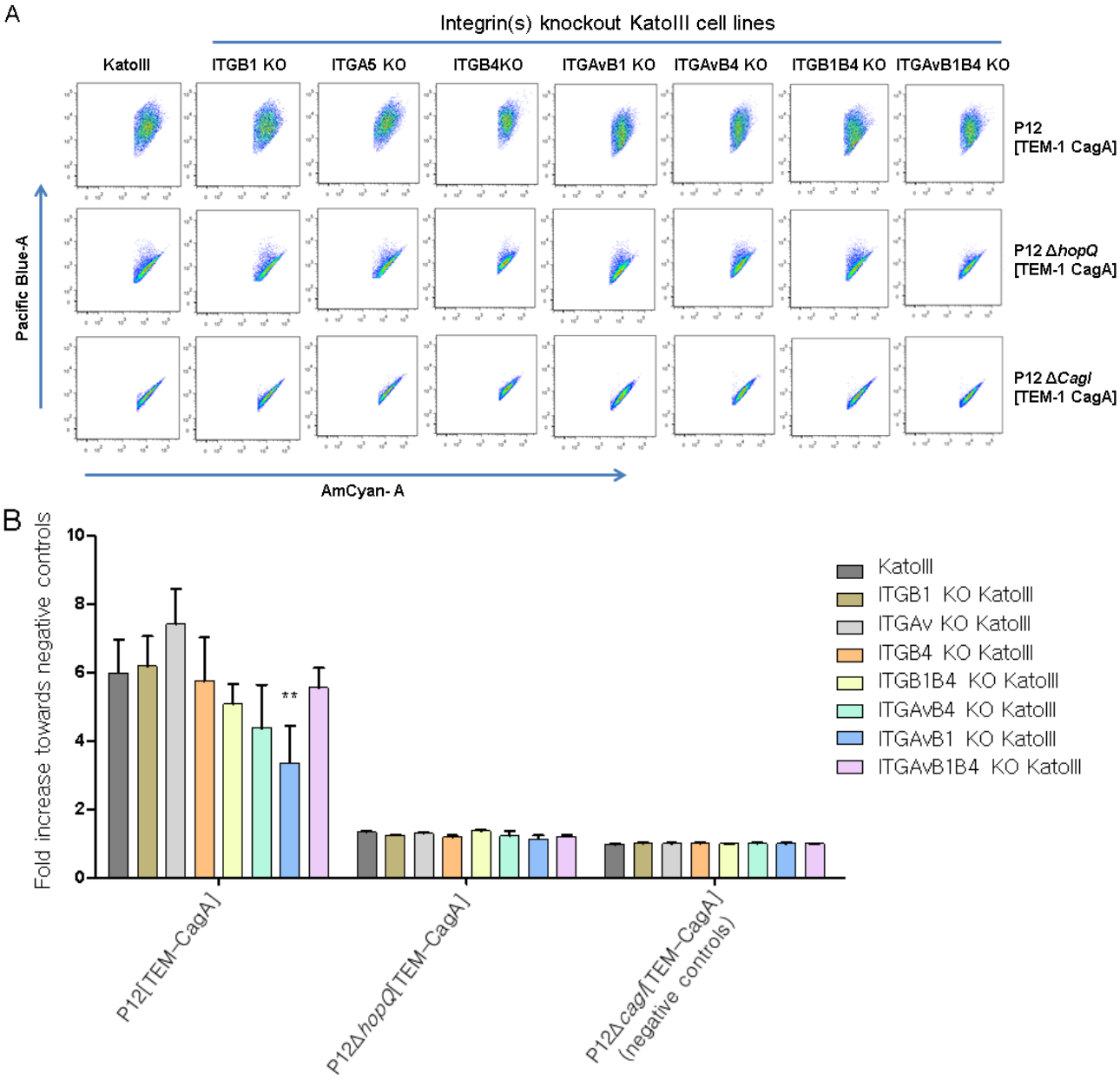

Figure 2.12 Quantitative evaluation of TEM-CagA translocation into KatoIII and its seven integrin-depletion cell lines (n=4). KatoIII and its integrin-depletion cell lines were infected with P12[TEM-CagA], its *hopQ* deletion mutant and its *cagI* deletion mutant with the MOI of 60 for 3h. Subsequently, substrate CCF4-AM was loaded into infected cells for 1.5h. A. Fluorescence of each cell was analyzed by flow cytometry in the AmCyan (green) and Pacific Blue (blue) channels. Different degrees of green-to-blue shifting indicated varying amount of TEM-CagA being translocated into cells. Plots are the representative of four independent experiments. B. After calculating the ratio of blue to green of each sample from four independent experiments, CagA translocation quantification was determined by normalizing the Blue/Green ratio to the mean of the respective negative controls. All values are shown as standard errors of the mean (±SEM). The significance of differences was analyzed using One way ANOVA (***P*<0.05).

2.3 Qualification of CagA translocation in integrin-depletion cell lines by detecting phosphorylated tyrosine (in collaboration with Katrin Gerrer)

In order to exclude the possibility that the observed integrin-independent CagA translocation was caused by strain-specific effects of *H.pylori* strain P12, other strains with distant geographical origin were used to infect the ITGAvB1 KO and the ITGAvB1B4 KO KatoIII cell lines. It is known that after translocation into host cells, CagA can be tyrosine-phosphorylated by host Src family kinases and C-Abl kinase at the host inner leaflet of the plasma membrane. Therefore, the traditional and classical way of CagA translocation qualification is the detection of tyrosine-phosphorylated CagA from infected cells.

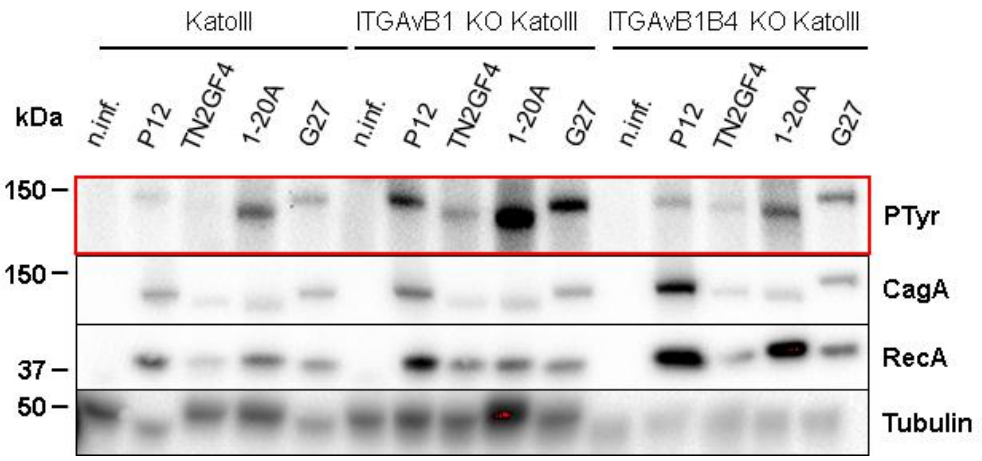

Figure 2.13 Qualification of CagA translocation in integrin-depletion cell lines by different *H.pylori* strains. KatoIII cells and the two integrin-depletion cell lines were infected with strain G27, 1-20A and TN2GF4 for 4 hours with an MOI of 60. Translocation of CagA was determined by detecting tyrosine-phosohorylated CagA with the antibody 4G10. n.inf.: non-infected control.

H.pylori strain G27 was originally isolated from Europe, 1-20A from Africa and TN2GF4 from East Asia. KatoIII cells infected with these *H.pylori* strains were used as the positive controls. As the negative controls, two integrin-depletion cell lines were left without infection. As indicated in **Figure 2.13**, a phosphorylated CagA band can be detected in each infected sample except the negative controls, as highlighted in the red box, indicating that all three different *H.pylori* stains had successfully translocated CagA into the two tested integrin-depletion cell lines, as well as into positive control cell line KatoIII. As the loading controls, the tubulin immunoblot demonstrates total cell lysates

loaded in the SDS gel, the RecA blot shows the amount of bacteria in the cell lysates and the CagA blot indicates the total CagA amount in the lysates. The sizes of the CagA bands as well as the phosphorylated CagA bands were slightly different, revealing that CagA from different strains has variable molecular weight.

Thus, independent *H.pylori* strains showed CagA translocation in two representative integrin-depletion cell lines. In summary, these data suggest that the observed host cell integrin-independent CagA translocation is a common phenomenon among diverse *H.pylori* strains and is not a specific effect of strain P12.

2.4 Integrin and CEACAM profiling in integrin-depletion cell lines

Since changes in the expressions of host receptors (integrins and CEACAMs) on the cell surface can alter the CagA translocation results, an integrin and CEACAM profiling in each integrin-depletion cell line was performed, in order to investigate whether depletion of integrin(s) can influence the expression levels of other receptors, such as the remaining integrins and the CEACAMs.

2.4.1 Integrin profiling in integrin-depletion cell lines

To profile integrin expression on each integrin-depletion cell surface, integrin α1, integrin α2, integrin α3, integrin α4, integrin α5, integrin αv, integrin β1 and integrin β4 expression was determined by flow cytometry using specific antibodies. In parallel, wildtype AGS and KatoIII cell lines were stained with the antibodies mentioned above as the positive controls. Furthermore, all cell lines were stained with secondary antibodies alone as the negative controls. As indicated in Figure 2.14 A, the remaining integrins expressed on the surface of each integrin-depletion AGS cell line exhibited similar expression levels as were found on wildtype AGS cells, and the statistical test (one way ANOVA) showed no significant deviation between each integrin-depletion cell line and AGS wildtype cell line.

However, the situation was different in integrin-depletion KatoIII cell lines as indicated by the following observations. As compared to wildtype KatoIII, the ITGB1 KO KatoIII cell line showed a significant reduction in integrin αv ($P<0.001$) and integrin β4 expression ($P<0.001$). The ITGAv as well as the ITGAvB4 KO KatoIII cell lines exhibited a significantly decreased integrin β4 expression ($P<0.05$) and ($P<0.001$), respectively. (Figure 2.14 B).

In conclusion, the integrin depletion did not affect expression levels of remaining integrins in AGS cells. On the other hand, depletion of certain integrins in KatoIII cells led to a significantly decreased expression of some remaining integrins. However, these changes did not influence the CagA translocation outcome, which will be discussed in section 3.3.

