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Studies on the role of the CagY protein of the type IV secretion system of *Helicobacter pylori* for the production of cytokines by host cells

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Content

List of Figures

List of Abbreviation

ASA ..... acetylsalicylic acid
ATPase ..... adenosinetriphosphatase
BabA ..... blood group antigen binding adhesion
bp ..... base pairs
CagA ..... cytotoxin-associated gene A
CagC ..... cytotoxin-associated gene C
CagL ..... cytotoxin-associated gene L
cagPAI ..... cag pathogenicity island
CagT ..... cytotoxin-associated gene T
CagX ..... cytotoxin-associated gene X
CagY ..... cytotoxin-associated gene Y
cDNA ..... complementary DNA
CEA ..... carcinoembryonic-antigen
CEACAM ..... carcinoembryonic antigen-related cell adhesion molecule
chrDNA ..... chromosomal DNA
ComB ..... DNA uptake B
CXCL ..... chemokine (C-X-C motif) ligand
DMSO ..... dimethyl sulfoxide
DNA ..... deoxyribonucleic acid
dNTPs ..... nucleoside triphosphate
EBV ..... Epstein-Barr-Virus
EDTA ..... ethylenediaminetetraacetic acid
EYFP ..... Enhanced Yellow Fluorescent Protein
FCS ..... fetal calf serum
H. pylori ..... Helicobacter pylori
H_2SO_4 ..... sulfuric acid
HCl ..... hydrogen chloride
HEK CC1 ..... Human embryonic kidney Ceacam 1
HEK CC5 ..... Human embryonic kidney Ceacam 5
HEK 293 ..... Human embryonic kidney 293
Hop Q ..... Helicobacter outer membrane protein Q
Hop ..... Helicobacter outer membrane protein
IFN ..... interferon
IFNβ ..... Interferon β

IFNγ ..... interferon γ
IKK ..... inhibitor of nuclear factor kappa-B kinase subunit
IKKε ..... IκB kinase-ε
IL-1β ..... interleukin 1β
IL-8 ..... interleukin 8
IRF7 ..... Interferon regulatory factor 7
ISFG3 ..... interferon-stimulated gene factor 3
IκB ..... inhibitor of nuclear factor kappa B
kbp ..... kilo base pairs
LMP1 ..... Late Membrane Protein 1
LPS ..... liposaccharide
MALT ..... mucosa-associated lymphoid tissue
MAP ..... mitogen-activated protein
$MgCl_2$ ..... magnesium chloride
NF-κB ..... nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIK ..... NF-κB inducing kinase
NOD1 ..... nucleotide-binding oligomerization domain-containing protein 1
NSAID ..... non-steroidal anti-inflammatory drug
PBS ..... phosphate-buffered saline
PCR ..... polymerase chain reaction
pDNA ..... plasmid DNA
PFA ..... paraformaldehyde
PGN ..... peptidoglycan
PI ..... propidium iodide
PMSF ..... phenylmethylsulfonyl fluoride
PPI ..... proton-pump inhibitor
PRM ..... pathogen-recognition molecules
PRR ..... pathogen-associated molecular pattern
RIP2 ..... receptor-interacting-serine/threonine-protein kinase 2
RNA ..... ribonucleic acid
Stat ..... signal transducer and activator of transcription 2
T4SS ..... type 4 secretion system
TBK1 ..... TANK-binding kinase 1
TEMED ..... tetramethylethylenediamine
TES ..... transformation effector site

Th1-cells ..... T helper 1 cells
TNFα .....tumor necrosis factor α
TRAF3 .....TNF receptor associated factor 3
TRL .....Toll-like-receptor
VacA .....vacuolating cytotoxin A

Abstract

Helicobacter pylori is a gram-negative bacterium populating the human stomach. Nearly 50% of the world's population is infected. It is known to be a major cause of medically relevant gastroduodenal diseases like peptic ulceration and chronic inflammation that can lead to gastric cancer.
The high pathogenicity is caused by the high number of virulence factors that are important for colonization and infection of the gastric mucosa. One of the best known pathogenic mechanisms is the type IV secretion system (T4SS), a pore-like structure that is needed for the bacterial oncogene CagA translocation leading to different effects on cellular signaling cascades. T4SS is able to induce the production of several cytokines responsible for the recruitment of immune cells and, probably, chronic inflammation. Proteins building the T4SS are encoded in the *cag*-pathogenicity island (*cagPAI*), a *Helicobacter pylori*-specific DNA region.
The VirB10 homologue CagY is a very important component of the T4SS. The exact function of the CagY protein in *Helicobacter pylori*'s pathogenicity is not fully clear. The protein structure is quite unique. The two sequences encoding the Repeat Regions are very variable and show no similarity to any known protein. One exception might be the Late Membrane Protein (LMP1) of the Human Herpes Virus 4 (HHP4) or Epstein-Barr-Virus, that is known for interaction with the transcription factor IRF7 in virus infections.

In this work, the role of CagY in stimulation of cytokine secretion by activation of the signal cascades Interferon and NF-κB in host cells was investigated. A T4SS-dependent activation of IFNβ- and the NF-κB-promoters E-selectin, was proven. Infection of HEK 293-, HEK CC1- and HEK CC5-cells with *Helicobacter pylori* wildtype strains and mutants with knockout of different T4SS-components caused different levels of promoter activation. Based on former experiments in the work group, a suggested interaction between CagY and IRF7 was examined. For further examination of the suggested direct interaction, a stable transfected cell line stably expressing the fusion protein EYFP-IRF7 was established.

Unexpectedly, this cell line lost its function, since the fusion protein was not expressed anymore. Sequencing of reisolated DNA from the cell line did not identify a reason for the loss of function, such as e.g. a mutation in the protein encoding gene sequence. Furthermore, a possible CagY-dependent translocation of IRF7 should be studied. For visualization under confocal microscopy and for best possible evaluation conditions, a staining method was established in this work. Different cell lines were first transfected with the self-constructed plasmid pEYFP-IRF7 and thereafter infected with *Helicobacter pylori* wildtype strains and mutants. Due to the ambivalent results, neither a confirmation, nor an exclusion of CagY-dependent IRF7-translocation into the nucleus was possible.
Due to the highly repetitive special gene structure of *cagY*, its correct amplification via PCR was very difficult. Therefore, no functional fusion proteins could be generated to prove direct protein-protein-interaction between CagY and IRF7.

Zusammenfassung

Helicobacter pylori ist ein gram-negatives Bakterium, das den menschlichen Magen infiziert. Die Durchseuchung in der Weltbevölkerung liegt bei etwa 50%. Das Bakterium ist als eines der wichtigsten Ursachen für gastrointestinale Krankheiten bekannt, wie Magenulzera und chronische Entzündung der Magenschleimhaut, welche zum Magenzellkarzinom führen können. *Helicobacter pylori* gilt somit als wichtiger Risikofaktor für Adenokarzinome im Magen. Die Infektion mit diesem Bakterium ist auch mit vielen extra- intestinalen Krankheiten, wie dem MALT- Lymphom, assoziiert.

Die hohe Pathogenität liegt an der Vielzahl an Virulenzfaktoren, welche für die Besiedlung und Infektion der Magenschleimhaut wichtig sind. Eines seiner bekanntesten pathogenen Mechanismen ist das Typ IV Sekretionssystem (T4SS), einer porenartigen Struktur zur Übertragung des Onkogens CagA, das intrazelluläre Signalkaskaden beeinflusst. Die Protein-Bestandteile des T4SS sind in der sogenannten *cag*-Pathogenitätsinsel (*cag*PAI), einer *Helicobacter pylori*-spezifischen DNS-Region, codiert.
Das Protein VirB10 homologue CagY ist eine sehr wichtige Komponente des T4SS. Die genaue Funktion des CagY Proteins in der Pathogenität von *Helicobacter pylori* ist noch wenig geklärt. Die Struktur dieses Proteins ist recht einzigartig. Die zwei Repeat Regionen der Protein-codierenden Sequenz sind sehr variabel und zeigen keinerlei Ähnlichkeiten zu denen bereits bekannter Proteine. Eine Ausnahme bildet das *Late Membrane Protein* (LMP1) des Humanen Herpes Virus 4 oder des Epstein-Barr-Virus, welches für die Interaktion mit dem Transkriptionsfaktor IRF7 (Interferon regulierender Faktor 7) bei Virusinfektionen bekannt ist.

In dieser Arbeit wurde die Rolle von CagY bei der Stimulierung der Zytokin-Sekretion durch Aktivierung der Signalkaskaden Interferon und NF-κB in Wirtszellen untersucht. Es wurde eine T4SS-abhängige Aktivierung der IFNβ- und NF-κB-Promotoren nachgewiesen. Bei der Infektion von HEK 293-, HEK CC1- und HEK CC5-Zellen mit *Helicobacter pylori*-Wildtyp-Stämmen und Mutanten mit Knockouts von Komponenten des T4SS wurden unterschiedliche Aktivierungsgrade dieser Promotoren sichtbar. Diese Gen-Promotoren sind Bestandteil der Signalkaskaden von Interferonβ und NF-κB. Basierend auf vorläufigen Experimenten in der Arbeitsgruppe galt es eine eventuelle direkte Interaktion von CagY und IRF7 zu überprüfen. Zur weiteren Untersuchung des vermuteten direkten Zusammenspiels wurde eine stabile transfizierte AGS Zelllinie hergestellt, die stabil das Fusionsprotein EYFP- IRF7 exprimieren sollte.

Unerwartet verlor diese Zelllinie jedoch ihre Funktion, da sie im Verlauf das Fusionsprotein nicht mehr exprimierte. Eine Sequenzierung der DNS-Rückisolate ließ keinen offensichtlichen Grund für den Funktionsverlust, wie z.B. eine Mutation, erkennen. Weiterhin sollte die CagY-abhängige Translokation des IRF7 in den Zellkern gezeigt werden. Für die Visualisierung unter dem Konfokalmikroskop wurde in dieser Arbeit eine Färbemethode etabliert, um eine bestmögliche Auswertung zu ermöglichen. Verschiedene Zellen wurden zunächst mit dem selbst konstruierten Plasmid pEYFP-IRF7 transfiziert und anschließend mit *Helicobacter pylori* Wildtyp-Stämmen und Mutanten infiziert. Da die

Ergebnisse ambivalent waren, konnte weder eine Bestätigung noch ein Ausschluss der CagY-abhängigen IRF7-Kerntranslokation abschließend geklärt werden.
Durch die hochrepetitive besondere Genstruktur des *cagY* gelang die exakte PCR-Amplifikation des Gens nicht. Dadurch konnten keine funktionellen Fusionsproteine zum Nachweis einer direkten Protein- Protein- Interaktion zwischen CagY und IRF7 hergestellt werden.

1 Introduction

1.1 *Helicobacter pylori*

1.1.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium [1] populating the stomach of half of the world population. It is known to be a major cause of medical relevant gastroduodenal diseases like chronic inflammation and peptic ulceration. Therefore, the infection with *H. pylori* is a risk factor for development of gastric carcinoma [2, 3], MALT (mucosa-associated lymphoid tissue) lymphoma [3, 4] but also extra-intestinal diseases [5]. Already more than 50.000 years ago, *H. pylori* was associated to humans. Therefore, it was possible to reconstruct human migration during the last thousands of years by analyzing their DNA [6].

H. pylori was first correctly described by Barry J. Marshall and J. Robbin Warren in the early 1980s and linked to the pathogenesis of active chronic gastritis and gastric ulcer, yet still termed “unidentified curved bacilli” [1, 7]. In order to proof that the new isolated bacterium is able to cause acute gastritis, Barry J. Marshall did a self-experiment by drinking a *H. pylori*-suspension with bacteria isolated from a patient with gastritis [8]. In 2005, Barry J. Marshall and J. Robbin Warren were decorated with the Nobel Prize in Physiology or Medicine for their work.

1.1.2 Diagnostics and Therapy

Since the pathogen *H. pylori* was described in the early 1980s, infection caused by this bacterium gained more attention in the medical society. There are different guidelines provided from institutions and associations dependent on the regional circumstances of infection with *H. pylori* [3, 9]. In Germany the German Society of Gastroenterology, Digestive and Metabolic Diseases (Deutsche Gesellschaft für Gastroenterologie, Verdauungs-und Stoffwechselkrankheiten; DGVS) published an updated guideline, the “S2k-Guideline *Helicobacter pylori* and gastroduodenal ulcer disease”, giving recommendation in handling infections by *H. pylori* [10, 11].

For diagnostic of *H. pylori* infection, there are different non-invasive and invasive methods: Urea breath test, stool antigen test and serology (IgG-antibodies) are non-invasive methods [12]. Rapid urease test, culture of *H. pylori*, histology and staining and PCR are invasive diagnostic methods requiring biopsies via endoscopy [10-12]. The German S2k-guideline recommends only those tests for clinical diagnostics that can directly prove a current infection: Rapid urease test, culture, PCR, stool antigen test, urea breath test. Two positive test results are needed for diagnosis of *H. pylori* infection [10, 11], with some exceptions, e.g. a histological examination confirming *H. pylori* and chronic active gastritis does not need a second test for diagnosis [3]. The opinions towards mass screening for *H. pylori* infection are controversial. The European guideline advises to test patients with dyspepsia to treat them while the German guideline dissuades from non-invasive testing for *H. pylori* infection in patients with dyspepsia symptoms [3].

A diagnosis of an infection with *H. pylori* is not obligatory followed by medical therapy. The aim is avoidance of over-treatment. The indications for eradication of the Maastricht V/Florence consensus report and the German guideline mention essentially the same points. The target groups with indication for eradication are patients with peptic ulcer, gastric MALT lymphoma, functional dyspepsia after esophagogastroduodenoscopy, idiopathic thrombocytopenic purpura and iron-deficiency anemia of unclear cause. Furthermore, eradication should be done before long-term treatment with ASA (acetylsalicylic acid) or NSAID (non-steroidal anti-inflammatory drug), or for patients with a history of ulcer disease, upper gastrointestinal hemorrhage under treatment with ASA or NSAID and as prophylaxis against gastric carcinoma in a patient at risk [3, 10, 11]. Eradication in order to reduce the risk [13, 14] and prevent gastric cancer is only for persons with special risk, i.e. positive family anamnesis due to low prevalence of gastric cancer in Germany [3, 10, 11].
There are different opinions concerning the treatment of *H. pylori*-infection [3, 15]. The S2k-guideline in Germany advises different therapy programs for first and second line therapy facing the increasing resistance especially to the antibiotic drug clarithromycin [3, 10, 11]:
Triple therapy for 7-14 days (PPI, Clarithromycin, Metronidazole; PPI, Clarithromycin, Amoxicillin), quadruple therapy (PPI, Bismuth-potassium salt, Tetracycline, Metronidazole) or combined (concomitant) quadruple therapy (PPI, Clarithromycin, Amoxicillin, Metronidazole) for 7 to 10 days [10, 11]. The medicaments have no major side effects [4]. Despite the thorough treatment, recurrence of *H. pylori* infection after eradication is still observed [16], about 2% each year in industrialized countries and 6-12% in developing countries [10, 11]. Therefore, follow-up care with breath test, stool antigen test is recommended [3, 4].

1.1.3 Clinical relevance

1.1.3.1 Epidemiology

Several studies were done in order to investigate the spread of *H. pylori* infection in the world's population. The prevalence in different regions and countries has high variations from about 10% up to more than 80% [6, 12, 16]. For example Northern Europe has a lower prevalence than the southern European countries [16]. Within a region higher prevalence was observed in migrants comparing to the population of their receiving countries [6, 10, 11]. The prevalence of *H. pylori* infections in Germany is about 3% for children and 48 % for adults [10, 11]. But during the last years, a decreasing prevalence was observed [12].
There are different factors assumed to be risk factors for infection with *H. pylori* like low socioeconomic conditions in childhood, lower education, crowded homes, etc. [16] In general, different aspects are discussed to be a risk factor for infection with *H. pylori*, i.e. drinking water, diet, etc. [12].
Different transmission routes are supposed like fecal-oral and oral-oral. Also the iatrogenic transmission by gastric intubation is described [6]. There seems to be a higher advent of intrafamilial transmission [6, 16], especially from mother to child [6, 12, 16] and also in

general from infected family members to child due to close contact [10, 11]. A higher infection rate in the early ages of childhood has been observed [17].

1.1.3.2 Associated diseases

The infection with *H. pylori* is able to induce chronic gastritis and in the following process also dyspepsia, peptic ulcers, and MALT-lymphoma [3]. *H. pylori* infection is also described as a main risk factor for gastric cancer [2]. In children, infection with this pathogen is more associated with nodular gastritis and less to atrophic gastritis and intestinal metaplasia compared to adults [17]
Next to the known gastrointestinal diseases caused by *H. pylori*, a lot of associated extra-gastroduodenal diseases are discussed. The infections are found to have links, or even an important role in the genesis of the extra-gastroduodenal illnesses. Diseases among this group to be mentioned are iron deficiency anemia, immune thrombocytopenic purpura, nonalcoholic fatty liver disease [5, 18], metabolic and vascular diseases etc. The last four diseases still have to be further examined since the study results are conflicting [5].

1.1.4 Virulence factors and Pathogenesis

The prevalence of *H. pylori's* virulence factors differs from strain to strain and is found in different regions and populations [19]. There is even a difference between adults and children [20]. The virulence factors have essential function in the pathogenesis of a *H. pylori* infection: colonization, immune escape and disease induction [19]. Patients can also be infected with one or multiple strains at the same time [6].

Virulence factors responsible for colonizing the host include urease, flagella, chemotaxis and adhesins [19].
For a successful colonization of the human stomach the pathogen *H. pylori* needs systems to survive in the gastric acid with a pH from 1 to 2. It rarely invades the mucosa, but binds and interacts with epithelial cells [4]. *H. pylori* also moves in the gastric mucus layer [21] and uses mucus pH gradient for spatial orientation [22]. It produces the enzyme urease, which hydrolyzes urea into carbon dioxide and ammonia and allows the bacterium to survive in the acidic stomach. Responsible for its binding to the gastric epithelial cells are adhesins like BabA [19] and other proteins from the Hop (*Helicobacter* outer membrane proteins) family [4]. BabA (blood group antigen binding adhesion) is a major adhesin on the outer membrane of *H. pylori*. It recognizes blood group antigens on host cells and strongly influences the density of *H. pylori's* colonization [19]. HopQ is an adhesin protein of *H. pylori* interacting with CEACAMs, proteins of the carcinoembryonic antigen-related cell adhesion molecule family, on host cells. Upon interaction between the human proteins CEACAM1, CEACAM3, CEACAM5 or CEACAM6 with HopQ, the virulence factor CagA translocates into the host cell [23].

H. pylori counts numerous virulence factors that are responsible for the pathogenesis and clinical outcome upon infection. Among them, there is the exotoxin VacA inducing apoptosis and probably responsible for *H. pylori*'s nourishment [4]. VacA (vacuolating cytotoxin A) is able to induce vacuole formation in cells. The physiologic role of vacuolation still needs to be determined, but several clinical studies have shown an association

between this virulence factor and the severity of the clinical outcomes, i.e. association with higher risk for gastric cancer [19].

Most *H. pylori* strains have a *cag*PAI, a pathogenicity island that indicates a region on the chromosomal DNA which encodes for virulence factors, i.e. components of the T4SS and the protein CagA [4, 19].
One of *H. pylori´*s best known pathogenicity mechanisms is the *cag* type IV secretion system (*cag*-T4SS). Its function is to inject the oncogenic toxin CagA into the cytoplasm of the host cell [24], where it disturbs several cellular processes [19]. Additionally, the machinery formed by the *cag*-T4SS is able to induce the production of several cytokines responsible for the recruitment of immune cells [4, 25, 26] and, most likely, chronic inflammation [20]. A rather large and one of the most important components of the system is the VirB10 homologue CagY [24]. It contains two repeat regions, which are highly variable and present no homology to any known protein [27], except small homology regions to the Late Membrane Protein 1 (LMP1) of the Human Herpes Virus 4, or Epstein-Barr-Virus (for details see 1.1.7).

CagA (cytotoxin-associated gene A) is a bacterial oncoprotein and is injected into host cells via the type IV secretion system (T4SS) [19, 28]. The protein leads to cellular immune response and cytokine production by host cells [4]. Dependent on its production, *H. pylori* strains can be divided into two types: Type I strains have the gene CagA and produce this protein together with VacA. Type II strains do not have the gene for CagA and do not produce neither CagA nor functional VacA. But VacA is CagA independent [29]. Once in the host cell, CagA modifies intracellular transduction pathways which may lead to malignant degeneration of epithelial cells in the stomach. CagA is strongly associated with gastrointestinal adenocarcinoma, myeloid leukemia and B cell lymphoma.

Phosphorylation seems to be essential for its oncogenic function [19]. After translocation in the host cell, CagA is tyrosine-phosphorylated [28]. It induces different cellular responses [26], i.e. the increase of inflammatory response of the host through the NF-κB pathway leading to IL-8 secretion [19]. The CagA and T4SS were shown to be important for IL-8 induction [19, 30]. It also influences epigenetics, that changes gene expression without changing the DNA sequences per se but by changing other circumstances of the DNA strains, e.g. DNA methylation [19]. Following the binding of *H. pylori* to the epithelial host cells and interference into their cell signaling, cell-cell junctions and cell proliferation get disrupted. CagA indirectly changes the tight junctions and adherent junctions of epithelial cells [26].

Activation of the immune system leads to recruitment of neutrophils, T- and B-lymphocytes, plasma cells, macrophages, etc. along with cell damage in the epithelium [4]. Epithelial cells then release different cytokines upon infection with *H. pylori* [31, 32], like Interleukin 1β, Interleukin 2, Interleukin 6, Interleukin 8, tumor necrosis factor α (TNFα). IL-8 plays a major role and is released after activation of NF-κB and transcription factor activator protein 1 [4]. IL-1β regulates expression levels of Fas, NF-κB, MAP-kinases and

influences hypochlorhydria, gastric atrophy and gastric carcinoma. The specific immune response upon *H. pylori* infection is Th1-cell- mediated [4].

The clinical outcome of *H. pylori* infection is very variable and depends on pathogenic characteristics and on characteristics of the host [4]. Only about 85% have mild asymptomatic gastritis, but 15% of the patients can get gastric ulcer and less than 1% can get gastric cancer [19]. So the clinical outcome of *H. pylori* infections depends on the interaction between bacterial virulence factors, host immune response and environment (like diet, etc.) [19]. Comparing *H. pylori* infection of children to adults, children develop reduced gastric inflammation, despite similar colonization levels and virulence factors of the pathogen. They also show a different gastric immune response regarding recruited immune cells and cytokine levels, e.g. less Th1, Th17 and immunosuppressive Treg cells and more IL10 and TGF-β1 [33].

1.1.5 *cag*PAI

PAI is the abbreviation of pathogenicity island. A pathogenicity island indicates a DNA region on the chromosomal DNA which encodes for virulence factors making a bacterium pathogenic, e.g toxins, adhesins, etc. Pathogenicity islands carry many genes coding for virulence factors which together form a DNA region with a size often over 30 kbp flanked by repeats. The G+ C content in the sequence differs from the rest of the chromosomal DNA. This is the reason why they are supposed to have been inserted from a phage or plasmid by horizontal gene transfer. Among one species the PAI region is present in pathogenic bacteria strains and absent or rarely present is less pathogenic strains [34].
The cag (cytotoxin associated gene) region in the chromosomal DNA of *H. pylori* presents the PAI of this bacterial species. Its G+ C content of about 35% is increased in comparison to the rest of the chromosomal DNA (38-45%). With a size of 40 kbp the *cag* region is inserted into a chromosomal racemase gene and flanked by a 31 bp direct repeat [35]. Next to CagA, proteins to form the T4SS are also encoded in the *cag* region [35]. This region consists of at least 11 operons and operon cag26 encodes CagA [36]. Figure 1 shows the transcriptional profile, which is in general conserved among the different strains of *H. pylori*. Most of the proteins encoded in the pathogenicity island have not-yet-known functions [37].

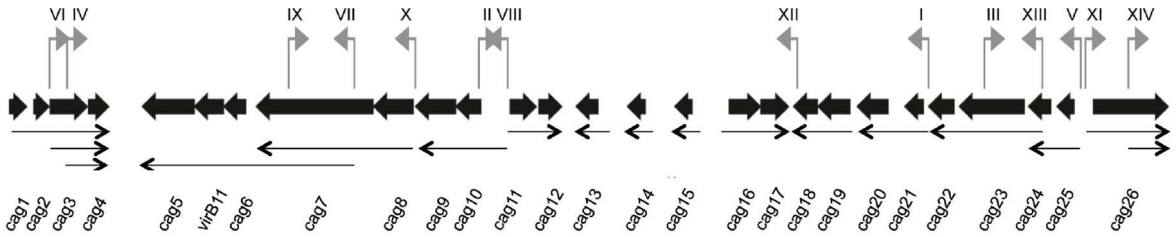


Figure 1: Transcriptional profile of the *cag*PAI of *Helicobacter pylori*
Each big black arrow represents a gene of the *cag*PAI region. Their length and placement in the construction is nearly proportional to the length and intergenic spacing of the genes they are representing. Modified from [36].