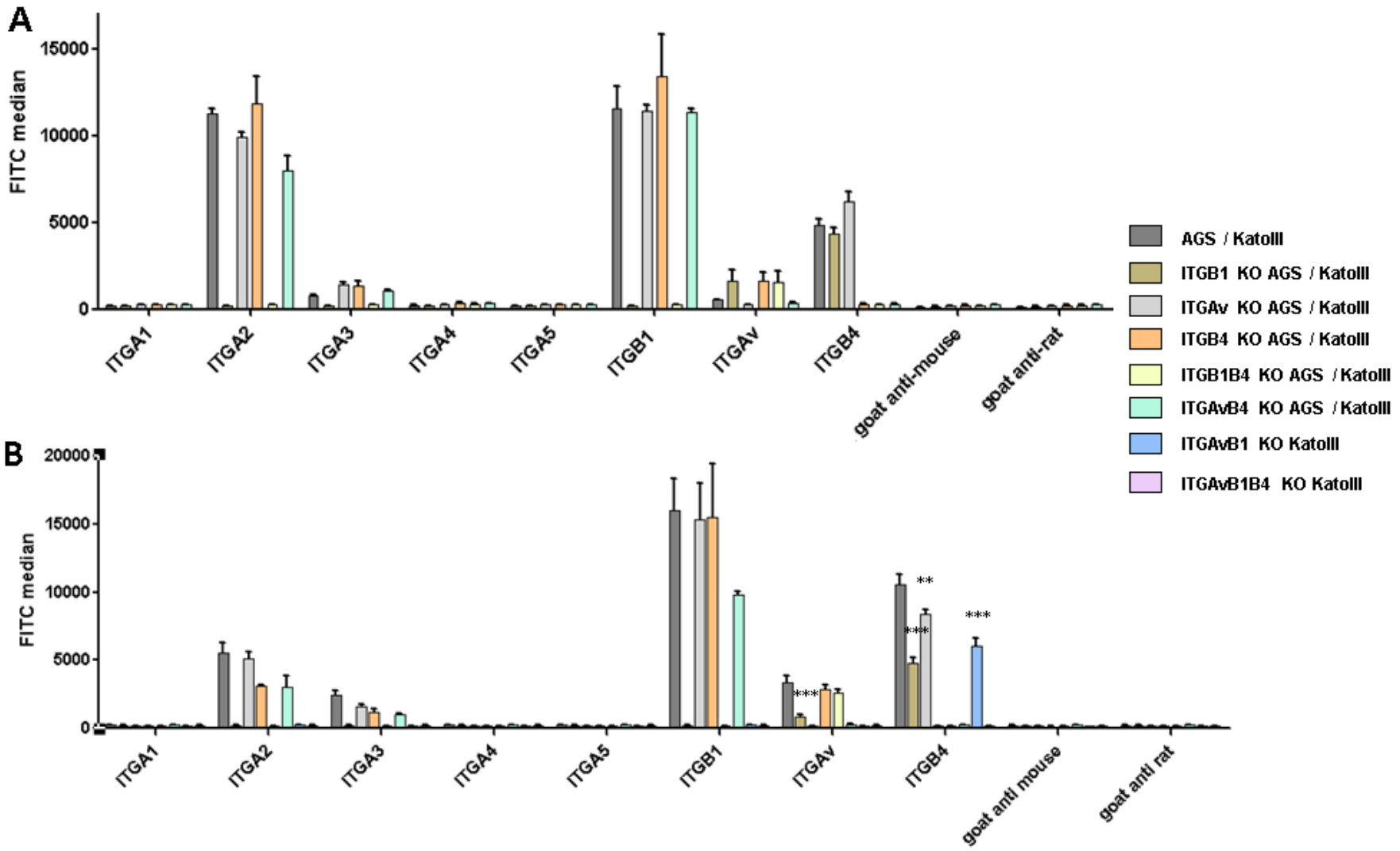

Figure 2.14 Integrin profiling in different integrin-depletion cell lines. Wildtype cell lines and integrin-depletion cell lines were stained with antibodies specific to ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGAv, ITGB1 and ITGB4, and were subsequently monitored by flow cytometry in the FITC-A channel. FITC median were obtained and analyzed with the Flowjo software. All values were indicated as standard errors of the mean (+SEM) from three independent experiments. The significance of differences was analyzed using One way ANOVA. A. Integrin profiling in integrin-depletion AGS cell lines (n=3). B. Integrin profiling in integrin-depletion KatoIII cell lines (n=3, ***P<0.001, **P<0.05)

2.4.2 CEACAM profiling in integrin knockout cell lines

To perform CEACAM profiling in wildtype AGS/KatoIII and their integrin-depletion cell lines, CEACAM expression was analyzed by flow cytometry after staining them with CEACAM1, CEACAM5 and CEACAM6 antibodies. Among them, wildtype AGS and KatoIII cells stained with CEACAM antibodies were used as positive controls, and all tested cell lines were stained with the secondary antibodies alone as the negative controls.

As indicated in **Figure 2.15 A**, CEACAM profiling in the wildtype AGS and the five integrin-depletion AGS cell lines revealed low expression levels of CEACAM1 and CEACAM5, and relatively high expression levels of CEACACM6. In addition, integrin depletion in AGS cells did not influence CEACAM1 and CEACAM5 expression levels, as each integrin-depletion cell line showed equally expressed CEACAM1 or CEACAM5 when compared to wildtype AGS cells. However, the ITGAv KO AGS cell line showed significantly reduced expression of CEACAM6 ($P<0.05$) when compared to AGS cells. Thus, depletion of the αv integrins expression on AGS cells can significantly affect CEACAM6 expression levels.

As shown in **Figure 2.15 B**, CEACAM profiling in the wildtype KatoIII cells and seven integrin-depletion KatoIII cell lines revealed low expression levels of CEACAM1, and relatively high expression levels of CEACACM5 and CEACAM6. In addition, depletion of one or more integrins in KatoIII cells did not lead to varying CEACAM1 expression. However, CEACAM5 and CEACAM6 expression varied markedly amongst different integrin knockout cell lines. Particularly, the ITGB4 KO KatoIII cells showed a drastic decrease in CEACAM5 ($P<0.05$) and CEACAM6 ($P<0.001$) expression and therefore had the lowest expression of CEACAM5 and CEACAM6 of all cell lines. Moreover, the ITGAvB1 KO KatoIII cells displayed a marked reduction in CEACAM5 ($P<0.05$) and CEACAM6 ($P<0.001$) as well, and represent the cells with the second lowest CEACAM5 and CEACAM6 expression. However, the ITGAvB1B4 KO KatoIII cells didn't show a significant decrease in CEACAM5 expression, but did in CEACAM6 ($P<0.001$). Taken together, the genetic inactivation of certain integrin genes in KatoIII cells did strongly influence the expression levels of certain CEACAMs, especially CEACAM5 and CEACAM6.

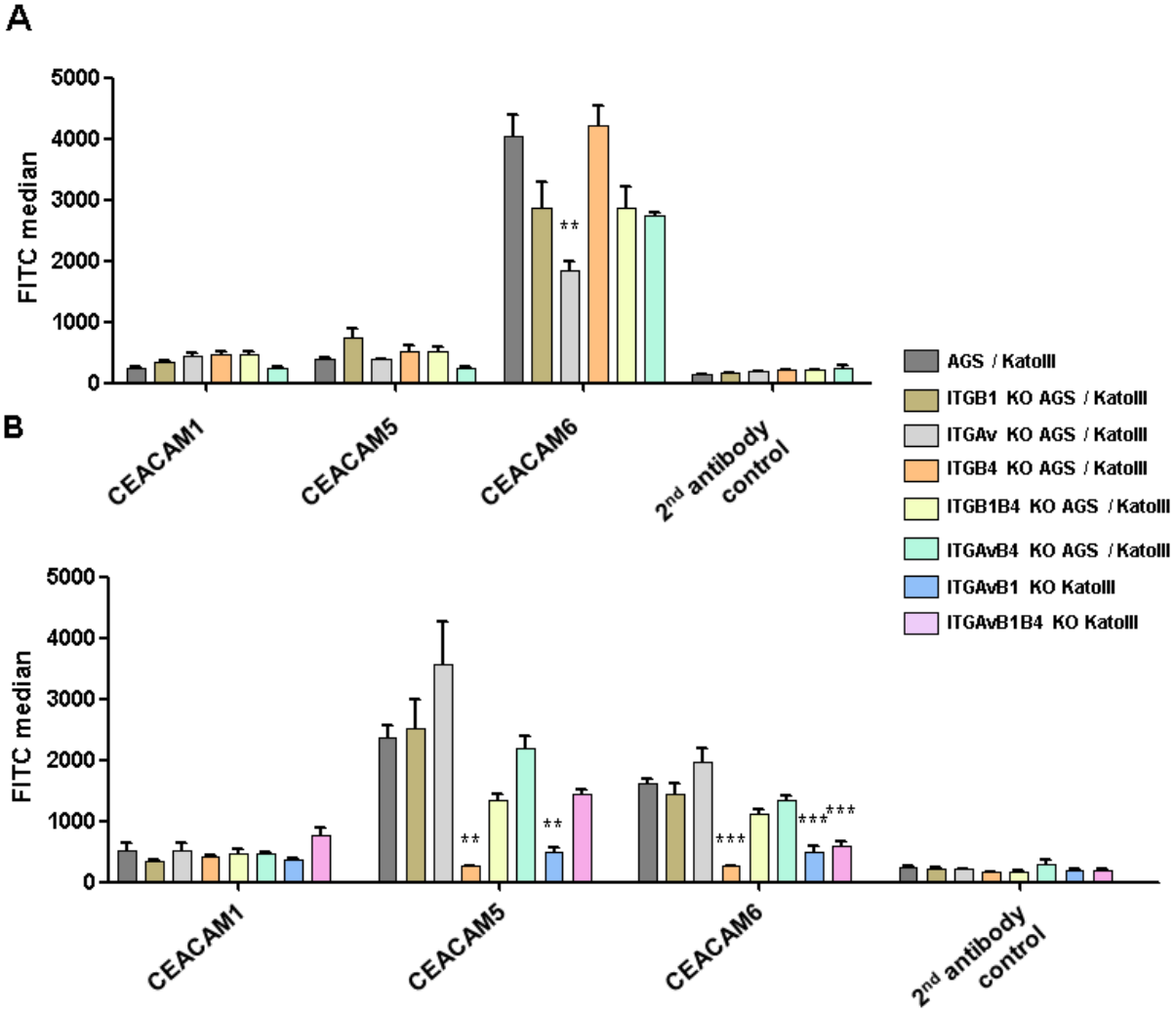

Figure 2.15 CEACAM expression profiling in different integrin-depletion cell lines. AGS or KatoIII wildtype cells and their integrin-depletion cells were stained with antibodies to quantify CEACAM expression by flow cytometry. Cells stained with secondary antibody alone were used as negative controls. **A**, CEACAM profiling in integrin-depletion AGS cell lines **(n=3)**. **B**, CEACAM profiling in integrin-depletion KatoIII cell lines **(n=3)**. All values in A and B are depicted as standard errors of the mean (±SEM) from three independent experiments. The significance of differences was analyzed using One way ANOVA (***P<0.001, **P<0.05).

A particularly interesting observation was that although the ITGB4 KO KatoIII cell line produced extremely low levels of all CEACAMs, it had a full capacity for CagA translocation, as compared to the KatoIII wildtype cell line. In addition, the ITGAvB1 KO KatoIII cells showed a significantly reduced CagA translocation, but the ITGAvB1B4 KO KatoIII cells didn't. These discrepancies led us to examine whether CEACAM expression might change upon *H.pylori* infection. To this end, KatoIII and its integrin-depletion cell lines were infected for 3h with P12 wildtype, P12Δ*hopQ* or left uninfected, and subsequently stained with different CEACAM antibodies. As shown in **Figure 2.16 A & C**,

after infection with either strain, CEACAM1 and CEACAM6 expression pattern in each cell line didn't change before and after *H.pylori* infection. However, as indicated in Figure 2.16 B, CEACAM5 expression in each cell line did increase after infection with P12 wildtype, but with varying degree. Among them, KatoIII, the ITGB1 KO KatoIII and ITGAv KO KatoIII cell lines showed a two to three fold increase of CEACAM5 expression. The ITGB1B4, ITGAvB4 and ITGAvB1B4 knockout KatoIII cell lines showed a one to two fold increase of expression. Moreover, CEACAM5 expression was certainly increased in ITGAvB1 KO KatoIII cell line, but not as much as in the ITGB4 KO KatoIII cell line. In addition, this increase or upregulation of CEACAM5 expression was HopQ dependent, since infection with the isogenic *hopQ* deletion mutant of strain P12 did not change the CEACAM5 expression pattern as compared to the non-infected control. These changed expression levels of CEACAMs influenced the CagA translocation efficiency accordingly. The details will be discussed in section 3.3.

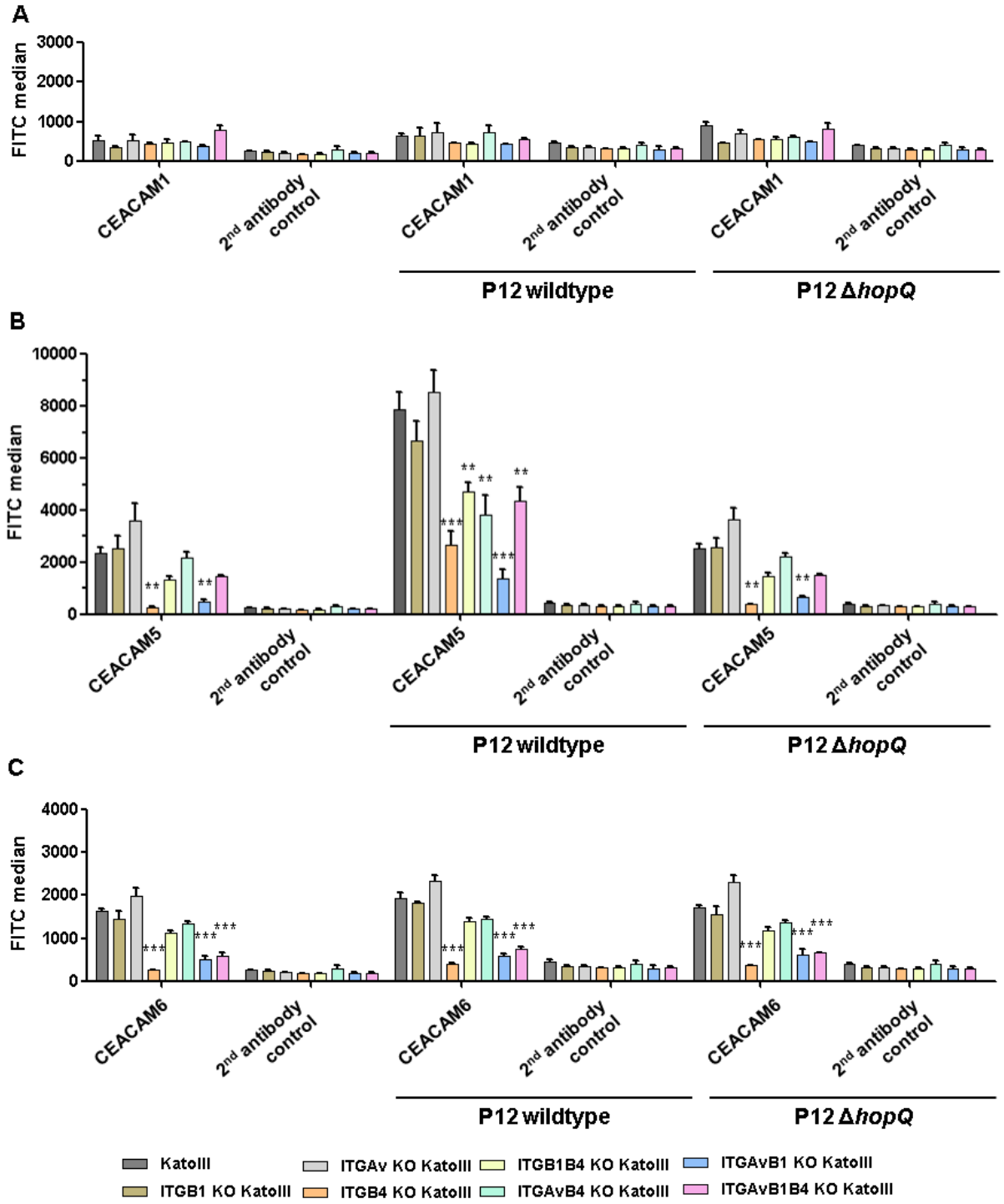

Figure 2.16 CEACAM profiling in different integrin-depletion cell lines upon *H.pylori* infection (n=3). KatoIII and its different integrin-depletion cell lines were infected either with P12 wildtype strain or P12Δ*hopQ* for 3h, or left without infection. Afterwards, cells were stained with specific antibodies for CEACAM surface expression detection by flow cytometry. Cells stained with only secondary antibody in different conditions were considered as negative controls. **A**, the CEACAM1 profiling. **B**, the CEACAM5 profiling. **C**, the CEACAM6 profiling. Error bars indicate ± SEM from three independent experiments and the significance was analyzed by one way ANOVA (***P<0.001, **P<0.05)

3 Discussion

3.1 Generation of integrin-depletion cell lines

3.1.1 Generation of integrin-depletion AGS cell lines

Generation of integrin-depletion cell lines was the first step to characterize the role of integrin in CagA translocation. Five of the planned integrin-depletion AGS cell lines (integrin β1, αv, β4, αvβ4, β1β4 KO AGS) were established successfully with the double-nicking strategy. Unfortunately, the other two of the planned integrin αvβ1 KO AGS and integrin αvβ1β4 KO AGS cell lines could not be generated. After looking into the integrin mediated functions, we realized that adhesion of integrins and their extracellular matrix ligands is essential for cell proliferation and survival [161-163]. This integrin-mediated cell survival can be disrupted by the absence of integrin-ECM ligation. As a result, an apoptosis pathway "anoikis" is triggered. Anoikis, a Greek word meaning "homelessness", is a tissue architecture surveillance mechanism to make sure that the dissociated and displaced cells are effectively removed, in order to prevent dysplastic growth of mainly epithelial and endothelial cells [163-166].

So, how does absence of integrin trigger anoikis? To understand this, we first need to understand how integrin-ECM adhesion supports cell survival. Integrins are intimately coupled with many growth factor receptors, and the correct ligation of ECM proteins with integrins is the prerequisite for cells to respond to the stimulation by growth factors. This phenomenon is therefore defined as the anchorage-dependent cell survival and proliferation, and integrin adhesion plays a fundamental role [167-170]. Integrin-ECM adhesion can elicit the integrin-mediated intracellular signal transduction pathways including anti-apoptotic pathways and pro-survival pathways in the context of supporting cell survival. For example, the focal adhesion kinase (FAK) is recruited to activate the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway to promote cell survival by inhibiting the mitochondrial release of cytochrome-C and by activating the NF-κB survival pathway [171, 172]. Furthermore, FAK can trigger an

anti-apoptotic MAPK (mitogen-activated protein kinases) pathway, as a result, pro-apoptotic proteins Bim and Bad are degraded and downregulated, respectively [173, 174]. Meanwhile, Integrin-linked kinase (ILK) is another key player in integrin mediated signalling pathway. It can activate the pro-survival PI3K/PKB pathway independent of FAK, suggesting that both FAK and ILK can support cell survival in parallel with different strategies [175, 176].

Loss of integrin engagement with ECM can directly disrupt above mentioned integrin mediated growth factor receptor activation, as well as several survival signalling events. In the absence of integrin-ECM engagement, the PI3K/Akt pathway and MAPK pathway are therefore disrupted. As a result, the pro-apoptotic protein Bim and Bad cannot be phosphorylated and degraded properly [177]. This can rapidly promote the oligomerization of two other pro-apoptosis proteins, Bax and Bak, within the outer mitochondrial membrane, which leads to outer mitochondrial membrane permeabilization and cytochrome-C release. Ultimately, the apoptotic pathway is activated [178]. Of note, the apoptotic pathway caused by absence of integrin-ECM and mitochondrial damage, as described here, is defined as the intrinsic pathway of anoikis [166].

In addition, the loss of integrin-ECM ligation can lead to anoikis via the extrinsic pathway. The extrinsic pathway is initiated by the ligation of the extracellular death ligand FasL and its transmembrane receptor Fas [179]. Absence of integrin anchorage can result in increased expression of the FasL and Fas, as well as decreased expression of Fas-mediated signaling antagonist c-Flip [180]. Ligation of Fas and FasL can recruit and activate caspase 8. The latter can further activate more caspases, like caspase-3 and -7 to trigger cell death [181]. Alternatively, caspase-8 can directly activate the pro-apoptotic protein Bid and finally leads to the release of cytochrome-C to trigger anoikis [182, 183]. Thus, the intrinsic and extrinsic pathways can be linked at this point.

Therefore, it can be speculated that the reason for the failed generation of integrin αvβ1 KO AGS and integrin αvβ1β4 KO AGS cells might be anoikis, triggered by the depletion of β1 and αv integrins at the same time in AGS cells, which probably led to cell death in the targeted cells.

3.1.2 Generation of integrin-depletion KatoIII cell lines

Interestingly, all the planned integrin-depletion KatoIII cell lines were successfully generated, suggesting that KatoIII cells confer anoikis resistance and can survive independently of integrin-ECM anchorage. Indeed, metastatic transformed cancer cells like KatoIII cells can develop sophisticated strategies to bypass anoikis, in order to facilitate the aggressive metastatic spread of cancer in foreign tissues and organs [184].

The most common strategy exploited by metastatic cancer cells to resist anoikis is to induce several oncogenes or activate different proteins in order to constitutively activate pro-survival or anti-apoptotic signaling pathways to compensate the loss of integrin anchorage. For example, the phosphatase and tensin homolog (PTEN), a major antagonist of pro-surviving PI3K/Akt signaling, is often mutated, down-regulated or inhibited in many aggressive cancers to elicit sustained survival signaling [185, 186]. Moreover, during epithelial-mesenchymal transition (EMT, a key event in metastatic spread of epithelial cells), several genes encoding transcription factors like Snail, Twist and NF-κB are induced to facilitate aniokis resistance by transcription of tumor promoting genes, which involved PI3K/Akt cascade activation [187-189]. Similarly, the tyrosine kinase receptor c-Met is activated during EMT and it plays an important role in stimulating PI3K/Akt signaling [190]. Furthermore, the tropomycin-related kinase B (TrkB), the most efficient suppressor of anoikis, is often overexpressed in many aggressive cancers, especially in gastric cancers [191]. Its anoikis resistance effect is achieved through activating PI3K/Akt and MAPK pathways, as well as downregulating E-cadherin expression via the induction of Snail and Twist [192-194]. Loss of E-cadherin can lead to decreased cell-cell adhesion and β-catenin release. The latter can function as a transcription factor to induce tumor promoting genes involved in anoikis insensitivity and increased cell motility [195].

Other strategies exploited by metastatic cancer cells for confering anoikis resistance include the exploitation of oxidative stress, hypoxia, and modifications of energetic metabolism [196-201]. We can see that cancer cells have developed diverse strategies to overcome anoikis and to facilitate its spread. Therefore it is not surprising that KatoIII cells, the gastric cancer cells derived from metastatic sites, can survive without integrin

expression, which makes it possible to generate integrin-depletion KatoIII cell lines without any integrin expression.