The absence or presence of some parts of the *cag*PAI region is one of the important criteria to divide *H. pylori* strains into two groups: type I strains and type II strains [29].

Type I carry the genes for CagA and VacA, produce both proteins and are associated with severe diseases [29]. The *cag*PAI takes an important position in the transcription and secretion of IL-8 [38]. With the ability of the induction of IL-8 secretion, this type belongs to the virulent *H. pylori* strains [35]. The lack of other genes encoded in the island turns the bacteria less harmful. AGS cells, e.g., are less responsive when infected with *H. pylori* mutants lacking genes of the *cag*PAI in comparison to the wildtypes [39]. Type II are attenuated in pathogenicity and only carry the gene for VacA but do not produce it. Also the gene coding for CagA is absent [29]. For more about the pathology of IL-8 see 1.1.2.

1.1.6 T4SS

Type IV secretion systems (T4SS) are complexes formed by different proteins. They are present in gram-negative and gram-positive bacteria as well as archaea. Depending on the function of the systems, they can be divided into three groups.
The secretion systems in the first group are to conjugate DNA from one cell to another. Conjugation helps the bacteria to fit better into their environment and is also one important reason for pathogens' spread of resistance against antibiotics [40]. In this group the best known system is the VirB/D system in the bacterial species *Agrobacterium tumefaciens* [41]. The secretion system of *Agrobacterium tumefaciens* consists of 12 different proteins, VirB1 to VirB11 and VirD4 [41]. This protein structure is conserved as subset in many bacteria species with a large diversity of subunit numbers and protein compositions [40]. The second group classifies T4SS, which allow the exchange of DNA between the cell and extracellular part. The third group transfers protein effectors, mostly toxic protein effectors from pathogens to host cells and therefore increase the virulence of pathogenic bacteria [40-42].

The protein complex of T4SS, which is placed in the bacterial membrane and protrudes into the extracellular milieu [43], has different parts: The inner membrane complex composes of three ATPases VirB4, VirB11, VirD4; VirB6 and VirB8 [41]. The core complex is formed by 14 copies of each protein VirB7, VirB9 and VirB10 [40, 41, 44]. The pilus is built by the two proteins VirB2 and VirB5 [41].
The VirB10 protein functions as a structural protein, but also as a sensor or transducer. Its transmembrane domain is necessary for pilus biogenesis [45]. The C-terminal region has an important role in the regulation of the protein passage through the bacterial outer membrane [46], which is pilus independent [45]. Lacking this protein, bacterial infection effects are decreased [47].

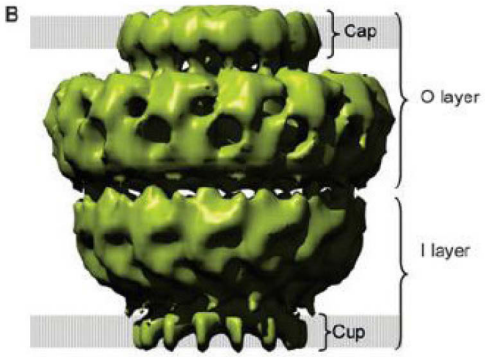


Figure 2: Cryo-electron microscopy structure of the core complex belonging to the plasmid PKM101 T4SS of *E. coli*
The I-layer lies in the inner membrane while the O-layer lies in the outer membrane, both together forming a pore-like channel to connect the cytoplasmic side with the extracellular side. Adapted from [41].

The T4SS systems of *H. pylori*, termed *cag*-T4SS, belong to all three groups of the type IV secretion systems mentioned above: The ComB system is the most conserved one and belongs to group two. The third group can be found in the most pathogenic *H. pylori* strains due to the production and injection of the CagA protein via T4SS into the host cells. Next to CagA the *cag*-T4SS also transfers peptidoglycan fragments [24]. The so-called type I bacteria (see 1.1.4 and 1.1.6) strain possesses both groups of secretion systems. One is to take up bacterial DNA (group comB system, group two), one is to insert effector proteins into host cells (Cag T4SS, group three) [44].
Consisting of numerous proteins, the *cag*-T4SS has conserved components which have sequence similarities to *A. tumefaciens*' VirB proteins, termed VirB homologous proteins and components that are unique for *H. pylori* T4SS [24]. The protein CagY, for example, has similarities to the protein VirB10 in the C-terminal portion [41] (see 1.1.7) and therefore is also called VirB10 homologue. CagE has similarities to VirB4. Furthermore, there are lots of VirB-homologous proteins in *cag*-T4SS [24].
The transmembrane core complex is formed by HP0532, HP0528, HP0527 corresponding proteins encoded by the genes *hp0532*, *hp0528* and *hp0527*, homologues to the proteins VirB 7, 9, 10 [41] also called CagT, CagX and CagY [24]. *H. pylori*'s *cag*-T4SS has a specific pilus which is more complex than other T4SS pili [41]. The VirB2 homologue HP0546 or also CagC [24] was suggested as the major pilus component [40, 48] and forms the pilus together with a VirB5 homologous protein HP0539, also called CagL [42].

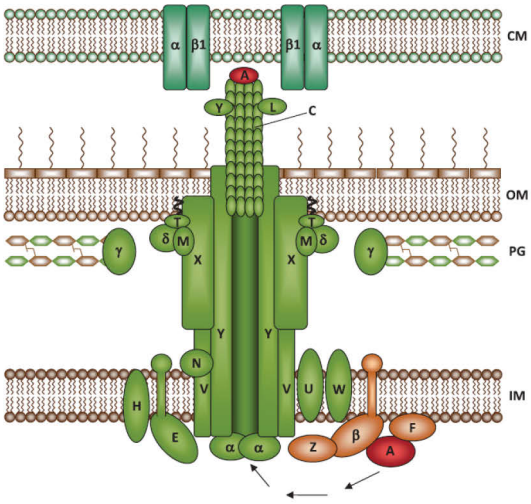


Figure 3: Scheme showing the assembly model of *cag*-T4SS of *Helicobacter pylori*
The green proteins stand for essential and supportive elements forming the secretion system. Translocation factors are marked in orange. The effector protein CagA is marked in red. CagD, CagG and CagI and non-essential components are not shown.
Abbreviations: IM: inner (bacterial) membrane; PG: peptidoglycan layer; OM: outer (bacterial) membrane; CM: cytoplasmic membrane of a eukaryotic target cell
Adapted from [24].

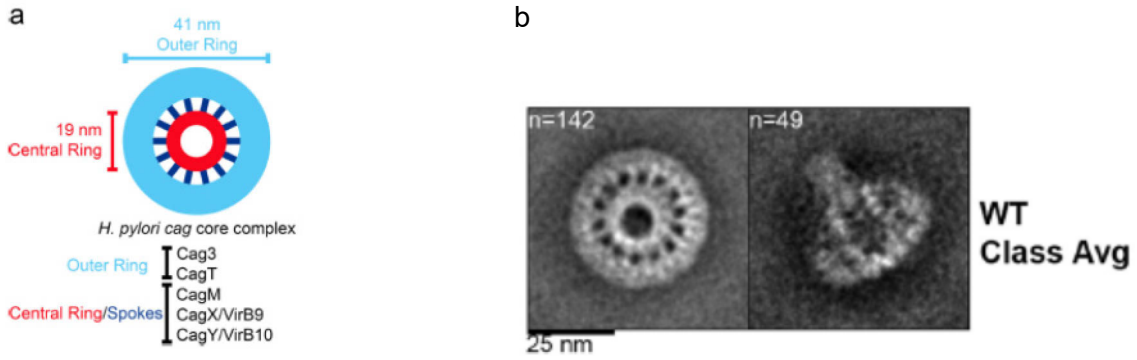


Figure 4: Structure of *cag*-T4SS of *Helicobacter pylori*
A A schematic model of the *cag*-T4SS core complex of *Helicobacter pylori* [49] show the complex from above. The outer ring consisting of two proteins Cag3 and CagT. The inner ring is formed by proteins CagM, CagX and CagY.
B A negative-stain EM picture shows the *cag*-T4SS from above (left) and from the side (right). Both are wildtype complexes.
A and B from [49].

The *cag*-T4SS is assumed to play an important role in the pathogenesis of diseases caused by *H. pylori* as infection experiments in mongolian gerbils have shown a decrease of pathogenicity effects in gerbil stomachs infected with bacterial mutants lacking a functioning secretion system compared to the wildtype strain [50, 51]. This secretion system shows a functional plasticity through the capability of rearrangements of the DNA sequence encoding for the CagY, one essential protein to form the secretion system [52].

1.1.7 CagY

As already mentioned in 1.1.6 the CagY protein is a component of the T4SS transmembrane core complex [24, 41, 53]. It is exposed to the bacterial surface and is part of the T4SS pilus having an important function in the interaction with host cells [43, 48, 54]. This protein is termed HP0527 or CagY.
The sequence encoding for the protein CagY contains two big repeat regions. Those regions are composed of lots of repeated amino acid sequences whose compositions [53] and sequence length vary between different *H. pylori* strains [53, 55]. There is also a VirB10 homologous region, which is at the C-terminal end of the sequence [56].
The DNA sequence that encodes the CagY protein is quite special. In the database no homology could be found to the repeat region II [27]. In *H. pylori* strain 26695, whose complete genome sequence was one of the first to be sequenced [56], repeat region I, which goes from amino acid position 9 to 325, can be divided into three parts, termed A, A* and A**. A comprises 130 amino acids and is followed by A* also with 130 amino acids. Compared to region A, region A* is similar except of 3 mismatches. Region A** has 57 amino acids, which is completely similar to the corresponding 57 amino acids in region A*. Repeat region II spans amino acid positions 477 to 1383. Since the size of this region is bigger than repeat region I, the composition is more complex: Six consensus sequences α, β, λ, μ, δ and ε with a length from 5 to 14 amino acids and each with a certain amino acid combination form 74 segments. The segments together form repeat region II [53]. Repeat region II contains a high number of cysteines, which are quite regularly distributed over the whole sequence [53]. This special arrangement of cysteines may indicate a special protein structure stabilized via disulfide bridges [27, 53]. Interaction within the repeating sequences was also proven [27].

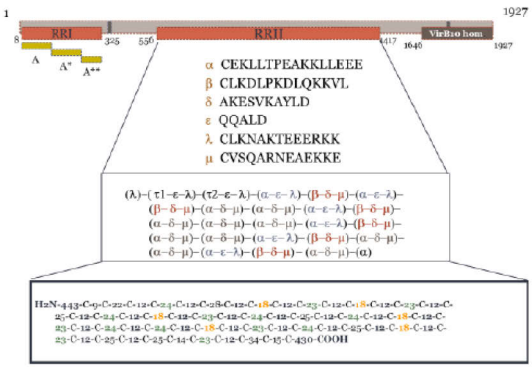


Figure 5: Modell of sequence assembly encoding the protein CagY
Adapted from Luisa Jimenez-Soto.

Sequence and amino acid analysis supposed the repeat region II (RRII) to encode for proteins forming an α-helical coiled-coil structure [27], which spans the whole bacterial membrane. CagY's N-terminus should be on the side of the cytoplasm [57]. Further studies also have shown repeat region II (RRII) to have surface and pilin association [58].
The protein fragment encoded by the RRII is inserted into the outer membrane of the bacterial envelope and extents of the outer membrane as an α-helical sheath covering the

pilus after a cell contact to AGS cells [59]. This might be the reason why the RRII-encoded protein is stable to physical parameters like pH and temperature [27].
Next to its role encoding the sequence of CagY, the repeating regions are also supposed to play a key role in the functional plasticity of the T4SS and therefore also in the pathogenicity of the entire bacterium [52, 54, 60]. Except of the two proteins Hp0523 and Hp0539 the functionality of CagY is independent from other *cag*PAI proteins [59] but it still seems to interact with some distinct cagPAI proteins [57]. Essential for CagA translocation is the binding of the β1-integrin, a subunit of the host cell adhesion receptor α5β1-integrin, with the C-terminal part of CagY [58]. Both, the surface-related expression of the RRII and also the CagY binding to β1-integrin are T4SS pilus independent [54].
The functionality of the T4SS can be mediated by recombination within the repeating sequences [52], e.g. in-frame deletions or duplications [55]. This process is enforced by an intact host immune system and is predominantly mediated by NF-κB [52]. Mice infection experiments have shown IFNγ, CD4-cells and T-cells to be essential for the selection of T4SS's loss of function that was CagY-induced [60]. The functionality change of T4SS is not caused by its structure modulation [52, 54] but probably by the alternation of binding between CagY and β1-integrin [54].
Assuming the importance of the CagY protein in the induction of the immune response, a fragment of this protein was tested with other cellular components, which are important for *H. pylori*'s pathogenicity, like the cytotoxin-associated gene A (CagA), adhesion, urease, vacuolating cytotoxin A (VacA) neutrophil-activating protein, etc. as a vaccine. In contrast to the mentioned cellular components, CagY was able to induce an immune response in mice showing the potency as a vaccine candidate for an oral vaccination generating a protective immunity to *H. pylori* [61].