3.2 Host cell integrin independent *H.pylori* CagA translocation in gastric epithelial cell lines

3.2.1 Host cell integrin-independent *H.pylori* CagA translocation in AGS cells

Translocation of *H.pylori* CagA into host cells via the type IV secretion system (T4SS) is considered as a major risk factor for gastric diseases including gastric cancer. To understand the CagA translocation mechanism, especially the transport of CagA across the host cell membrane, previous studies have shown that instead of being randomly injected into the host cells, CagA is translocated via the host receptor integrin β1. However, different interaction mechanisms were proposed. Kowk and colleagues proposed that CagL, a type IV secretion system component protein which harbors the classical integrin interaction RGD motif, locates on the tip of the T4SS and interacts with host cell integrin β1 to trigger CagA translocation into host cells [127]. However, the mechanism of how the CagL-integrin β1 interaction leads to CagA translocation still remains a mystery. Independently, the study from Jimenez-Soto and colleagues showed that instead of CagL, other T4SS components, like CagI, CagY and CagA, can directly interact with host cell integrin β1 in an RGD-independent fashion [128]. This discrepancy led us to the first hypothesis that integrin β1 might not be the only receptor exploited by *H.pylori* for CagA translocation. Indeed, more lines of evidence came from the observation described below. Firstly, besides integrin β1, CagL can interact with other integrins, like integrin αvβ3, αvβ5 and αvβ6 [202-205]. Secondly, many integrin β1 antibodies targeting different epitopes, integrin β1 ligands and integrin β1 binding proteins could not reduce or block CagA translocation. Together, we supposed that *H.pylori* can exploit more host factors than integrin β1, and the possible candidates can be other host cell integrins. Therefore, we generated integrin-depletion cell lines with the CRISPR-Cas9 system in order to identify other integrins engaged by *H.pylori* during CagA translocation.

Surprisingly, AGS cells showed CagA translocation independent of many host cell integrins upon wildtype *H.pylori* strain P12[TEM-CagA] infection, as shown in section 2.2.1. Different integrin-depletion cell lines exhibited comparable CagA translocation

efficiency. Most notably, it is the first study to our knowledge to investigate the role of different integrins in *H.pylori* CagA translocation, which challenged the existing theory. The two previous studies identified integrin β1 as the receptor mainly by evaluating CagA translocation via detecting phosphorylated CagA in human integrin β1 deficient cell lines and human integrin β1 expressing cell lines. For example, the mouse fibroblast cells GD25 and mouse epithelial-like cells GE11 were deficient in detecting phosphorylated CagA but the human integrin β1 stably transfected GD25β and GE11β cells were translocation competent [127, 128]. Similarly, the human promyelocytic leukaemia cells HL60 were capable of CagA translocation but the differentiated HL60 cells lost the ability, since differentiated HL60 showed "integrin switch" from integrin β1 to integrin β2 [128]. These results strongly suggested that *H.pylori* can use integrin β1 as the receptor for CagA translocation. However, the results shown in this study revealed that depletion of integrins did not have a significant effect on CagA translocation. Neither integrin β1, nor other host epithelial-specific integrins are essential as receptors for CagA translocation. To this end, we propose that integrin β1 can function as a receptor for *H.pylori* CagA translocation, but it is not the only one that the bacteria exploit for this important pathogenicity mechanism.

As mentioned in the Introduction section 1.4, host cell integrin is not the only known receptor involved in CagA translocation, our group recently found that CEACAMs also play an important role in this process. So far the molecular mechanism of how HopQ-CEACAM interaction can contribute to CagA translocation via the type IV secretion system is not well understood. There are many possibilities and one of the major hypotheses is that the HopQ-CEACAM interaction can further assist T4SS-integrin interaction to promote the CagA translocation via a CEACAM-integrin clustering in the lipid rafts. Therefore, a *hopQ* isogenic deletion mutant of strain P12 was used to infect integrin-depletion AGS cell lines in order to disrupt the HopQ-CEACAM interaction, since HopQ is the only *H.pylori* interacting partner for CEACAMs identified so far. We hypothesized that host cells can be deprived of CagA translocation ability when both integrins and CEACAMs do not function properly as receptors for *H.pylori*. However this was not the case. Again, all five integrin-depletion cell lines showed comparable CagA translocation efficiency as compared to AGS cells, meaning that integrin depletion did not affect CagA translocation outcome. The results consistently suggested that host cell integrins are not essential for CagA translocation. Of note, AGS and the integrin-

depletion cell lines exhibited reduced CagA translocation efficiency upon P12Δ *hopQ*[TEM-CagA] infection, suggesting that beside CEACAMs, *H.pylori* must have exploited other host factors instead of host cell integrins to translocate CagA.

Nevertheless, some limitations in this assay are worth noting. The CagA translocation efficiency in integrin αvβ1 and integrin αvβ1β4 knockout AGS cell lines could not be investigated due to the anoikis effects in these two cell lines, as mentioned before in section 3.1.1. Therefore, CagA translocation was evaluated in integrin-depletion KatoIII cell lines.

3.2.2 Host cell integrin-independent *H.pylori* CagA translocation in KatoIII cells

Similarly, CagA translocation efficiency was evaluated and quantified in seven integrin-depletion KatoIII cell lines in order to assess the role of different host cell integrins in CagA translocation. One of the cell lines, integrin αvβ1 knockout KatoIII, showed a significantly reduced CagA translocation as compared to AGS cells upon wildtype P12[TEM-CagA] infection. All other depletion cell lines exhibited approximately similar level of CagA translocation as AGS cells. It was difficult to understand the observation that depletion of αv and β1 integrins in KatoIII cells can lead to reduced CagA translocation, but depletion of all possible integrins in the integrin αvβ1β4 knockout KatoIII cells cannot. This discrepancy led us to presume that depletion of certain integrins can affect expression levels of other receptors, like the remaining integrins or the CEACAMs, and the CagA translocation outcome can be changed. Therefore, we checked integrin and CEACAM expression in each integrin-depletion KatoIII cell line to see whether the cells can compensate the loss of certain integrins with other receptors, like remaining integrins or CEACAMs. Indeed, the expression levels of integrin and CEACAM were affected, especially CEACAMs. The detailed information will be discussed in the next section 3.3.

However, all integrin-depletion cell lines as well as KatoIII cells, showed extremely low levels of CagA translocation when infected with the isogenic *hopQ* deletion mutant strain, suggesting that *H.pylori* mainly utilizes the outer membrane protein HopQ to interact with host CEACAMs for CagA translocation in KatoIII cells. This was different

from AGS cells, where the HopQ-CEACAM interaction only accounted for roughly half of the CagA translocation. However, the molecular mechanism for this phenomenon is unclear. A possible explanation could be the lack of (an) unknown co-receptor(s) in KatoIII cells. Nevertheless, the results indicated that neither individual integrin nor combination of different integrins were essential for CagA translocation.

Taken together, the results provided compelling and convincing evidence that host cell integrins were not essential for *H.pylori* CagA translocation, as demonstrated by the highly sensitive β-lactamase reporter assay. Next, the question was addressed whether the host cell integrin independent CagA translocation was a strain specific effect from *H.pylori* strain P12.

Therefore, three other *H.pylori* strains with distant geographic origins were tested in two representative integrin-depletion cell lines, integrin αvβ1 knockout and integrin αvβ1β4 knockout KatoIII cell lines, to examine CagA translocation by detecting phosphorylated CagA in the infected cell lysates via immunoblot. After infection with three different *H.pylori* strains, the phosphorylated CagA band can be detected in each sample (except the negative controls), suggesting that the obtained integrin-independent CagA translocation was not a P12 strain-specific effect, but a common phenomenon of diverse *H.pylori* strains. However, the use of immunoblot as a quantification method to define the subtle differences in expression of target proteins is still under debate. It is reported that approximately 25% of published papers contained misleading or inappropriate western blotting quantifications [158]. Therefore, the western blot results presented here was not quantified, and the CagA translocation quantification in each integrin-depletion cell line was performed using the highly sensitive β-lactamase reporter system in this study.

3.3 Influence of host cell integrin depletion on other host cell receptors and the consequences for the CagA translocation.

To investigate whether depletion of integrins can affect the expression level of other receptors which are potentially involved in CagA translocation, like CEACAMs and the remaining integrins, we performed expression profiling of CEACAMs and integrins by flow cytometry. We were interested whether the expression level of αv integrins, β4 integrin and/or CEACAMs might be increased in the context of β1 integrins depletion. If this would be the case, it could be explained that the elevated expression level of other host receptors compensated the loss of β1 integrin to maintain CagA translocation efficiency. However, the outcome was different than the above proposed scenario.

3.3.1 Expression profiling of integrins and CEACAMs in integrin-depletion AGS cell lines

The β-lactamase reporter assay revealed that integrin depletion did not affect CagA translocation efficiency in AGS cells. Meanwhile, integrin profiling results showed that remaining integrin expression levels in each integrin-depletion AGS cell line did not change significantly as compared to AGS cells. This result confirmed that the depletion of certain integrins did not have an influence on CagA translocation efficiency. Moreover, it excluded the possibility that depletion of certain integrins led to elevated expression levels of remaining integrins, which might have compensatory effects on CagA translocation.

The next question was whether the depletion of integrins in AGS cells can lead to elevated expression levels of CEACAMs? Unexpectedly, the CEACAM6 expression in one of the integrin-knockout cell lines was significantly reduced. To begin with, the CEACAM1 and CEACAM5 detection by flow cytometry revealed very low and nearly equal expression levels in AGS cells and the integrin-depletion cell lines. However, CEACAM6 expression in each cell line was high, suggesting that HopQ may exploit CEACAM6 to support CagA translocation in AGS cells. Among depletion cell lines, integrin αv knockout AGS cells exhibited a significantly decreased CEACAM6 expression. In theory, CagA translocation efficiency should be compromised when the

receptor CEACAM6 has reduced expression. However, like other cell lines, the integrin αv knockout cell line showed normal CagA translocation without reduction. This was unexpected but not surprising, since the HopQ-CEACAM interaction only accounts for half of the CagA translocation in AGS cells. A 50% reduced CEACAM6 expression can lead in theory to one fourth of the CagA translocation reduction, which might be statistically insignificant. In addition, the host pathogen interaction is complicated in the infection process. It is possible that the HopQ-CEACAM interaction can assist the interaction of the T4SS and some unknown receptors other than integrins, and the unknown receptor can compensate the reduction of CEACAM6. Furthermore, it is also possible that the reduced CEACAM6 expression was still above the threshold needed for CagA translocation, therefore the CagA translocation efficiency was not compromised.

Taken together, depletion of β1 integrins, αv integrins or β4 integrin (integrin α6β4) did not influence CagA translocation in AGS cells. More importantly, depletion of combinations of β1 integrins and β4 integrin, or αv integrins and β4 integrin did not influence as well.

3.3.2 Expression profiling of integrins and CEACAMs in integrin-depletion KatoIII cell lines

Similarly, integrin and CEACAM profiling was also performed in integrin-depletion KatoIII cell lines to understand the obtained CagA translocation result. Unlike AGS cells, KatoIII cells showed a largely changed expression level of other receptors in the context of depletion of integrin(s). To begin with, integrin β1 knockout KatoIII cells showed a deceased integrin αv and integrin β4 expression; Integrin αv knockout KatoIII cells showed reduced integrin β4 expression; Moreover, integrin αvβ1 knockout KatoIII cells showed declined integrin β4 expression. Interestingly, CagA translocation efficiency was compromised in integrin αvβ1 knockout KatoIII cell line, but not in integrin β1 and integrin αv knockout cell lines. Taken together, these results revealed that depletion of β1 integrins, and reduced expression of αv integrins and β4 integrin in the integrin β1 knockout KatoIII cell line had no influence on CagA translocation. Similarly, depletion of αv integrins and reduced expression of β4 integrin in the integrin αv knockout cell line had no influence in CagA translocation. So far these results indicated that either absence or decreased expression of integrins did not have an effect on CagA

translocation. Therefore the question was addressed whether depletion of αv and β1 integrins and reduced integrin β4 expression in the integrin αvβ1 knockout KatoIII cell line was accounted for the reduced CagA translocation efficiency in the integrin αvβ1 knockout KatoIII cell line. Obviously, this was not the reason since the integrin αvβ1β4 knockout KatoIII cell line exhibited statistically non-reduced CagA translocation efficiency. Thus, there must be other reasons for the reduced CagA translocation in the integrin αvβ1 knockout KatoIII cell line. Therefore, the CEACAM expression profiles were next analyzed.