In my work group, the protein LMP1 was observed to have structural similarities to the CagY protein. At first, a Yeast-2-Hybrid screen against all proteins of the human genome has shown different potential interactives with the repeat regions of CagY. The most interesting one among them was the IRF7 protein that interacted with the repeat region I of CagY. Since IRF7 and LMP1 interact naturally, further investigations were performed by Luisa Jimenez-Soto. Preliminary data showed LMP1 and repeat region I of CagY to carry partially homologies (data unpublished).

1.2 Interferon and NF-κB pathways

The infection with *H. pylori* induces different pathways [62]. Interferon type I plays an important role in controlling *H. pylori*. Mucosal defense against *H. pylori* infection is at least partly induced by NOD1 and ISFG3 after activation of protective Th1 immune response [63]. NF-κB also takes an important role in the immune response to *H. pylori* infection [64]. Its activation seems to be CagA-dependent [65] and requires physical contact between *H. pylori* and epithelial cells [66].

1.2.1 Interferon pathway

The innate immune response in general is induced by different molecules and different cells [67]. Therefore "pathogen-associated molecular patterns" (PAMPs) which can be

lipopolysaccharides (LPS), peptidoglycans (PGN), flagellins, DNA, RNA, etc. and are localized on microorganisms, can be recognized by “pathogen-recognition molecules” (PRMs) of the host. The best known PRMs are the Toll-like-receptors (TRLs) [67]. TLRs can be localized to the cell surface, but also the intracellular part of the cell [68]. Bacterial fragments are recognized directly on the cell surface or after having been internalized into the cell by bacterial secretion systems or endosomes [68].
The best characterized pathway leading to IFN type I production upon bacterial infection is the TLR4-initialized one. TLR4 especially recognizes LPS of gram-negative bacteria [68]. But TLR4 seems not to be essential for gastric epithelial cells to respond to *H. pylori* [67, 68].
A relatively new family of PRMs, termed nucleotide-binding oligomerization domain (NOD) [69], is assumed to play a key role in the induction of an innate immune response upon infection with *H. pylori* [67]. The nucleotide-binding oligomerization domain 1 (NOD1) is mainly a sensor for products of gram-negative bacteria [70]. As a pathogen recognition receptor, NOD1 has an essential role in the induction of the innate immune response in early stages [71]. It is expressed in epithelial cells, i. a. in the gastrointestinal tract [72]. NOD1 is a intracellular sensor which leads to the activation of IFN type I pathway after having recognized activation factors like peptidoglycans (PGNs) [73] or LPS [72].

Interferons function as one group of important regulators of the innate immune system, a complex system to be activated, regulated and controlled [74-76]. They can be divided into three subgroups: Interferon type I, Interferon type II and Interferon type III [74]. Type I Interferon comprises lots of members. IFNα, IFNβ, IFNε, IFNω and IFNκ are encoded in the mammalian genome [77]. In humans, there exist only one IFNβ whereas many IFNα species can be found [78]. IFNγ forms the second Interferon subgroup, Interferon type II while type III interferon includes IFNλ1, IFNλ2 and IFNλ3 [74]. There is not much known about the recently recognized interleukins of type III interferon, but there is a suggestion that they are secreted by different cells upon viral infection [79].
Three IFN types were first recognized depending on the cell types secreting them: IFNα and IFNβ, one produced by leukocytes and the other by fibroblasts [79]. IFNγ is mainly produced by T-cells and natural killer cells [79]. But in general, most of the mammalian cells are able to secret IFNα and IFNβ [79]. IFNα and IFNβ are the main interferon type to be produced upon viral and bacterial infection [77]. They are also produced after fungal and parasitic infections [74]. They can be induced through different receptor recognizing bacterial fragments, like Toll-like-receptors (TLRs), RIG-I like receptors (RLRs), nucleotide-binding and oligomerizing domain (NOD) like receptors (NLRs), DNA sensors, AIM-like receptors (ALRs) [36] and then transduce signal through different pathways [78]. IFNα and IFNβ are important cytokines to modulate macrophages’ and dendritic cells’ functions [74]. Affecting CD4+- and CD8+-T-lymphocytes and many other immune cells, IFN type I also influences the adaptive immune response to infections [74, 77]. The establishment of the adaptive immune response to *H. pylori* infection is also facilitated by IFN type I’s support of the maturation of gastric dendritic cells, which then leads to IFNγ secretion by T cells [80] and natural killer cells (NK-cells) [77]. But on the other hand, IFN type I was already observed to suppress IFNγ [81]. Next to the induction of Type I IFN signaling, IFNα and IFNβ also influence tissue integrity and cell metabolism. They are able

to inhibit antimicrobial peptides as well as proinflammatory molecules [82]. They also play an important role in the maturation process of dendritic cells [77]. Upon infection with *H. pylori*, Th1 cells secretion of IFNγ is primarily induced by gastric dendritic cells [80]. Among other things, IFNγ can activate macrophages in order to ensure the immune response to infections. Besides, IFNγ play an notable role in the inflammation of the stomach upon infection with *H. pylori* [83] and is correlated with gastritis severity and gastric tumors [84]. Despite an induction of IFNγ through NOD1 via IRF1 [84], the adaptive immune response to *H. pylori* infection is mainly TLR-controlled via the activation of dendritic cells [85].

As already described above, the host's innate immunity response to *H. pylori* is type I interferon initialized and the activation of type I Interferon signaling upon *H. pylori* infection is NOD1-induced. NOD1, the cytosolic PRM of gastric epithelial cells recognizes peptidoglycans (PGNs) of *H. pylori,* which are transferred into the host cells via the T4SS [73], but also *cag*PAI-independently [82, 86, 87]. The *cag*PAI-dependent insertion of PGNs into the host cell is not bacterial invasive [73].
NOD1-activation by *H. pylori* leads to its interaction with RICK, also called RIP2. RICK leads to activation of TRAF3 (TNF receptor associated factor 3). TRAF3 activates TBK1 (TANK-binding kinase 1) which together with IKKε activates IRF7 by phosphorylation, a translocation factor of the interferon pathway. Binding to the promoter in the nucleus IRF7 initializes the translation of the cytokine Interferon β. After being secreted, this cytokine binds to Type I IFN-receptor which causes the activation of ISGF3 (interferon-stimulated gene factor 3, a transcription factor for Th1 chemokines), a complex formed by Stat1-Stat2-IRF9 [63, 64]. The following induction of Th1 chemokines like CXCL9, CXCL10 and CXCL11 take part in the mucosal host defense against the *H. pylori* infection [64].

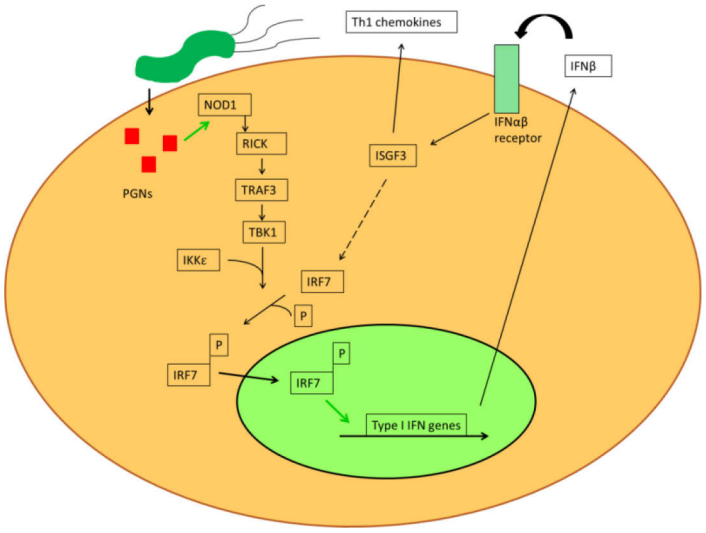


Figure 6: Interferon type I pathway upon activation by *Helicobacter pylori*
Diagram based on [63, 64, 77].
First NOD1 recognizes PGNs internalized from *Helicobacter pylori* by the host cell. In the following RICK, TRAF3 are induced and lead to IRF7-phosphorylation by TBK1 together with IKKε. After translocation to the nucleus, IRF7 binds to the IFN type I promoter to induce gene transcription. Produced IFNβ cytokines bind to

IFNαβ receptors, which lead to the translocation of ISGF3 in order to induce transcription of Th1 chemokines and also IRF7 to enhance the immune response.
NOD1: nucleotide-binding oligomerization domain 1; RICK: a caspase-recruitment domain-containing kinase [88]; TRAF3: TNF receptor associated factor 3; TBK1: TANK-binding kinase 1; IKKε: inhibitor of NFκ-B kinase-ε; IRF7: Interferon regulatory factor 7; ISGF3: IFN-stimulated gene factor 3

Another important role of IFN type I is the protection of tissue upon infection on the one hand and the regulation of immune response on the other hand [86, 89, 90], innate and adaptive [91]. Balancing the killing of pathogens and protecting the tissue against the own immune system is a very important task done by IFN type I. The effect of IFN type I is ambivalent. Depending on the bacterial species causing the infection, type I interferon has protective or detrimental function [86].
During *H. pylori* infection, IFN type I protects the host by decreasing the bacterial burden in the gastric mucosa with secretion of the cytokine CXCL10 [63, 86]. Dysfunction of the IFN type I can cause autoinflammation and autoimmunity [92].
It is important to note that IFNα and IFNβ are not only produced upon infection by microorganisms, but a constitutive weak production of IFNα and IFNβ could have been proven [93].

1.2.1.1 IRF7

IRFs are interferon regulatory factors and perform a function as transcription factors. In this group, there are nine members identified in mammalian cells. They possess an important function in host immune response and also have a noteworthy effect in the establishment of autoimmune diseases and in cell differentiation [94].
The IRF7 gene was first cloned in 1997 in the context of Epstein-Barr-Virus (EBV) latency, i. a. Late Membrane Protein (LMP1) [95], an oncogenic protein of EBV [96]. The IRF7 protein can be activated by LMP1 [97, 98], most likely by phosphorylation [94]. At the same time, it can induce the expression of LMP1 [94, 96]. Therefore it is seen as the most relevant IRF for the activation of LMP1 [99].
As already mentioned in 1.2.1, IRF7 is an important regulator of the type I Interferon pathway [100]. IRF3 also belongs to the IRF family and the closest IRF to IRF7 [94]. Functional interaction between these two IRFs was observed [101]. IRF3 also has a key function in the Interferon-α and -β regulation [102]. Both together are of essential role of IFNα/ β induction upon viral infection [103]. Upon infection, IRF7 translocates into the nucleus after being phosphorylated (see figure 5) and binds to the Interferon-promoter. Next to LMP1, IRF7 can be activated by NOD1 and other pathogen-associated molecular pattern (PRRs), among them also different receptors, e.g. Toll-like receptors [94], but also upon virus infection, or LPS from bacteria and IFN [101]. The IRF7 has a positive feedback effect on its own production in the cell upon infection [77, 94]. IRF7 is present in most cells, but not in all [77]. This protein is mostly expressed in lymphatic cells [95]. In most immune cells, it is present on a basic level in the cytoplasm and causes a basic secretion of IFNα and IFNβ. This effect is essential for the initial induction of the mentioned cytokines after infection, which is called priming [94].
Together with transcription factor IRF3, IRF7 is of great importance to enhance the induction of IFNα/ β to a high range in a so-called feedback loop [104]. IRF7 also seems to influence oncogenesis with relation to EBV infection. In contrary, IRF7 also shows tumor

prevention effects. It is supposed that the oncogenic properties of IRF7 depend on the cell type [94]. Until now, it is shown that upon virus infection, IRF7 is more critical for the late induction phase. But when exposing the cells to IFN and then infect them, IRF7 is getting expressed and is also active in the early induction phase. [103]. Thus, IRF is described to have an upregulating function of the detrimental effect of IFN type I reaction [104].