Indeed, both CEACAM5 and CEACAM6 expression were markedly decreased to almost background level in the integrin αvβ1 knockout KatoIII cell line. Moreover, CEACAM6 expression was significantly reduced in integrin αvβ1β4 knockout KatoIII cells. Of note, the CEACAM expression pattern in KatoIII cells was different from AGS cells. A very low expression level of CEACAM1, but a high expression of CEACAM5 and CEACAM6 suggested that *H.pylori* principally uses CEACAM5 and CEACAM6 to translocate CagA in KatoIII cells. Therefore, dramatically decreased CEACAM5 and CEACAM6 might be the reason for reduced CagA translocation efficiency in the integrin αvβ1 knockout KatoIII cell line. Moreover, although CEACAM6 expression was strongly reduced in the integrin αvβ1β4 knockout KatoIII cell line, CEACAM5 did not change significantly and this could be the reason for maintaining CagA translocation at the normal level in the integrin αvβ1β4 knockout KatoIII cell line.

However, it was discussed in section 3.2.2 that the HopQ-CEACAM interaction accounted for most of the CagA translocation in KatoIII cells. In this case due to poorly expressed CEACAM1, CEACAM5 and CEACAM6, CagA translocation in integrin αvβ1 knockout KatoIII cells should be diminished to very low levels instead of an only approximately 40% reduction. In addition, it was surprising to observe that integrin β4 knockout KatoIII cells expressed equal but extremely low levels of CEACAM5 and CEACAM6, which were similar as the background level, although the cells exhibited indistinguishable CagA translocation efficiency as the positive control KatoIII cells.

These discrepancies led us to assume that CEACAMs expression might be changed during the *H.pylori* infection. Indeed, CEACAM5 expression levels in all cell lines increased two to four folds after three hours of infection with strain P12, and the effect was HopQ-dependent, since the *hopQ* deletion mutant failed to increase CEACAM5

expression. Among them, CEACAM5 expression elevated two fold in the integrin αvβ1 knockout cell line and four fold in the integrin β4 knockout cell line. Therefore, it was highly possible that CEACAM5 expression reached the threshold needed for full capacity of CagA translocation in integrin β4 knockout cells, but not in integrin αvβ1 knockout cells.

Taken together, we have shown a host cell integrin independent CagA translocation in AGS and KatoIII cells. Furthermore, integrin depletion in KatoIII cells can change CEACAM expression levels and CagA translocation efficiency can be influenced by fluctuations in CEACAM expression.

3.3.3 Changing expression levels of host receptors in certain integrin-depletion cell lines

As mentioned above, it was surprising to discover that the CEACAM expression level, especially CEACAM5 and CEACAM6, was decreased in the absence of certain integrins. interestingly, CEACAM5 expression was increased during *H.pylori* infection. Which molecular events underlie these two different outcomes in the cellular state?

In order to explain why integrin depletion can lead to decreased expression level of CEACAMs, a literature search was performed, but unfortunately, no direct evidence was found. However, it is known that integrins are full-fledged signal transduction receptors mediating many important cellular events, as mentioned in section 1.3 and section 3.1. Of note, integrin is linked with many transcription factors involved in up- or down-regulation of many genes, which are linked to cell proliferation, cell motility, cytoskeletal organization and cancer progression [101, 107, 109, 206]. Depletion of integrin surface expression can certainly disrupt many signal transduction pathways and therefore affect transcription of many genes. Therefore, it is feasible that integrin depletion may result in changing expression levels of CEACAMs. For example, an affymetrix GeneChip assay identified 538 genes the expression of which was regulated by integrin α6β4, including genes involved in cell motility, adhesion molecules, metastasis, metabolism, apoptosis, and other signaling molecules [207]. Although CEACAMs were not on this affymetrix GeneChip list, it is possible that CEACAMs are regulated by integrins indirectly. Furthermore, it is puzzling that integrin β4 knockout

KatoIII cells markedly suppressed CEACAM5 and 6 expression, but integrin β1β4 knockout cells failed to suppress. Similarly, Integrin αvβ1 knockout KatoIII cells exhibited significantly decreased CEACAM5 expression, but integrin αvβ1β4 knockout cells didn't. Again, this study is the first to report these observations and no published data are available to explain the exact mechanisms underlying these phenomena. However, it is definitely interesting for future researches.

On the other hand, it is observed that *H.pylori* infection could induce elevated CEACAM5 expression in KatoIII cells and this effect was HopQ dependent. This was an exciting finding, suggesting that not only can HopQ bind to CEACAM5, but also induce the CEACAM5 upregulation for a better binding and enhanced *H.pylori* pathogenesis. Unfortunately, this effect was not reproducible in other gastric epithelial cells, like AGS, MKN45 and ST23132 cell lines [142], suggesting that this was a KatoIII-specific effect which may not be true in the *in vivo* infection process.

3.4 Proposed models for transport of CagA across the host cell membrane

In this study, we report for the first time an integrin-independent CagA translocation of *H.pylori*. CagA translocation efficiency was not affected by presence or absence of host cell integrin, but by CEACAM expression levels. These results confirm that HopQ can function as a non-T4SS contributor in CagA translocation, and meanwhile suggest that host cell integrin is not essential in this process.

Other than integrins, independent studies identified different host factors which were involved in CagA translocation. For instance, Lai and colleagues proposed that host membrane cholesterol was essential in CagA translocation, since depletion of cholesterol resulted in significantly reduced CagA translocation in AGS cells, as introduced in section 1.4.1 [129]. However, depletion of host cholesterol can have many side effects. Therefore it is hard to distinguish whether reduced CagA translocation was caused by absence of cholesterol, or other side effects. Moreover, depletion of host cell cholesterol results in lipid raft disruption, and many cellular receptors are lipid raft associated like integrins, growth factor receptors and CEACAMs. Thus it is likely that cholesterol depletion affected raft-associated receptors which are important for CagA translocation. In addition, Murata-Kamiya and colleagues reported that CagA can directly interact with host phosphatidylserine to translocate CagA into MDCK cells [131], as introduced in section 1.4.1. However, the molecular mechanism of CagA-bound phosphatidylserine flipping back to the inside of cells is not well understood, and the follow-up researches should be included to explain the interaction mechanisms.

Based on the current literature and the data from this work, the following working models regarding *H.pylori* host-pathogen interaction for CagA translocation are proposed (Figure 3.1). Outer membrane protein HopQ, by binding host CEACAMs, provides one of the initial signals to recruit other unknown receptors for binding T4SS and/or CagA to translocate CagA into host epithelial cells. Such unknown host cell receptors may be the reported cholesterol, phosphatidylserine, or other uncharacterized raft-associated cellular receptors (Figure 3.1 A).

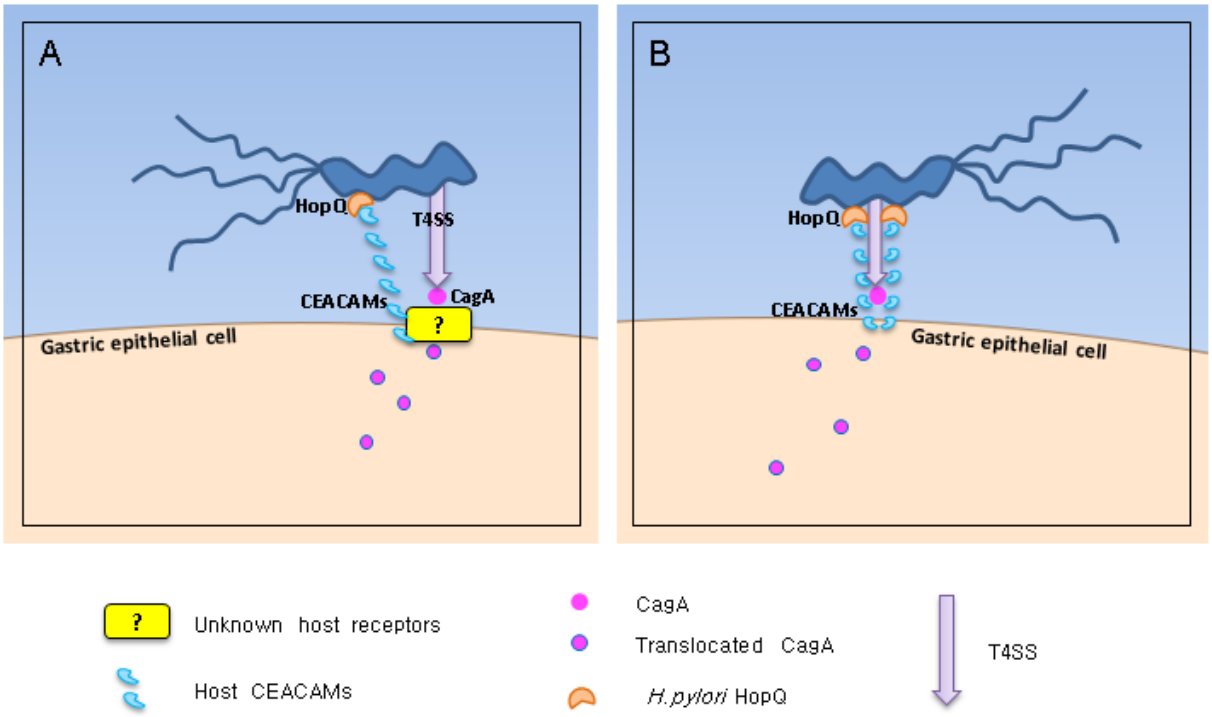

Figure 3.1 Proposed working models for *H.pylori* CagA translocation regarding host-pathogen interaction. **A**, Outer membrane protein HopQ, by binding host CEACAMs, provides one of the initial signals to recruit other unknown receptors for binding T4SS and/or CagA to translocate CagA into host epithelial cells. The unknown host receptors may be the reported cholesterol, phosphotidylserine or other uncharacterized raft-associated cellular receptors. **B**, HopQ. CEACAMs and certain T4SS components can form an interaction complex stabilizing each other, to facilitate CagA translocation via host CEACAMs.

However, in some cases the situation is different. The HopQ-CEACAM interaction appears to be the major prerequisite in CagA translocation. For example, CagA translocation efficiency is diminished to extremely low level in KatoIII cells, as revealed by CagA translocation BLA reporter assay upon infection with *hopQ* deletion mutant in this study. This is consistent with the finding that CagA cannot be translocated into MKN45 and HEK293 cells when the HopQ-CEACAM interaction was interrupted, or CEACAMs were not expressed on the cell surface [142]. Besides the possibility proposed above, it is also likely that these cell lines lack additional receptors and CEACAMs become the major receptors for CagA translocation. Therefore, we propose that HopQ, CEACAMs and certain T4SS components may form an interaction complex stabilizing each other, to facilitate CagA translocation via host CEACAMs. In this case, the absence of HopQ can lead to an unstable complex of T4SS-CEACAM. As a result, CagA translocation is disrupted or abolished (**Figure 3.1B**). However, future work is needed to consolidate and optimize these models.