1.2.2 NF-κB pathway

NF-κB takes an important role in mediating the components of inflammatory reactions [105, 106]. It causes different immune responses due to different activators e.g. LPS, TNF [105] or by LMP1 [94] and due to different infected cells [107]. It influences the coordination of innate but also adaptive immune responses [108]. There are two types of NF-κB pathways: canonical and non-canonical (see Figure 7) [109]. The non-canonical one is mainly activated through molecules belonging to the TNF receptor superfamily [105]. To activate NF-κB, there are numerous different stimuli (chemokines, cells), receptors and accordingly different cascades to follow [110]. Activated genes are proinflammatory cytokines and chemokines [106]. In resting cells most of the NF-κB dimers are located in the cytoplasm and bound to IκB [108]. NF-κB proteins in mammals have different names: Rel (c-Rel), RelA (p62), RelB, NF-κB1 (p50), NF-κB2 (p52) [108].

NF-κB takes an important role in the immune response to *H. pylori* infection [64]. For the induction of NF-κB by *H. pylori* in gastric epithelial cells, CagA was shown to be essentially required [65] and physical contact between *H. pylori* and epithelial cell seems to be necessary [66]. Furthermore, the induction of NF-κB is influenced by *cag*PAI [66, 111]. The pathway is induced *cag*PAI-dependently in epithelial cells that are unresponsive to LPS [112].
Upon infection with *H. pylori*, the NF-κB pathway is rapidly activated [111] e.g. by NOD1 [64]. This activation is quite weak [63]. Downwards the pathway, NOD1-activation leads to interaction with RICK, then TGFβ-activated kinase 1 and results in the induction of NFκ B and MAPK [64]. Active NF-κB translocates into the nucleus. The active form is found in the cytoplasm and nucleus [113]. Upon infection with *H. pylori* especially p50/p65 dimers and p50 homodimers play an important role as transcription factors [113]. Then activated genes are those from proinflammatory cytokines, chemokines like IL-1β, IL-8, TNFα, etc. [66, 106]. The opinions on the role of NF-κB upon *H. pylori* infection remain controversial. In one research, NOD1 is described to be important for release of proinflammatory cytokines during infection with *H. pylori*, in particular NF-κB, but also further transcription factors [62]. In another research, NOD1 seems not to be the principal signal transducer in epithelial cells infected with *H. pylori* [112].
Upon *H. pylori* infection, IL-8 is also increased next to IFNβ [63]. Produced and released upon infection, IL-8 is a potent chemoattractant that recruits neutrophils [113]. IL-8 is important for transvenular cell migration in acute inflammation [114]. IL-8 production depends on NF-κB on a high level [113], but its production is also dependent on other factors than NF-κB [62]. In infected epithelium, increased IL-8 levels could be shown. But since biopsies from uninfected gastric mucosa show IL-8 at low level, the assumption came up that there is a constitutive IL-8 expression in uninfected cells [115]. After eradication IL-8 and TNFα decrease, IL-8 does more than TNFα [116].

Probably there is an interaction between the IFNβ and NF-κB pathway, since NF-κB is also capable to activate the IFNβ-promoter [93] and TRAF3, a part of the IFN pathway is a known inhibitor of NF-κB [64].

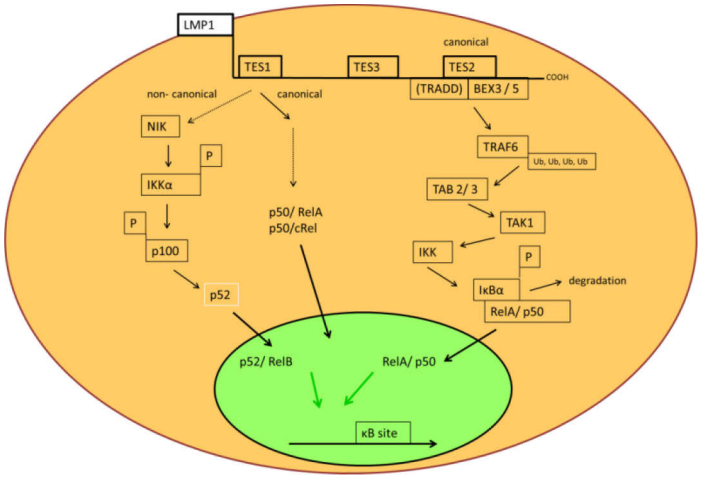


Figure 7: LMP1-mediated activation of the NF-κB pathway
The figure shows the activation of NF-κB by the integral membrane protein, LMP1: the TES1-mediated canonical (also called classic [110]), non-canonical NF-κB pathway (also called alternative [110]) and the TES2-mediated canonical NF-κB pathway [96].
The canonical pathway induces rapid and reversible immune response. The cascade is IKKγ (= NEMO)-dependent [109]. IKKγ is one member of the IKK complex next to IKKα and IKKβ. TRADD probably is not directly required for TRAF6 activation via TES2, but needed in some cells; BEX3/5 seems to be important for downstream pathway: TRAF6 is ubiquitinated; recruitment of TAB 2/3; activation of TAK1; activation of IKK complex comprising IKKγ, IKKα and IKKβ; phosphorylation of IκBα and therefore its following degradation; translocation of dimer RelA/ p50 into the nucleus. TES1-mediated canonical NF-κB activation in an yet unknown cascade [96].
Non-canonical pathway induces slower and irreversible immune response. It is IKKγ (= NEMO)-independent [109]. TES1 indirectly activates NIK by protecting NIK from degradation. NIK is constitutively produced and controlled via degradation mechanisms in unstimulated cells. The phosphorylation of IKKα leads to phosphorylation of protein p100 and turns it into p52. Forming different heterodimers, e.g. with RelB, p52 translocates into the nucleus [96].

Researches have shown that the NF-κB pathway does not always go straight to production of proinflammatory cytokines as described above. IKKα also has repressive function on target genes and therefore for downregulation of inflammatory processes [105].

1.3 Cells

1.3.1 HEK 293

The HEK 293 cell line is a hypotriploid cell line having a complex karyotype and was generated from human embryonic kidney cells in the 1970s. Normal embryonic kidney cells were transfected with sheared adenovirus 5 DNA. The number 293 is attributed to the 293rd experiment of Graham leading to the final result. It was first established for studies with adenoviruses [117]. Since then it is often used in research especially in those on processes inside the cell [118], due to its robustness, simple and uncomplicated handling

(growth, cell culture, etc.), good transfection results and efficient protein production [118, 119]. HEK 293 cells are also often used for production of biotherapeutic proteins [119].

1.3.2 HEK CC1 and HEK CC5

HEK 293 cells do not express CEACAM receptors on their cell membrane. *H. pylori* failed to inject the CagA protein into those cells. But after functional expression of CEACAM 1 or CEACAM 5 on the HEK 293 cell surfaces, CagA injection became possible [23, 120].
In our work group stably transfected cell lines with HEK 293 cells expressing CEACAMs, in particular CEACAM 1 and CEACAM 5, were established.

1.3.2.1 CEACAMs

The carcinoembryonic-antigen-related cell-adhesion molecules (CEACAMs) belong to the CEA (carcinoembryonic-antigen) family. They are glycoproteins on the cell membrane of different cell types. CEACAM 1 proteins for example are expressed on the surface of epithelial cells, endothelial cells, lymphocytes and myeloid cells while CEACAM 5 proteins are only expressed on the surface of epithelial cells. In humans, 11 different CEACAM1 splice variants were identified [121]. As target for bacterial and viral adhesins [121], CEACAMs enable cellular interaction and influence cellular growth and differentiation by initializing intracellular processes [122]. Like CEACAM 1, CEACAM 5 is able to downregulate immune responses [121]. But increasing immune response due to expression of CEACAM was also described [123]. As well, CEACAMs are involved in physiological processes [122]. Since CEACAMs are linked to cancer disease, e.g. progression and metastasis, they are used as tumor markers for melanoma, lung, colorectal and pancreatic cancers [122].
CEACAM 1, CEACAM 3, CEACAM 5 and CEACAM 6 were identified as receptors for *H. pylori* with HopQ having a high interaction affinity [23]. It was shown that the interaction of *H. pylori* with CEACAM receptors is essential for the translocation of CagA into the host cells via T4SS [124].

1.3.3 AGS

The AGS cell line is a human gastric adenocarcinoma epithelial cell line [125] generated from human gastric cancer cells from a biopsy. The patient had an untreated human adenocarcinoma of the stomach [126]. This cell line was first established to serve as model to do research on human tumor therapy and test tumor drugs and radiation [126]. Then it also was used as an in vitro system to investigate the impact of infections with *H. pylori* [127]. After attachment of *H. pylori* to AGS cells, rearrangement of the cytoskeleton, protein phosphorylation [125] and IL-8 secretion [127] were observed.

1.3.4 TNFα

The tumor necrosis factor α (TNFα) is a cytokine produced during inflammation as part of the immune response. It is involved in induction of different intracellular signaling. Next to its role in fighting infection, negative influence on cancers was also described [128].
Relations to other cytokines and signaling pathways are quite various: TNFα is activated by NF-κB but TNFα also can activate the NF-κB [106] IKKβ-dependently [108]. LMP1 and

TNFα induce the same NF-κB pathway [129]. IRF7 is induced by TNFα [94]. On the other hand, NOD1 stimulation did not induce TNFα [71] and vice versa NOD1 expression is not activated by TNF [63]. Therefore, a unique and overlapping innate immune response initiated by both NOD1 and TNFα was suggested [71]. Upon infection with *H. pylori*, TNFα was found significantly higher in inflammation [31].

1.4 Aim of these studies

The aim of these studies was to determine the role of the protein CagY in the production of cytokines, Interferon β and IL-8, associated normally with the Cag T4SS and the relevance of its homology to LMP1:
Since Repeat Region I (RRI) of the CagY protein has homologous regions to LMP1, which is able to activate the Interferon pathway in host cells through interaction with IRF7, interaction between CagY and IRF7 should be investigated. The focus was on CagY-dependent nuclear translocation of the transcription factor IRF7 and on their possible direct protein-protein interaction. Also, the impact of the suggested interaction on Interferon pathway, such as the ability of this interaction to start the pathway by promoter activation and downstream cytokine secretion of IFNβ, was examined. In parallel, possible CagY-dependent activation of the NF-κB pathway resulting in the production of IL-8 in the host cells should be examined, since LMP1 is able to activate the NF-κB pathway.

2 Material and Methods

2.1 Materials

2.1.1 Chemical compounds

2.1.1.1 Reagents

Ethidium bromide (Sigma), $MgCl_2$ (2mM) (Thermo Fisher, Thermo Scientific, NEB), DMSO (Sigma), methylene blue (Sigma), PFA (Sigma), mounting media (Invitrogen™ ProLong™ Gold), 100%-ethanol (Roth), 70%-ethanol, x-gal (Roth), Bis-Acrylamide (Rotiphorese® NF-Acrylamid/ Bis-Lösung 30% (29:1)) (Roth), ammonium persulfate (Roth), N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma), 2,2,2-Trichlorethanol (Aldrich), isopropanol (Roth), Tween 20 (Sigma), Phalloidin Texas Red (1:2000) (Life Technologies), Phalloidin Alexa 647 (Life Technologies), DAPI (1:10000) (Sigma), IL-8 Standard (800pg: 10µl with 80 pg/µl) (BD), Triton 100x (Merck), Type F immersion oil liquid (Leica), DNA water (Roth), distilled water, HBSS (Hank balanced salt solution) (gibco), PI (propidium iodide) (Miltenyi Biotec), Q5 high CG enhancer (NEB), glycerol (Roth), phenol-chloroform (1:1) (Roth); NaAc (Roth), EDTA (Roth), HCl (Roth), methanol (Roth), H_2SO_4 (Roth), phosphoric acid (Roth), IFNβ Standard (Immunotools); dNTPs (Thermo Fischer), agarose (Roth), bovine serum fraction (BSA) Fraction V (biomol), powdered milk (Roth)

2.1.1.2 Buffers and Solutions

2.1.1.2.1 *Buffers*

HiFi buffer 10x (Thermo Fischer), Phusion buffer 5x (Thermo Scientific), Tango buffer 10x (Fermentas), Tango 2x (Fermentas), T4 ligase buffer (10x) (Fermentas/ NEB), Q5 reaction buffer (NEB), buffer Orange (Fermentas)

Agarose gel loading buffer (6x):	0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 30% (v/v) glycerol; TAE 1x to be added to reach final volume
TAE buffer 50x:	242 g/l Tris; 57.1 ml/l acetic acid; 50 mM EDTA; pH 8
Cracking buffer (5x):	sucrose (saccharose) 25g; sDDW (distilled water) to be added until the mixture is 40ml to dissolve the sucrose at 65°C; 5 M NaOH 5ml and 10% SDS buffer 2.5ml to be added; sDDW to the mixture until the volume reaches 50ml

For Western Blot:

2x single- gel buffer:	152 mM Tris-HCl; 0.2 M serine; 0.2 M Glycine; 0.2 M asparagine; pH 7.4
2x SDS buffer (for loading):	100 mM Tris, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol
2x SDS buffer (for loading) with mercaptoethanol	100 mM Tris, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; 10% β-mercaptoethanol
5x SDS running buffer	25 mM Tris; 250 mM glycine; 0.1% SDS; pH 8.3
Anode I buffer:	300 mM Tris; 10% methanol; pH 10.4
Anode II buffer:	25 mM Tris; 10% methanol; pH 10.4
Cathode buffer:	25 mM Tris; 40 mM 6- aminocaproic acid; 10% methanol; pH 9.6
Blocking buffer:	TBS buffer+ 3% BSA
Washing buffer:	total volume of 2 liters: 200ml TBS 10x buffer+ 0.075% Tween 20 + distilled water
10x TBS buffer:	1.5 M NaCl; 200 mM Tris; pH 7.5
10x Stripping buffer:	250 mM glycine; 10% (w/v) SDS; pH 2

For ELISA:

Coating buffer IL-8 ELISA (& IFNβ ELISA protocol 2):	100 mM Na_2HPO_4; pH 9. 6
Coating buffer IFNβ ELISA (protocol 1):	0.1 M sodium bicarbonat buffer; 3.56g Na_2CO_3 add H_2O to 1 l; pH 9.5
Wash buffer IL-8 & IFNβ ELISA:	PBS+ 0.05% Tween-20
ELISA buffer IL-8 & IFNβ ELISA:	50 mM Tris/ HCl ; pH 7. 6

2.1.1.2.2 *Solutions*

PBS 10x:	2 g/l KCl; 80 g/l NaCl; 2 g/l KH_2PO_4; 14.4 g/l Na_2HPO_4*2 H_2O or 28.9 g/l Na_2HPO_4*12 H_2O
PBS*:	PBS buffer; Leucin (1:1000); Pepstatin (1:1000); PMSF (1:100)
PMSF:	100 mM PMSF in isopropanol
$T_{10}E_1$:	10mM Tris; 1mM EDTA; pH 8.5
GEBS	20% glycerol; 50 mM EDTA; 0.05%

bromophenol blue; 0.5% sarkosyl; pH 8

DPBS 1x (Dulbecco's Phosphate Buffered Saline) (gibco)

2.1.1.3 Kits

Chromosomal DNA extraction	QIAamp® DNA Mini Kit (250) (Qiagen)
Plasmid DNA extraction:	QIAprep® Miniprep QIAprep® Midiprep

mi-Gel Extraction (metabion)

Champion™ pET151 Direktionales TOPO™ Expressionskit mit BL21 Star™ (DE3) One Shot™ Chemisch kompetente *E. coli* (Invitrogen)

Immobilon™ Western, Chemiluminescent HRP Substrate Peroxide Solution (Millipore)

illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare)

Dual Luciferase Kit (Promega)

Vectastain ABS Kit, Peroxidase Standard PK 4000 (Biozol)

TMB Substrate Reagent Set (BD OptEIA™)

2.1.1.4 Ladders

ladder for protein gel:	Precision Plus Protein™ Standards All Blue (BioRad)
ladder for agarose gel:	GeneRuler 1kb DNA ladder (Thermo Scientific™)

2.1.2 Bacteria

2.1.2.1 *Escherichia coli* strains

Top10	(Thermo Fisher)
BL21 Star™ (DE3)	(included in kit Champion™ pET151 Direktionales TOPO™ Expressionskit mit BL21 Star™ (DE3) One Shot™ Chemisch kompetente *E. coli*) (Invitrogen)

2.1.2.2 *H. pylori* strains

Name	Characteristics	Reference
P12	Strain isolated from a patient with duodenal ulcer in Germany	[130]
P12ΔcagA	Deletion of *cagA*-sequence in P12 strain	lab group

P12ΔcagY	Deletion of *cagY*-sequence in P12 strain	lab group
P12ΔcagE	Deletion of *cagE*-sequence in P12 strain	lab group
KG-HP2	Deletion of *cagY*-sequence in P12 strain	Katrin Gerrer
26695	Strain isolated from a patient with gastritis in the United Kingdom	[56]
26695Δhp0547	Deletion of *cagA*-sequence in 26695 strain	lab group
26695Δhp0527	Deletion of *cagY*-sequence in 26695 strain	lab group
26695Δhp0544	Deletion of *cagE*-sequence in 26695 strain	lab group
26695ΔCagPAI	Deletion of *cagPAI* region in 26695 strain	lab group

2.1.3 Cell lines

Cell type	Characteristics	Media
AGS	Human adenogastric carcinoma cell line; from ATCC	RPMI Medium 1640 (1x) (gibco® by life technologies™) +10% FCS; PenStrep (1:100)
AGS pEYFP-IRF7 #4	AGS cells transfected with plasmid for expression of EYFP-IRF7-fusion proteins in eukaryotic cells pEYFP-IRF7 clone 4, Kanamycin-resistance	RPMI Medium 1640 (1x) (gibco® by life technologies™) +10% FCS; PenStrep (1:100); G418 (1:50)
AGS pEYFP-IRF7 #5	AGS cells transfected with plasmid for expression of EYFP-IRF7-fusion proteins in eukaryotic cells pEYFP-IRF7 clone 5, Kanamycin-resistance	RPMI Medium 1640 (1x) (gibco® by life technologies™) +10% FCS; PenStrep (1:100); G418 (1:50)
HEK 293	Human embryonic kidney cell line; from DSMZ	DMEM 1x (Dulbecco's modified Eagle Medium) (gibco® by life technologies™) + 15% FCS + 1% L-glutamine 200mM

HEK CC1	HEK 293 expressing CEACAM 1, from lab group	DMEM 1x (Dulbecco's modified Eagle Medium) (gibco® by life technologies™) + 15% FCS + 1% L-glutamine 200mM; G418 (1:100)
HEK CC5	HEK 293 expressing CEACAM 5, from lab group	DMEM 1x (Dulbecco's modified Eagle Medium) (gibco® by life technologies™) + 15% FCS + 1% L-glutamine 200mM; G418 (1:100)
Jurkat	Human T- cell leukemia cells, from DSMZ	RPMI Medium 1640 (1x) (gibco® by life technologiesTM) +10% FCS; PenStrep (1:100)
HeLa	Cervix carcinoma cells, from DSMZ	RPMI Medium 1640 (1x) (gibco® by life technologies™) +10% FCS; PenStrep (1:100)

2.1.4 Media

Bacteria:

Brucella Broth (BD), LB Agar (Lennox L Agar) (Invitrogen), GC Agar Base (Oxoid), SOC media (Life Technologies)

Freezing media (for *Hp*): 35ml Brucella Broth, 5ml fetal calf serum (not heat inactivated), 10ml glycerol, all filtrated through a 0.22µl filter into a 50ml falcon

Cells:

AGS: RPMI Medium 1640 (1x) (gibco® by Life Technologies™) +10% FCS

HEK 293, HEK CC1, HEK CC5: DMEM 1x (Dulbecco's modified Eagle Medium) (gibco® by Life Technologies™) + 15% FCS + 1% L-glutamine 200mM

2.1.5 Antibiotics and Additives

Ampicillin (Sigma), kanamycin (Sigma), PenStrep (penicillin and streptomycin) (Thermo Fisher)

Antibiotics	Resolvent	Concentration for *E. coli* in mg/l	Concentration for *H. pylori* in mg/l
Ampicillin	water	100	/
Kanamycin	water	50	8

Table 1: Antibiotics

vitamin mix — 100 g/l α-D-glucose; 10 g/l L-glutamine; 26 g/l L-cysteine; 1.1 g/l L-cystine; 0.15 g/l L-arginine; 0.1 g/l cocarboxylase; 3 mg/l thiamine; 0.5 g/l uracil; 13 mg/l p-aminobenzoic acid; 0.25 g/l nicotinamide adenine dinucleotide; 10 mg/l vitamin B12; 1 g/l adenine; 30 mg/l guanine; 20 mg/l iron(III) nitrate

G418 disulfate salt solution (Geniticin 50ng/ml) (Sigma), Lipofectamine® 2000 Reagent (Invitrogen), Opti-MEM® (gibco® by Life Technologies), RNAiMax (Thermo Fisher), recombinant human TNFα (PreproTech) (in PBS final concentration 10µg/ml), fetal bovine serum (FCS) (Thermo Fisher) at 56°C for 20 minutes heat inactivated, L-glutamine (Life Technologies), cholesterol (Thermo Fisher)

2.1.6 Primers

Name	Sequence 5‘ to 3‘	Reference
pEGFP-FP	TTTAGTGAACCGTCAGATC	commercial primer from GATC
pEGFP_C2-FP	GATCACATGGTCCTGCTG	commercial primer from GATC
pEGFP_C2-RP	TTTAAAGCAAGTAAAACCTC	commercial primer from GATC
CMV-F	CGCAAATGGGCGGTAGGCGTG	commercial primer from GATC
LJ 136	CACCATGAATGAAGAAAACGATAAACTTGAAACT	Jimenez-Soto
LJ 132	CTCGAGttatttttcgaactgcgggtggctccaATTGCCACCTTTGGGGCTTGTGGTGATTTCTT	Jimenez-Soto
LJ 134	CACCggatccATGAATGAAGAAAACGATAAACTTGAAACTTCTAAAAAAG	Jimenez-Soto
LJ 214	CAG TAA GCT TCG ATG GCC TTG GCT CCT GAG AGG	This work+ LJ
LJ 215	CTG AGG TAC CCT AGG CGG GCT GCT CCA GCT CC	This work+ LJ
LJ 223	CAGTAAGCTTcgGACGAACTGTTCCCCCTCATCTTCCCGGCAGA	Jimenez-Soto
LJ 224	AGTCGGTACCTTAGGAGCTGATCTGACTCAGCAGGGCTGA	Jimenez-Soto

LJ 216	AACAACCCCTTCCAAGAAGAGCAGCGTGGGGAC	This work+ LJ
LJ 217	GTCCCCACGCTGCTCTTCTTGGAAGGGGTTGTT	This work+ LJ
LJ 227	CAGTGGATCCATGGCCTTGGCTCCTGAGAGG	This work+ LJ
LJ 228	CGATGCGGCCGCCTAGGCGGGCTGCTCCAGCTC CATAAGG	This work+ LJ

2.1.7 Plasmids and Vectors

Plasmids	Characteristics	Reference
pRLNull	Commercial, ampicillin resistance	Promega
p125-luc	Insertion of Interferon β promoter [131]	[132]
pELAM-luc	Insertion of E-selectin promoter [133]	[134]
pGFP-RelA	Plasmid pEGFP-C1 with RelA-sequence [135], kanamycin resistance	[136]
pRenilla-IRF7	Plasmid pRenilla with IRF7-sequence, ampicillin resistance	Luisa Jimenez-Soto
pHDN1 (pEYFP-IRF7)	Insertion of IRF7 into pEYFP-C1, kanamycin resistance	This work
pRFPLamin	Insertion of fluorescent-marked protein binding to the Lamin of the nuclear membrane	Xaver Sewald

Vectors	Characteristics	Reference
pEYFP-C1	Commercial 4.7 kbp, kanamycin resistance	Clontech
pECFP-C1	Commercial 4.7 kbp, kanamycin resistance	Clontech
pET151	Commercial (included in kit), ampicillin resistance	One Shot™ Chemisch kompetente *E. coli* (Invitrogen)
pGEX-4T-3	Commercial, 4.97 kbp, ampicillin resistance	GE Healthcare

2.1.8 siRNA

control siRNA: AllStars Negative Control siRNA (Qiagen)

siRNA	Sequence 5‘ to 3‘	Reference
S2-IRF7-Bosco2016	CUGGAAGCACUUCGCGCGCAA	Commercial from Eurofins Genomics