3.5 Outlook

The data presented in this thesis suggest an integrin-independent CagA translocation process in AGS and KatoIII cells. However, the possibility that non-epithelial integrins, like leukocyte specific integrins and/or even the platelet integrin are expressed on these gastric epithelial cancer cell lines cannot be excluded. It is a common strategy for cancer cells to change in their pattern of integrin expression in order to express the correct integrins to survive in a different environment for their aggressive metastatic growth and spread. For example, while normal squamous cells express integrin α2β1, α3β1 and α6β4, the squamous carcinoma cells express high level of integrin αvβ5, αvβ6 [208, 209]. Melanoma cells are reported to over-express integrin αvβ3, which is not the case in normal melanocytes [210, 211]. However, we don't have direct evidences that epithelial cancer cells can "switch" from epithelial integrins to leukocyte integrins. Although not very likely, possibility that leukocyte integrins or even the platelet integrin α IIbβ3 are expressed on the AGS and KatoIII cells which might take over the responsibility for CagA translocation has to be excluded in future experiments.

Similarly, the presence of unusual or undiscovered αβ association of integrin in the two cancer cell lines (AGS and KatoIII) was not determined yet. Although the chance is extremely low, it is worth checking by using more integrin antibodies in future to make sure that no unexpected integrins are expressed on the AGS and KatoIII cell lines.

Disruption of HopQ-CEACAM interaction can lead to moderate to strong reduction of CagA translocation [142]. Besides the fact that CEACAMs can function as receptors for CagA translocation, they also act as the adhesin receptors of HopQ for *H.pylori* to adhere on the cell surface [141]. Bacteria cannot bind to the cells properly when the HopQ-CEACAM interaction is interrupted, thus the T4SS cannot translocate CagA appropriately. Therefore, to better understand the role of bacteria adhesion and its effect on CagA translocation, more binding data and mutagenesis studies should be included in future to elucidate the role of CEACAMs for CagA translocation.

Most importantly, as illustrated in Figure 3.1A, the identification of the unknown host receptors exploited by *H.pylori* for CagA translocation is important. However, the search for the binding partner(s) for the *H.pylori* T4SS is complicated, the future work should

be performed in a more cooperative way to reveal the molecular mechanisms underlying the host–pathogen interaction and CagA translocation.

4 Materials and Methods

4.1 Materials

4.1.1 Bacterial strains

4.1.1.1 *Helicobacter pylori* strains

Table 4.1 *H. pylori* strains used in this study

H. pylori strains	Description
P12	The Clinical isolate from the University of Hamburg (isolate number: 888-0) [212]
G27	The clinical isolate from an endoscopy patient in Tuscany, Italy [213]
P12Δ*hopQ*	*hopQ* gene knockout mutant in P12 strain
P12Δ*cagI*	*cagI* gene knockout mutant in P12 strain
P12[TEM-CagA]	CagA fusion protein fused with β-lactamase TEM-1 in P12 strain
P12Δ*cagI*[TEM-CagA]	*cagI* gene deletion mutant in P12[TEM-CagA]
P12Δ*hopQ*[TEM-CagA]	*hopQ* gene deletion mutant in P12[TEM-CagA]

4.1.1.2 *Escherichia coli* strains

Table 4.2 *E.coli* strains used in this study

E.coli strains	Genotypes

DH 5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA–*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*–1 *gyr*A96 *rel*A1
One shot® Top 10	F– *mcr*A Δ(*mrr*–*hsd*RMS–*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
One shot® Stbl3	F^- *mcr*B *mrrhsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*–14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*–5 λ^- *leumtl*–1

4.1.2 Plasmids

Table 4.3 Plasmids created in this study

Plasmid	Genotype/description
PX462	pSp–Cas9n(BB)–2A–Puro (Addgene ID: 48140)
pZQ30	PX462 + 8081 (CRISPR constructs targeted on integrin β1 *exon 5*)
pZQ31	PX462 + 8283 (CRISPR constructs targeted on integrin β1 *exon 5*)
pZQ32	PX462 + 9596 (CRISPR constructs targeted on integrin αv *exon 4*)
pZQ33	PX462 + 9798 (CRISPR constructs targeted on integrin αv *exon 4*)
pZQ34	PX462 + 12 (CRISPR constructs targeted on integrin β4 *exon 6*)
pZQ35	PX462 + 34 (CRISPR constructs targeted on integrin β4 *exon 6*)

4.1.3 Oligonucleotides

Table 4.4 Oligonucleotides used in this study

Name	Sequence (5' to 3')	Description

ZQ66	GAGGGCCTATTTCCCATGATTCC	U6 forward primer, for sequencing of sgRNA constructs
ZQ80	CACCGAGTTCTGTTCACTTGTGCAA	top oligo for targeting integrin β1 exon 5 (guide A)
ZQ81	AAACTTGCACAAGTGAACAGAACTC	bottom oligo for targeting integrin β1 exon 5 (guide A)
ZQ82	CACCGGTGCTCAGTCTTACTAATAA	top oligo for targeting integrin β1 exon 5 (guide B)
ZQ83	AAACTTATTAGTAAGACTGAGCACC	bottom oligo for targeting integrin β1 exon 5 (guide B)
ZQ93	CACCGGTGCAGTCCGAGTTGCTAAT	top oligo for targeting integrin αV exon 5
ZQ94	AAACATTAGCAACTCGGACTGCACC	bottom oligo for targeting integrin αV exon 5
ZQ95	CACCGCAGTTCTCCAATGGTACAAT	top oligo for targeting integrin αV exon 4 (guide A)
ZQ96	AAACATTGTACCATTGGAGAACTGC	bottom oligo for targeting integrin αV exon 4 (guide A)
ZQ97	CACCTAAACAGGAGCGAGAGCCTGT	top oligo for targeting integrin αV exon 4 (guide B)
ZQ98	AAACACAGGCTCTCGCTCCTGTTTC	bottom oligo for targeting integrin αV exon 4 (guide B)
CU1	CACCGAAATCCAATAGTGTAGTCGC	top oligo for targeting integrin β4 exon 6 (guide A)
CU2	AAACGCGACTACACTATTGGATTTC	bottom oligo for targeting integrin β4 exon 6 (guide A)

CU3	CACCGGCGTCCCGCAGACGGACATG	top oligo for targeting integrin β4 exon 6 (guide B)
CU4	AAACCATGTCCGTCTGCGGGACGCC	bottom oligo for targeting integrin β4 exon 6 (guide B)

4.1.4 Antibodies

4.1.4.1 Primary antibodies

Table 4.5 Primary antibodies used in this study

Name	Specificity	Origin	Supplier
Anti-phosphotyrosine antibody (clone 4G10)	phosphotyrosine-containing proteins	mouse	Millipore
Anti-phosphotyrosine antibody (PY99)	phosphotyrosine-containing proteins	mouse	Santa Cruz Biotechnology
Anti-integrin β1 (clone LM534)	human integrin β1 (aa 588-706)	mouse	Chemicon
Anti-CD29,FITC (clone Ha2/5)	extracellular domain of human integrin β1	hamster	BD Biosciences
Anti-β1 chicken Integrin (Clone W1B10)	extracellular part of the chicken integrin β1	mouse	Sigma
Anti-Integrin α1 antibody (Clone FB12)	human Integrin α1	mouse	Chemicon
Anti-Integrin α2 antibody (clone P1E6)	human Integrin α2	mouse	Chemicon
Anti-Integrin α3 antibody (clone P1B5)	human Integrin α3	mouse	Chemicon
Anti-Integrin α4 antibody (clone P1H4)	human Integrin α4	mouse	Chemicon

Anti-Integrin α5 antibody (clone P1D6)	human Integrin α5	mouse	Chemicon
Anti-Integrin α6 antibody (clone NKI-GoH3)	human Integrin α6	mouse	Chemicon
Anti-Integrin αV antibody (clone P2W7)	human Integrin αv	mouse	Santa Cruz Biotechnology
Anti-integrin β4antibody (clone 439-9B)	human integrin β4	rat	Abcam
Anti-CEACAM1 antibody (clone 8G5)	human CEACAM1	mouse	Genovac
Anti-CEACAM5 antibody (clone 26/3/13)	human CEACAM5	mouse	Genovac
Anti-CEACAM6 antibody (clone 9A6)	human CEACAM6	mouse	Genovac

4.1.4.2 Secondary antibodies

Table 4.6 secondary antibodies used in this study

Name	Specificity	Origen	Supplier
Anti-mouse POX (horseradish peroxidase)	mouse IgG	Goat	Sigma
Anti-rabbit POX	rabbit IgG	Goat	Sigma
Anti-mouse-Alexa488	mouse IgG	Goat	life technologies
Anti-rat-Alexa488	rat IgG	Goat	life technologies
Anti-rabbit-Alexa488	rabbit IgG	Goat	life technologies

4.1.5 Cell lines

Table 4.7 Cell lines used in this study

Name	Description
AGS	Human Caucasian gastric adenocarcinoma (ATCC® CRL-1739™)
KatoIII	Human stomach cancer cell line derived from metastatic site (ATCC® HTB-103™)

Table 4.8 Stable cell lines created in this study

Name	Description
ITGB1 KO AGS	Integrin β1 knockout AGS cell line with depletion of all 12 potential β1 integrins expression on the cell surface
ITGAv KO AGS	Integrin αv knockout AGS cell line with depletion of all 5 potential αv integrins expression on the cell surface
ITGB4 KO AGS	Integrin β4 knockout AGS cell line with depletion of integrin α6β4 expression on the cell surface
ITGB1B4 KO AGS	Integrin β1 and β4 knockout AGS cell line with depletion of all 12 potential β1 integrins and integrin α6β4 expression on the cell surface
ITGAvB4 KO AGS	Integrin αv and β4 knockout AGS cell line with depletion of all 5 potential αv integrins and integrin α6β4 expression on the cell surface
ITGB1 KO KatoIII	Integrin β1 knockout KatoIII cell line with depletion of all 12 potential β1 integrins expression on the cell surface
ITGAv KO KatoIII	Integrin αv knockout KatoIII cell line with depletion of all 5 potential αv integrins expression on the cell surface
ITGB4 KO KatoIII	Integrin β4 knockout KatoIII cell line with depletion of integrin α6β4 expression on the cell surface
ITGB1B4 KO KatoIII	Integrin β1 and β4 knockout KatoIII cell line with depletion of all 12 potential β1 integrins and integrin α6β4 expression on the cell surface
ITGAvB4 KO KatoIII	Integrin αv and β4 knockout KatoIII cell line with depletion of all 5 potential αv integrins and integrin α6β4 expression on the cell surface
ITGAvB1 KO KatoIII	Integrin αv and β1 knockout KatoIII cell line with depletion of all 12 potential β1 integrins and 5 potential αv integrins expression on the cell surface

ITGAvB1B4 KO KatoIII	Integrin αv, β1 and β4 knockout KatoIII cell line with depletion of all possible integrins on KatoIII cell surface

4.1.6 Enzymes and proteins

Table 4.9 Enzymes and proteins used in this study

Name	supplier
T4 polynucleotide kinase	New England Biolabs
PlasmidSafe ATP-dependent Dnase	Epicentre
T4 DNA ligase	New England Biolabs
High Fidelity PCR Enzyme Mix	Life technologies
Ex Taq DNA Polymerase	Takara
LA Taq DNA Polymerase	Takara
PageRuler Plus Prestained Protein Ladder	Pierce
Rabbit IgG	Pierce
restriction enzymes	NEB/Roche/Fermantas

4.1.7 Standard buffers

Table 4.10 Standard buffers used in this study

Buffer	Composition
PBS	137mM NaCl, 2.7mMKCl, 10mMNa2*HP*O4, 1.8mM KH2PO4, 1mM CaCl2, 0.5mM MgCl2, pH 7.4
TBS	50mM Tris-HCl pH 7.4, 150mM NaCl