2.1.9 Enzymes

Proteinase K (Sigma), HiFi polymerase (Thermo Fisher), Phusion High-Fidelity DNA polymerase (Thermo Scientific), Q5 polymerase (NEB), Trypsin-EDTA (TE) (Thermo Fisher), HindIII (Fermentas), KpnI (Fermentas), T4 ligase (Fermentas/ NEB), RNase (Sigma), Leucin (Sigma), Pepstatin (Sigma), BamHI (Fermentas), NotI (Fermentas), DpnI (Fermentas), HindIII (NEB), KpnI (NEB), NdeI (Fermentas)

2.1.10 Antibodies

2.1.10.1 ELISA

ELISA coating antibody	purified mouse anti-human IL-8 (BD) anti-human IFNβ antibody (Acris/ OriGene)
ELISA detection antibody	biotinylated anti-IL-8 (human) (BD) biotinylated anti-IFNβ (Acris/ OriGene)

2.1.10.2 First antibodies

Western Blot

Anti-CagA 257:	polyclonal antibody against C-terminal part of CagA (rabbit) [28]
Anti-CagY 273:	polyclonal antibody against the Repeat Region II of CagY from *H. pylori* (rabbit) [59]
Anti-IRF7:	Abcam ab109255, aliquoted to 1.7mg/ml

Cell staining

Ms mAB to panLamin ab20740 (abcam)

2.1.10.3 Second antibodies

Western Blot

Anti-rabbit-pox (Sigma)

Cell staining:

Alexa Flour® 555 goat anti-mouse IgG (Life Technologies)

2.1.11 Instruments

UV-GelDoc (BioRad); PCR cyclers (Eppendorf, Thermo, peqlab); NanoDrop® (peqlab); incubator for *E.coli* (37°C) (binder); incubator for *H. pylori* for short time (37°C, 10% CO_2) (Thermo Scientific); incubator with microaerophilic conditions (37°C, 5% O_2, 85% N_2, 10 % CO_2) (Scholzen); centrifuges: mini centrifuge (neoLab), table centrifuges (Hettich Zentrifugen & Thermo Scientific), swing rotor centrifuges (Thermo Scientific & Heraeus), centrifuge for PCR tubes (Nippon Genetics Europe); SP5 microscope (Leica); light microscope (hund); Protein GelDoc (BioRad); shaking platform (GFL); shaking platform in an incubator (Infors HT); balance (Fischer); DNA SpeedVac (Savant); direct reading

spectrophotometer (Hach); vortex machine (Vortex-Genie 2 from Scientific Industries); Thermomixer (Eppendorf); microwave (AEG); voltage source (Power Pac 1000)/ (Power Pac 300) (BioRad); water bath small and big (FGL); round spirit level (BioRad), pipettes (for 1000µl, 250µl, 20µl, 10µl) (Gilson); electric pipette (accu-jet® pro) (Brand); multichannel pipette (Thermo); ELISA plate reader (Tecan); stir bar (Omnilab), magnetic stirrer with heating plate (Heidolph); bunsen burner (WLD-TEC.); loop (Barkey); Drigalski glass spatula (Omnilab); Western Blot roller (Assistent, Karl Hecht); fridge (Liebherr); freezer (-20°C) (Liebherr, Siemens); freezer (-70°) (Thermo Scientific); paper cut system (Dahle); light box (Kaiser); fume hood (Kottermann); bench (BDK Luft-und Reinraumtechnik GmBH); glass bucket with indentations (Merck); SDS-gel plate system (BioRad) with spacer (1mm) + glass plate (biostep) with comb (25, 15); electrophoresis chamber (BioRad), blotting chamber (Fischer Biotec); plastic beaker (VitLab); plastic cylinder (VitLab); incubator for infected cells (37°C, 5% CO_2) (Binder); cell incubator (37°C, 5% CO_2) (Binder); cell counter (Casy®); glass bottles and glass bucket (Schott); CLARIOstar (BMG Labtech); agarose gel form + comb (Bio-Rad); flow sorter (BD); tweezers (Omnilab)

transparencies (Kabuco); tissue culture flask (75cm^2) (Falcon®); cell culture plates (6-well-plate, 12-well-plate, 24-well-plate, 48-well-plate) (Corning Incorporated); microscope cover glasses (A. Hartenstein); microscope slides (Menzelgläser); 1.5ml safe-lock tubes/ microcentrifuge tube (Eppendorf); cotton swab (deltalab); scalpel (Braun); petri dish (greiner bio-one); tips for the pipettes (Sarstedt); glass tips for the pipettes (EM); falcon (25ml, 50ml) (greiner & sarstedt); 96-well plate (NUNC); Parafilm (Bemis); 0.2ml PCR tubes (Brand); plates with deep wells to keep supernatant for ELISA (Omnilab); gloves (Safegrip® Nitril 25cm); disposable cuvettes (Brand); Millex® GP filter (0.22µm) (Millipore); syringes (60ml (infuject®), 10ml (Braun)); Pasteur pipettes (Assistant, Karl Hecht); tips for electric pipette (costar® stripette® 50ml, 25ml, 10ml, 5ml, 2ml, 1ml) (Corning Incorporated); tips for multichannel pipette (Sarstedt); PDVF-membrane (Millipore); Whatman blotting paper (Omnilab); dry ice

2.2 Methods

2.2.1 Working with bacteria

2.2.1.1 Defrosting of *E. coli*

To defrost *E. coli* a tube was taken out of the -70°C-freezer. The frozen bacteria solution was scratched with a loop that had been sterilized before by heating up. The piece of ice was then spread on the agar plate. During the whole process the bacteria tube was kept in dry ice. The plate was incubated in the incubator (37°C) for 2 days, normally over the weekend, until the bacteria could be passed on a new plate.

2.2.1.2 Defrosting of *H. pylori*

To defrost *H. pylori* a tube was taken out of the -70°C-freezer. The frozen bacteria solution was scratched with a loop that had been sterilized before by heating up. The piece of ice was then spread on the agar plate. During the whole process the bacteria tube was kept in dry ice. The plate was incubated in the incubator with microaerophilic conditions (37°C, 5% O_2, 85% N_2, 10 % CO_2) for 2 days, normally over the weekend, until the bacteria could be passed on a new plate.

2.2.1.3 Culture of *E. coli*

Once defrosted, the bacteria cells have to be passed on a fresh plate every day. To pass the bacteria, a loop was used to pick a little pellet from the agar plate and then to distribute the pellet on the new plate. By doing this the loop was moved in circles over the agar. To avoid contamination of *E. coli* with other bacteria the loop was sterilized before using by heating up and then cooled down by waiting some seconds and putting the loop into the agar. Depending on the selection aimed, agar plates with different antibiotics were taken. After having passed the bacteria, the new plate was placed into the incubator until the next day to be passed again. The old plate was discarded into a special container for bacteria.
To do experiences with the bacteria the cells were passed two times after defrosting to be sure that they are fit.

2.2.1.3.1 Plates

The plates to grow *E. coli* were poured with LB Agar. Therefore glass bottles were filled with 16mg LB agarose and 500ml distilled water. Then they were given to the autoclave team of the institute to be autoclaved. After cooling down the liquid agar and before pouring the agar into plates, antibiotics could be added, e.g. ampicillin, kanamycin, etc. To eliminate bubbles after pouring the plates were flamed shortly.

2.2.1.3.2 Liquid media

To culture *E.coli* in liquid culture, 5ml of Brucella Broth media was put in one glass tube. A pellet of bacteria was taken from the agar plate with a cotton swab and resuspended in the media. Depending on the selection aimed, the corresponding antibiotic was added. The tube was incubated in the incubator with a shaking platform at 37°C and 180rpm until the next day. The bacteria then had to be passed into a new liquid culture. Therefore 50µl of bacterial solution was pipetted in another tube with fresh media.

To culture bacteria in order to isolate DNA with a midi-prep kit a big pellet of bacteria was needed. To have this amount of bacteria *E.coli* was cultured in a glass bucket with four special indentations. Those indentations strengthened the swirling up effect of the shaking platform that more air could enter the mix to support the growth of the bacteria.

2.2.1.4 Culture of *H. pylori*

The bacteria had to be passed on a new plate every day. Therefore a loop was used to pick up a little of the grown bacteria from the agar plate to spread it on the new plate as it was done for *E.coli*. Distributing the pellet on the new plate the loop was moved in circles over the agar. To avoid contamination of *H. pylori* with other bacteria the loop was sterilized before using by heating up and then cooled down by waiting some seconds and putting the loop into the agar. Depending on the selection aimed, agar plates with different antibiotics were taken. After having passed the bacteria, the new plate was placed into the incubator with microaerophilic conditions (37°C, 5% O_2, 85% N_2, 10 % CO_2) until the next day to be passed again. The old plate was discarded into a special container for bacteria. To store *H. pylori* for a short time of up to 1 hour (e.g. until the next experiment) the bacteria plates could be stored in another incubator at 37°C, 10% CO_2.
To do experiences with bacteria cells they were passed two times after defrosting to be sure that they were fit.

2.2.1.4.1 Plates

H. pylori was cultured on cholesterol plates. Therefore 18g of GC Agar Base is mixed with 500ml of distilled water and autoclaved. Before pouring the plates, the mixture had to be cooled down to add 2ml of cholesterol, 5ml of vitamin mix and eventually antibiotics. Everything was mixed well by using a stir bar before pouring. To eliminate the bubbles the plates were flamed.

2.2.1.5 Freezing of *E. coli*

Freezing as pellet:
To freeze bacteria from a plate the whole bacteria on the plate was taken with a cotton swab and resuspended in PBS. Then the microcentrifuge tube was centrifuged for 3 minutes at 8000rpm. Supernatant was discarded and the pellet frozen in -20°C.
To freeze bacteria from liquid culture 1ml of the bacterial liquid culture was applied to one microcentrifuge tube. The tube was centrifuged for 3 minutes at 8000rpm. Afterwards, the supernatant was discarded and the pellet was stored in -20°C.

As solution:
Bacteria were collected with cotton swab an put into a tube already filled with 500µl of Brucella Broth media and 50µl with 70%-glycerol. The tube was immediately frozen in dry ice and then stored in the -70°C freezer.

2.2.1.6 Freezing of *H. pylori*

Bacterial cells were collected with a cotton swab and resuspended in a tube which was filled with 1ml of freezing media. The solution has to be well mixed. The tubes were immediately frozen in dry ice before storing in -70°C.

2.2.1.7 Transformation of *E. coli*

The *E. coli* strains for transformation were the Top10 strain and the BL21 Star™ (DE3) strain. The bacteria pellets were stored in -70°C.
First the ligation/ plasmid had to be added to the bacteria pellet. The mixture was then incubated in ice. After 30 minutes the bacteria cells were heat-shocked in a water bath with 42°C for 45 seconds and then put in ice immediately. 1ml of Brucella Broth media was added and the bacteria cells were incubated at 37°C for 55 minutes. The tube was centrifuged for 12 seconds to collect all the cells at bottom of the tube. The supernatant was discarded. The pellet was dissolved in the liquid still left in the tube. The mixture was to be distributed on a LB Agar plate. Depending on the resistance against antibiotics encoded on the transformed DNA, the corresponding agar plate containing this antibiotic had to be taken in order to select only the transformed bacteria cells.

Transformation of the CagY gene:
For the growth of bacteria transformed with the CagY gene in plasmid pET151 the LB agar plate with ampicillin needed special preparation. The plates got 50µl of x-gal (20 mg/ml) on the surface and were incubated at room temperature in the dark to dry. This reagent would be turned over into a blue color by bacteria which do not contain the right vector with the *cagY* gene inserted. To continue the white colonies having the right plasmid were picked and obliterated on a new plate.

2.2.1.8 Measuring of the optical density (OD)

The bacteria were collected with a cotton swab and suspended in 700µl of PBS. The bacterial solution was diluted 1:100 (10µl of this bacterial solution added to 990µl PBS) to measure the OD with a photo spectrometer. The measurement was done with a wave length of 550nm (OD_{550}) in a plastic cuvette with a diameter d= 1cm against the blank (1ml of PBS).

2.2.1.9 Making bacterial cell lysates

Bacteria was collected with a cotton swab from an agar plate and resuspended in PBS. The microcentrifuge tube containing the cells was centrifuged for 4000rpm for 10 minutes. The supernatant was exhausted and replaced by 20µl of PBS* and 25µl of 2x SDS. The cells were boiled at 95°C for 10 minutes and placed in ice immediately to freezing in -20°C.