50X TAE buffer	242 g/l Tris Base, 57,1 ml/l Glacial Acetic Acid, 50 mM EDTA
PFA 10X	Paraformaldehyde 27%, PBS 10X, pH 7,4
Anode I buffer	300 mM Tris-HCl pH 10.4, 10% methonal
Anode II buffer	25 mM Tris-HCl pH 10.4, 10% methonal
Cathode buffer	25 mM Tris-HCl pH 9.6, 40mM 6-amino caproic acid, 10% methonal
2X SDS loading buffer	100 mM Tris HCl pH 6,8; 4% SDS; 0,2% Bromophenol blue, 20% Glycerol, 10% β-Mercaptoethanol (optional)
5X SDS loading buffer	10% SDS; 0,5 M Tris HCl (pH 6,8); 50% Glycerol, 5% Bromophenol blue. Store at room temperature.
GEBS (Agarose Loading buffer 6X)	0,25% Bromophenolblue; 0,25% Xylene Cyanol FF, 30% Glycerol; in TAE buffer

4.1.8 Growth medium, antibiotics, reagents, supplements

Table 4.11 Growth medium, antibiotics, reagents and supplements used in this study

Name	supplier
Dulbecco's PBS (DPBS)	Life technologies
Opti-MEM I reduced-serum medium	Life technologies
RPMI 1640	Life technologies
DMEM hign glucose	Life technologies
L-Glutamine	Invitrogen

Fetal Calf Serum(FCS)	Invitrogen
Trypsin-EDTA (TE)	Invitrogen
Penicillin-streptomycin, 100×	Life technologies
Gentamycin	Invitrogen
Puromycin dihydrochloride	Life technologies
Hygromycin	PAA laboratories
G418	Invitrogen
Lipofectamine 2000	Life technologies

4.1.9 Commercial kits

Table 4.12 Commercial kits used in this study

Commercial kit	Supplier
SURVEYOR mutation detection kit	Transgenomic
Amaxa cell line Nucleofector Kit V	Lonza
TOPO TA Cloning kit	Life technologies
QIAamp DNA mini kit	Qiagen
QIAprep spin miniprep kit	Qiagen
QIAprep spin midiprep kit	Qiagen

illustra GFX PCR DNA and gel band purification kit	GE Healthcare
Gateway® BP Clonase® II Enzyme mix	Life technologies
Gateway® LR Clonase® Enzyme mix	Life technologies

4.2 Methods

4.2.1 Working with *H.pylori*

4.2.1.1 *H.pylori* cultivation and maintenance

H. pylori are capable of growing when cultured on nutrient-rich agars or medium. In this study, serum plates and liquid BB medium were used for cultivation. For the serum plates, GC agar base was autoclaved and supplemented with horse serum, nystatin, trimethoprim, Cholesterol, vitamin mix and/or appropriate antibiotics, while liquid BB medium were autoclaved before adding supplements as same as for the serum plates. The respective concentration of the supplements is listed below.

H. pylori strains were defrosted from -70ºC and plated on serum plates. The plates were inverted and incubated at 37 ºC under micro aerobic conditions (85% N_2, 10% CO_2 and 5% O_2) for three days. From the fourth day on, the bacteria were passaged everyday on new and fresh plates and 2 passages were needed before infection experiments. After 6 passages, the bacteria plates were autoclaved and discarded. *H. pylori* liquid culture was made by inoculating bacteria from plate to 30-50ml BB medium and shaking gently under microaerobic condition at 37ºC, 90rpm.

4.2.1.2 Generation of *H.pylori* frozen stocks

Freshly grown *H. pylori* after the second passage were collected by sterile cotton swabs and resuspended in sterile filtrated BB medium with 10% FCS and 25% glycerol. Stocks were kept at -70ºC for the long term storage.

4.2.2 Working with *Escherichia coli*

4.2.2.1 *E.coli* cultivation and maintenance

E.coli is cultivated either on Luria-Bertani (LB) plates or LB liquid medium with appropriate antibiotics. From frozen stocks, *E.coli* were streaked out on LB plates and incubated at 37°C for over night. For liquid culture, single colony from plate was inoculated to 100ml LB broth with shaking at 200 rpm at 37°C.

4.2.2.2 Plasmid extraction from *E.coli*

Plasmids were extracted from DH5α or Top10 with the QIAprep Spin Miniprep kit (Qiagen) following the Qiaprep users handbook. Instead of preparing plamid from liquid culture, bacteria grown on LB plates were resuspended in PBS to start the protocol. Concentration of the plasmid DNA was quantified by Nanodrop ND-1000 spectrometer (Peylab).

4.2.2.3 Restriction digestion

Vectors and PCR amplicons were treated with according restriction enzymes before ligation. The actual reaction conditions vary from one enzyme to the next, from incubation times, buffers to temperature, etc. Generally, total volume of 10μl was prepared for each reaction including 300ng to 500ng DNA, 0.5U/μl enzyme and corresponding buffer provided by the manufacture. The reaction usually incubated at 37°C for 1h.

4.2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method of separating DNA molecules by length in base pairs for demands of DNA visualization and purification. Negative charged DNA can move in an electoral field from negative electrode to positive electrode. The agarose matrix provided different migration rate for short DNA and long DNA. Typically, DNA is mixed with GEBS loading buffer before pipetting to 1% - 2%

agarose gel which soaked in the TAE buffer. 80V of voltage was applied until the dye line is 2/3 of the way to the bottom.

4.2.2.5 Ligation

Digested vectors and insert DNA were ligated by T4 DNA ligase (NEB) at 16 °C for 4h or at room temperature for 1h. For efficient ligation, the molar ratio of the insert and vector was from 3:1 to 5:1. Generally, 5U of ligase was used for the overall concentration of 3–8μg/ml of vector and insert in a total reaction volume of 10μl.

4.2.2.6 Transformation

Transformation can allow the introduction of foreign DNA into a cell for plasmids storage and replication. Briefly, 10ng to 100ng ligated DNA was added to 20μl ice cold competent cells. After 10 min incubation on ice, the mixture was heat shocked at 42°C for 30s, followed by immediately returning back to ice for 10min. 200μl LB medium was added to the mixture for the outgrowth of the competent cells at 37°C for 1h. Cells were then plated on LB plate with appropriate antibiotics depending on the plasmid being transformed.

4.2.2.7 TOPO TA cloning

TOPO TA cloning provides an efficient method to directly clone PCR fragment into TOPO vector without purification of the PCR product and ligation. For each sample, 50ng fresh PCR product and 1μl salt solution provided by the kit were mixed. Water was added to each mixture to a total volume of 5μl. 1μl of the provided Topo vector was then added to the mixture with thorough mixing after addition. Each reaction was then incubated at room temperature for 5min followed by placing the reaction on ice. 2μl of the reaction were directly transformed to Top 10 competent cells by heat shock. After the outgrowth, 20μl and 100μl of each transformation were plated on ampicillin or kanamycin LB plates.

4.2.3 Working with the CRISPR-Cas system

4.2.3.1 Design of short guide RNA

Design of short guide RNA (sgRNA) for gene targeting was accomplished in an online design tool http://toolsgenome-engineering.org developed by Feng Zhang's lab in Broad Institute of Massachusetts Institute of Technology and Harvard. Firstly, a 23 to 250bp genome fragment from the target region was input to the design tool. After computationally analysis, suitable targets were identified and listed with ranking and scores, according to the prediction of their off-target potential. Usually, the input region of interest should be selected from promoter region or early exons of the target gene. Designed sgRNA were modified as described in Figure 2.4 and ordered as oligos commercially. It is worth noting that although unusual, certain sgRNAs could not properly work as expected for reasons yet unknown [214] . Therefore, in order to obtain functional sgRNA, more than one (pair) sgRNAs were designed for each target gene and their efficiencies were tested in the intended cell line(s).

4.2.3.2 CRISPR constructs preparation

4.2.3.2.1 sgRNA oligos annealing

In order to acquire sgRNA expressing constructs, top and bottom oligos were annealed before cloning (Figure 2.3.B). For each reaction, 100μM top oligo, 100μM bottom oligo and 2μl 10×T4 ligation buffer were mixed thoroughly. Water was added to each mixture to a total volume of 20μl. Each reaction was incubated in a heating block at 95°C for 5min followed by placing the block in a cooling room (4-8°C) to allow the temperature ramping down naturally. The total annealing time took around 90min.

4.2.3.2.2 Cloning of sgRNA inserts into CRISPR vector.

CRISPR vectors used in this study is the Cas9 nickase vector (pSpCas9n(BB)-2A-Puro, PX462). The vectors contained the conserve region of the remained sgRNA scaffold, *S. pyogenes* Cas9 nickase, along with the 2A-Puro for transfection selection. Cloning started with restriction digestion of the vector. 1μg PX462 were mixed with 1μl FastDigest BpiI (Thermo Fischer Scientific) and its 10×FastDigest buffer. Water was

added to the mixture to reach volume of 20µl in total. Digestion mixtures were incubated at 37°C for 1h. Ligation of vectors with inserts was followed immediately, by adding following ligation components directly into digestion reaction: 2.5µl 10XT4 Ligation buffer (NEB), 0.4µM annealed oligo, and 1.5µl T4 DNA ligase (NEB). After incubation at 37ºC for 1h, 2µl of the ligation reaction were directly transformed to 20µl ice cold Stbl3 competent cells by heat shock. After the outgrowth, 100µl of each transformation were plated on ampicillin LB plates. Colony growth was inspected the next day; two to three colonies from each transformation were restreaked for sequence verification from the U6 promoter on the vector using U6-Fwd primer (section 4.1.3, ZQ66).

4.2.3.3 Functional validation of sgRNAs

Functional validation of sgRNAs was completed by transfection of sgRNA expressing constructs into target cell lines, followed by verification of genome modification from transfected population. In this study, host cell surface expressing integrins are the targets of CRISPR-Cas9 mediated gene knockout. Therefore, verification of genome modification can be accomplished by flow cytometry surface integrin detection. If the transfected population showed two distinct populations with different phenotypes, in this case, one of the population with a specific integrin expression and the other without, sgRNA(s) were considered valid and efficient.

4.2.3.3.1 Cultivation and maintenance of cells.

Cells are generally cultured in RPMI 1640 or DMEM supplemented with 10% (vol/vol) FCS at 37°C and 5% CO_2. To passage, the medium were removed, and the cells were washed once with DPBS gently. To detach cells, 2ml Trypin-EDTA was added to a 75cm^2 flask for 3 to 5min incubation at 37°C. When detachment was observed under microscope, 8ml of the pre-warmed RPMI1640 medium were added to neutralize the trypsin. After being gently pipetted up and down, cells were dissociated and were then reseeded into new flasks. Passages taken place in every 2-3 days with a split ratio of 1:5 or 1:8. Cells were discarded when the passage number reached 80.

4.2.3.3.2 Transfection of adherent/semi-adherent cells by Lipofection

Generally, one day before transfection, 0.5 x 10^5 to 2 x 10^5 cells were plated in 500µl of growth medium without antibiotics in a 24-well plate so that they would be 90-95% confluent at the time of transfection. For each transfection sample, 500µg DNA was diluted in 50µl of Opti-MEM® I Reduced Serum Medium without serum and mixed gently. Meanwhile, 2µl lipofectamine 2000 was diluted in 50µl of Opti-MEM® I Medium. After 5min incubation at room temperature, the diluted DNA was combined with the diluted Lipofectamine 2000 (total volume is 100µl). After gently mix, the mixture was incubated for 20min at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. Add the 100µl of DNA-Lipofectamine 2000 complexes to each well containing cells and medium. To ensure thorough mixing, the plate was rocked back and forth. The cells were incubated at 37°C in a CO_2 incubator for 24-48 hours until they were ready to analyse for transgene expression. Usually, it is necessary to remove the complexes or change the medium 4 to 6 hours after transfection to improve the efficiency. AGS cells were different, and the medium should be changed 45 min after transfection.

4.2.3.3.3 Detection of proteins on cell surface by flow cytometry

For adherent cells, cells were firstly detached by washing once with DPBS and incubated with trypsin-EDTA for 2 to 6min. Trypsinized cells were then neutralized by growth medium and 1×10^6 cells were taken in ice cold FACS buffer (DPBS containing 5% FCS and 0.1% sodium azide). For suspension cells, the cell suspension was adjusted in a concentration of $1\times10^6 ml^{-1}$. Around 2×10^5 cells were add to each well with round bottom of a 96-well plate and centrifuge at 4°C of maximum 300×g for 5 min. Primary antibodies with the concentration of 0.1 to 10µg ml^{-1} were added to each well. Dilutions of antibody, if necessary, were made in FACS buffer. Cells and antibodies were incubated at 4°C for 1 hour in the dark. Primary antibody stained cells were washed 3 times by centrifugation at 200-300×g for 5 minutes and were resuspended in 200µl to 1ml of ice cold FACS buffer. Subsequently, fluorochrome-labeled secondary antibodies were diluted in FACS buffer at the optimal concentration (according to the manufacture's instructions) and were added to each well, followed by 3 times washing as described above. Cells were analyzed by the flow cytometer right

after washing or kept in the dark on ice or at 4°C in a fridge until the scheduled time for analysis.

4.2.3.4 Generation of clonal gene-knockout cell lines

4.2.3.4.1 FACS sorting for desired population

Generation of clonal cell lines started from FACS sorting for integrin negative population from transfected cells. After sorting, most of the cells with undesired phenotype were removed, in a way to markedly simplify the time- and labor-consuming selection works. Each transfected population was stained with specific antibodies for sorting preparation, and the FACS sorting experiments were conducted in Microbiology Department of Technical university of Munich. After sorting, cells were cultured in the presence of penicillin and streptomycin for one or two weeks until they reached 1×10^6 for long term storage by freezing in liquid nitrogen.

4.2.3.4.2 Isolation of clonal cell lines

Clonal cell lines which arose from single knockout cells were obtained by performing serial dilutions from sorted population. Sorted cells were detached and dissociated by pipetting up and down carefully to prevent clumping. Afterwards, cell number was determined by counting with a hemocytometer. In order to dilute the cells in a final concentration of 1 cell per well in a 96-well plate, 100 cells were resuspended in 22 ml complete medium and 200 µl diluted cells were added to each well with multichannel pipette. At least two 96-well plates were plated for each sorted population. One to two weeks later, colonies in each well were inspected with the microscope, and those wells with more than one colony were marked off. Plates were returned to the incubator to allow them to grow for another 1 to 2 weeks. The wells with single clones were marked and expanded to 48- well plates, then 24-well plates, then 6-well plates and finally $25cm^2$ flasks for examine and freezing. For each cell line, four to five single clones were selected and examined for specific surface integrin expression by flow cytometry. For each cell line, only one of the single clones which showed complete knockout phenotype was kept for further culture and investigation.

4.2.4 Working with proteins

4.2.4.1 SDS-PAGE

SDS-PAGE, with full name of sodium dodecyl sulfate polyacrylamide gel electrophoresis, is the most widely used technique to separate proteins according to their molecular weight. Protein samples were collected from transfected or infected cells followed by denaturing at 95°C for 10 min and loaded on to a separated gel with 6% to 12% polyacrylamide percentage separating gel containing a 5% stacking gel. After loading, the gel was placed in BioRad electrophoresis chamber filled with SDS running buffer. An electrical current was applied to allow the negative charged proteins moving toward the positive electrode. Generally, 80 voltage was applied for 30 min to allow the proteins to go through the stacking gel, and the voltage was then raised to 120μl for 60-90 min. Gels were further used for coomassie staining or transfer to PVDF membranes.

4.2.4.2 Transfer

Proteins with an electoral charge provided by the SDS bounded to them have the ability to migrate through the gels into an electoral field. Therefore, transfer of proteins from polyacrylamide gels to PVDF membranes was achieved in semi-dry electric transfer chamber (Biotech-Fisher). Briefly, the membrane (closed to positive electrode) and the gel (closed to negative electrode) were together in between filter papers which were previously soaked with Anode I, II or cathode buffers. The current of the electoral transfer was maintained at 0.8 mA per 1 cm^2 of the gel area for 75 min.

4.2.4.3 Western blot

Western blot becomes routine in most biochemistry and biology lab. After separating proteins by SDS-PAGE and transferring proteins from the gel to PVDF membranes, proteins on the membrane were probed by appropriate antibodies for the demand of visualization. Briefly, the membrane was activated by methanol before being blocked with 5% skim milk for 2 hours at room temperature. Blots were then incubated with

primary antibody with appropriate dilution in 1% slim milk at 4 °C overnight. After proper wash with TBS-Tween (TBS-T, 0.075%) for 3 times, blots were incubated with secondary antibody with appropriate dilution in 1% slim milk at room temperature. After carefully wash with TBS-T for 4 to 5 times, blots were developed by ECL Western Blot Developer. Development was done either digitally by Gel Doc (Biorad) or by X-ray film developer. Blots were incubated with stripping buffer for 30min after development to remove the antibodies bounded to the membrane. After 3 times wash, Blots were then dried and reactivated for next round of immunoblotting.

4.2.4.4 Dot blot

A dot blot is a fast and easy way to determine the presence and effectiveness of the target protein in samples without separating electrophoretically by SDS-PAGE. To start, a PVDF membrane was prepared and activated in methanol for 5 sec and kept wet in TBS. The total volume of 2 to 5μl sample (10-50ng) was spotted onto the membrane by using a pippet tip. After totally dried, the membrane was then briefly activated again by methanol before being blocked in 3% BSA in TBS-T for 1 h in room temperature. The subsequent procedures like incubation with primary antibody and secondary antibody as well as development were the same as described in western blot.

4.2.4.5 Infection of eukaryotic cells with *H.pylori* and CagA phosphotyrosine assay

Two days before infection, 2.5×10^5 cells were seeded in each well of a 6-well plate to reach the confluence of 70-90% on the day of infection. *H.pylori* strains on agar plates which were around 24 hours old were resuspended in DPBS using cotton swabs. After measuring the OD_{550}, cells were infected using a Multiplicity Of Infection (MOI) of 60 (approx. 60 bacteria per cell) for 4 hours at 37°C and 5% CO_2. The infection was stopped by placing the plates on ice. The supernatants containing bacteria were discarded and autoclaved. The infected cells were washed twice with cold PBS to remove swimming bacteria. Subsequently, cells were collected carefully by cell scrapers after the addition of 1ml fresh made PBS*. After gentle centrifugation at 500×g for 10 min in 4°C, cell pellets were concentrated in small amount of PBS* solution containing 2×SDS loading buffer (1:1 ratio). After boiling at 95°C for 10min, cell samples were stored at -20°C for later immunoblotting detection of phosphorylated CagA protein.

Usually, a mouse polyclonal antibody PY99 or a mouse monoclonal antibody 4G10 was used to detect phosphorylated tyrosine in our lab.

PBS*: PBS with the addition of different proteinase inhibtors as follows: 1mM sodium vanadate, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin

4.2.4.6 Quantification of CagA translcaotion with plate-reader detection

One day before infection, adherent cells were detached and 2.5×10^4 cells were seeded in each well in a 96-well plate with black wall and transparent bottom with low fluorescence background (Fortitude). The confluence of the cells was 80% to 90% on the day of infection. Before infection, *H.pylori* strains with fusion protein of beta-lactamase TEM-1 and CagA were collected as described before. Ideally, bacteria were resuspended and pre-incubated in sterile PBS containing 10% FCS at 37°C, 10% CO_2 for 1.5h. Subsequently, cells were infected by bacteria with the MOI of 60 for 4 h at 37° C, 5% CO_2 as described above. Shortly before the infections were done, beta-lactamase substrate was prepared by mixing the fluorescent substrate CCF4-AM with solution B, solution C and PBS according to the manufacture's instruction (Live BLAzer™ FRET - B/G loading Kit, Invitrogen). Infections were stopped by placing the plates on ice and all the supernatants were removed. Prepared substrates mix was loaded immediately on the cell surface, followed by incubation at room temperature for 120min in dark. Before going to the plate reader, dust was removed from the bottom of the plate to make sure the accuracy. Plate reader filters were set to allow excitation of wavelength around 410nm, and detection of blue emission around 450nm and green emission around 520nm. After reading, data were normalized and analyzed following manufacture's instruction to obtain the blue to green fluorescence ratio.

4.2.4.7 Quantification of CagA translocation with flow-cytometry detection

For suspension and semi-adherent cell lines, CagA translocation is possible to be detected by flow cytometry as an alternative. The method of CagA translocation assay with flow-cytometry detection is very similar to the plate-reader detection except following procedures. Firstly, semi-adherent cells were detached after infection with

room-temperature trypsin-EDTA before incubation with CCF4-AM fluorescence substrate mix. Secondly, incubation of cells with CCF4-AM mix were implemented at 27 ˚C with constant shaking condition to allow even loading of cells with substrate and avoid cell sedimentation. Last but not the least; cells were washed at least 2 times with PBS by centrifugation at 200-300×g for 5 minutes after incubation with CCF4-AM substrate. Cells were then analyzed by flow cytometry for pacific blue fluorescence and amCyan green fluorescence.

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Qing

Curriculum Vitae

Personal data

Ms. Qing Zhao

Born in 18.01.1986, Qinghai province, P.R.China

Education

11/2011–present: Ludwig Maximilian University of Munich (LMU), Munich, Germany

- ✧ Ph.D, Bacteriology

09/2008–07/2011: Chinese Academy of Agricultural Sciences (CAAS), Beijing, China

- ✧ MS, Preventive Veterinary Medicine
- ✧ GPA: 3.2/4.0

09/2004–07/2008: Northwest Agriculture & Forest University (A&F), Shaanxi, China

- ✧ BS, Veterinary Medicine
- ✧ Ranking: graduated with the marks that ranked at top 8% (14/180) in my major.

Research Experience

11/2011– present

Bacteriology department, Max von Pettenkofer Institute

- ✧ Main focus: *Helicobacter pylori* host–pathogen interaction

09/2010–07/2011

National Foot–and–Mouth Disease Reference Laboratory, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute.

- ✧ Thesis: A novel T–cell immunogen enhances the immune responses for inactivated vaccine and subunit vaccine of foot–and–mouth disease virus in mice.

Publications

Verena Königer, Lea Holsten, Eva Loell, Benjamin Busch, Ute Breithaupt, Qing Zhao, Daniel A. Bonsor, Alexandra Roth, Arnaud Kengmo-Tchoupa, Stella I. Smith, Susanna Mueller, Eric J. Sundberg, Wolfgang Zimmermann, Wolfgang Fischer, Christof R. Hauck and Rainer Haas: *Helicobacter pylori* exploits human CEACAMs for adherence and translocation of CagA. *Nature Microbiology* 2016, 2:16188.

Professional seminars

"Immune cells, vessels and beyond" annual retreat, 2016, Kloster Schöntal, Germany

10th *Helicobacter pylori* workshop, 2015, Herrsching, Germany

67.Jahrestagung der Deutschen Gesellschaft für Hygien und Mikrobiologie (DGHM) e.V., 2015, Münster, Germany

9th *Helicobacter pylori* workshop, 2013, Kloster Wennigsen, Germany