2.2.2 Working with DNA

2.2.2.1 Isolation of chromosomal DNA out of bacteria

For extraction of chromosomal DNA out of bacteria the QIAamp® DNA mini Kit (250) (Qiagen) was used.
To prepare the extraction, first, a pellet of bacteria was collected from the plate by using a cotton swab. The pellet was resuspended in 600µl PBS buffer which was already filled in a microcentrifuge tube. The tube was centrifuged at 5000rpm for 10 minutes. The supernatant was discarded.
The DNA was extracted as it is described in the instructions of the kit:

For a pellet of bacteria cells taken from one plate 180µl of ATL buffer is needed to dissolve the pellet. Then 20µl of proteinase K was added. After vortexing, the tube was put on a thermomixer (3 minutes of shaking followed by a 2-minutes episode of pause) and incubated at 56°C until the pellet was completely lysed. 4µl of RNase (concentration 100 mg/ml) was added to the solution. Then the tube was vortexed for 15 seconds and incubated for 2 minutes at room temperature.
The tube was shortly centrifuged and 200µl of AL buffer was added. The mixture was vortexed for 15 seconds and then incubated at 70°C for 10 minutes. Again the tube was shortly centrifuged and 200µl of 100%-ethanol was added, the tube was then vortexed for 15 seconds and shortly centrifuged.
The whole mixture was applied to the QIAamp Mini spin column which then was put into a 2ml collection tube (both included in the kit). This construction was centrifuged at 6000g for 1 minute. The tube containing the filtrate was discarded and the spin column was placed in another clean 2ml collection tube. 500µl of AW1 buffer was added to the spin column which again was centrifuged at 6000g for 1 minute. The tube containing the filtrate was discarded. 500µl of AW2 buffer was added and the tube was centrifuged at 13000rpm (the kit instruction was 14000rpm) for 3 minutes and again for 1 minute to eliminate the entire AW2 buffer.
The tube with the filtrate was discarded and the spin column was placed into a new microcentrifuge tube. 200µl of AE buffer was added. The tubes were incubated for 1 minute and then centrifuged for 1 minute at 6000g (8000rpm). The extracted chromosomal DNA was ready to use or stored in -20°C.

2.2.2.2 Isolation of plasmid DNA out of bacteria

To isolate plasmid DNA out of bacteria there are kits in different sizes available. Depending on the amount of plasmid DNA to extract the mini-prep, midi-prep and maxi-prep kits can be chosen.
Using the kit QIAprep® Miniprep plasmid DNA was extracted out of bacteria cells. The mini-prep is to isolate small DNA amounts, e.g. to clone vectors. The pellet had to be resuspended in 250µl of P1 buffer (containing RNase). After having added 250µl of buffer P2 the content was well mixed before adding 350µl of N3 buffer. The tube was mixed well and centrifuged for 10 minutes at 13000rpm. The supernatant was transferred to a clean microcentrifuge tube. 500µl of isopropanol was added and the tube again was mixed and centrifuged for 10 minutes at 13000 rpm. Then the supernatant was discarded and 500µl of 70% ethanol was added. Again the tube was centrifuged, for 5 minutes at 13000rpm. The supernatant was discarded. The little pellet inside the tube was dried in the SpeedVac. The dried pellet was dissolved in 100µl $T_{10}E_1$.

To isolate bigger amounts of plasmid DNA, e.g. in order to transfect them, QIAprep® Midiprep was needed. The DNA was washed with chloroform to get the DNA completely clean. So the transfection into cells can work much better.
Since the number of cells from agar plates was not enough, the bacteria pellet to prepare had been taken from a 50ml-liquid culture. The kit from Qiagen was constructed for 25ml-liquid cultures so the amount of the reagent was doubled. First, the pellet was resuspended in 8ml of P1 buffer. After 8ml of P2 buffer was added, the falcon was well

mixed by inverting several times and incubated at room temperature for 5 minutes. After addition of 8ml prechilled P3 buffer the tube was again well mixed and incubated on ice for 15 minutes. Then it was centrifuged at more than 20000g for 30 minutes at 4°C. Meanwhile the Qiagen-tip 100 (included in the kit) was equilibrated with 8ml of QBT buffer. After the 30 minutes of centrifugation the supernatant was applied to the Qiagen-tip and passed by gravity flow. The Qiagen-tip was washed two times with 10ml of QC buffer, also by gravity flow. DNA was eluted with 5ml of QF buffer. The last three steps were done twice because the reagents used were doubled and could not fit into the tip to run the steps only one time. Using 7ml of room-temperature isopropanol the DNA was precipitated. Then the falcon was centrifuged at more than 15000g for 30 minutes at 4°C. The supernatant was discarded, the pellet washed with 4ml of room-temperature 70%-ethanol and centrifuged at more than 15000g for 10 minutes. The supernatant was discarded and the pellet was air-dried, usually overnight.
The next day, 350µl of $T_{10}E_1$ was applied to the dried DNA pellet. It took some day for the pellet to be resuspended.
The phenol chloroform extraction / ethanol precipitation was used to clean the plasmid DNA and concentrate it. First, the plasmid DNA solution was transferred to a 1,5ml tube. The tube was vortexed to mix the DNA solution with the phenol-chloroform (1:1) solution with a relation of 1:1. Then the tube was centrifuged for 10 minutes, 14000rpm at room temperature. From the two phases in the tube, the upper phase, consisting of TE and water, was taken to a new collection tube. 1/10 of volume of 3 M NaAc and 2,5 of volume of 100%-ethanol were applied and mixed by inversion. After having centrifuged the tube for 1 minute, 14000rpm at room temperature the supernatant was discarded, the pellet washed with 300µl of 70% ethanol and again centrifuged for 1 minute. The ethanol was removed with a pipette. $T_{10}E_1$ 10% solution (or water) was added. The tube was left at room temperature for 20-42 minutes until the pellet was resuspended without vortexing.

2.2.2.3 PCR

2.2.2.3.1 Amplification of *cagY*

Because of the large size of 5700bp the amplification of this DNA part had to be done with a 2-step-PCR. Therefore a sample mixture of 50µl in total was prepared. In the mixture there was 1µl of chromosomal DNA, 0.1µl of the forward and 0.1µl of the reverse primer, 5µl of HiFi buffer (10x), 1µl of dNTPs, 0.25µl of HiFi polymerase. Then the sample was filled with DNA water up to 50µl. To amplify *cagY* out of chromosomal DNA isolated from *H. pylori* strain 26695 the primer pair LJ134 and LJ132 was used. To amplify *cagY* out of chromosomal DNA isolated from *H. pylori* strain P12 the primer pair LJ136 and LJ132 was used.
The amplification process which was programmed in the PCR machine:

The first step:	initial denaturation	94°C for 3 minutes
The second step:	denaturation	94°C for 30 seconds
The third step:	annealing	48°C for 30 seconds
The fourth step:	extension	68°C for 6 minutes

This step lasts 6 minutes following the instruction of the HiFi polymerase that says 1 minute per kbp. The circle between step 2 and 4 was repeated 10 times.

The fifth step:	denaturation	94°C for 30 seconds
The sixth step	annealing	48°C for 30 seconds
The seventh step	extension	68°C for 6 minutes with an autoextension of 2 seconds per cycle

The process between step 5 and step 7 was repeated 20 times.

The eighth step	final extension	68°C for 10 minutes

The PCR reaction was hold at 10°C before taking out of the cycler.
After the amplification, the samples were stored in -4°C.

Since the described cycling program did not always work one modification was tried: the annealing temperature was reduced to 47°C.

2.2.2.3.2 Amplification of IRF7

The size of the sequence to amplify is 1512 bp. The total volume of one reaction is 25µl. 0.5µl of the plasmid pRenilla-IRF7 out of BL21 Star™ (DE3) was taken as template to be amplified with the primers LJ214 and LJ215 (each 0.1µl). 2.5µl HiFi buffer, 1µl of dNTPs, 0.2µl HiFi polymerase, 2.0µl $MgCl_2$ and 18.9µl of DNA water were added. The mix was placed into a cycling machine to run the following program:

The first step:	initial denaturation	95°C for 4 minutes
The second step:	denaturation	95°C for 30 seconds
The third step:	annealing	53°C for 30 seconds
The fourth step:	extension	72°C for 1.5 minutes

The cycle from step 2 to 4 was repeated 34 times.

The fifth step	final extension	72°C for 10 minutes

The PCR reaction was hold at 10°C before taking out of the cycler.
After the amplification, the samples were stored in -4°C.

When IRF7 was to be amplified out of cDNA, 2µl of DNA was taken. Corresponding to the increase of the template amount the DNA water amount was reduced. Since the IRF7 had to be cloned into the pGEX-T4-3 vector the primer pair LJ227 and LJ228 was taken.

The HiFi polymerase ran out, so the new Q5 polymerase was used with a modified protocol: For the PCR mix: 0.5µl pDNA or 2µl chrDNA, 0.25µl primers, 5µl Q5 reaction buffer (5x), 8µl dNTPs, 5µl Q5 high CG Enhancer, 0.25µl Q5 polymerase. The cycling program was kept except for the annealing temperature, which was changed to 63°C.

2.2.2.3.3 Amplification of RelA

4µl of 5x Phusion buffer, 0.4µl of dNTPs, 0.5µl of each primer (LJ216 and LJ217), 0.5µl of the plasmid pGFP-RelA as template, $MgCl_2$ (1mM, 1.5mM and 2mM were tested) and 0.2µl of Phusion DNA polymerase were mixed in a PCR tube. DNA water was added up to a total volume of 20µl.
The polymerase chain reaction was run with the following program:

The first step	98°C for 30 seconds
The second step	98°C for 5 seconds
The third step	70°C for 10 seconds
The fourth step	72°C for 3.5 minutes

Step 2 to 4 were repeated 32 times.

The fifth step	72°C for 10 minutes

The PCR reaction was hold at 10°C before taking out of the cycler. The tube was then stored in 4°C.

Since the PCR reaction was not successful several variations were tried: using GC buffer instead of HF buffer, decreasing the annealing temperature to 69°C.

Since the reparation of pGFP-RelA was not successful an amplification of RelA with cDNA as template was tried. The cDNA was a gift from Dr. Baldauf from the Virology Department of the Max-von-Pettenkofer-Institut München. The PCR reaction was set up with both primers LJ223 and LJ224 and the cycling program for IRF7-amplification.

2.2.2.4 Gel electrophorese

Pour agarose gel:
To control the size of a PCR product an agarose gel of 0.8% was necessary. For a total volume of 120ml mixture, TAE buffer was added to 0.96g agarose. The mixture was microwaved until the agarose powder was completely melted and the mixture clear. The hot liquid was cooled down with cold water and then poured into a form with a comb that would form the pockets in the gel. Afterwards, control of the form to be straight with a water balance.

Running PCR-products in agarose gel:
The gel was put into a chamber filled with TAE buffer. The ladder having the function of a leveling board that allows reading the size of the DNA fragments running through the gel was load into the first pocket. Before loading the samples into the following pockets they were mixed with the 6x loading buffer to get them visible and easier to apply to the pocket due to the weight of the loading buffer. Then the chamber was connected to a voltage source and ran for about 1 hour at 80V.

Detection under UV-light:
To see the DNA band in the gel, the gel was put into an ethidium bromide bath for 20 minutes to stain the DNA. Then the gel was placed in the GelDoc under UV-light to detect the stained bands. If the gel is intended to control the size of the DNA fragment it could be discarded right after the detection.

2.2.2.5 Extraction of DNA fragments out of agarose gel

If a cloning process is intended, clean DNA fragments without any enzymes and primers from the PCR reaction are needed. Running the PCR samples through an agarose gel as described above is a possible method to get clean DNA. In this case the pockets in the gel are bigger to contain a bigger amount of sample.
To detect the DNA bands, the gel was stained in a bath of 0.1% methylene blue for 30 minutes on a shaking platform. The shaking is to distribute the color equally on the gel. After 30 minutes the methylene blue could be discarded and the completely blue gel was cleaned in a water bath for 20 minutes, also on the shaking platform, to destain the gel itself to see the DNA bands. The water was then tipped away and the gel was put on a transparency and both on an illuminated platform. The DNA bands were cut out of the gel with a scalpel, put into microcentrifuge tubes, either to use right away or stored in -20°C.
To extract the DNA out of the agarose gel the kit “mi-Gel Extraction Kit” by metabion was used. First the piece of gel was weighted. Then the instruction of the kit was followed to extract the DNA, here shortly summarized:
First, 0.5ml of GEX buffer is to add to a 50-200mg-gel slice and then incubated in 60°C for 5 to 10 minutes until the gel is completely melted. Afterwards, 0.25 volume of isopropanol was added if the fragment was bigger than 4 kb. Then the melted gel was applied to the GP™ column that had been placed in a collection tube (both given in the kit). This construction was centrifuged for 1 minute at 10000g. In the following the columns were washed two times, first with 0.5ml of WN buffer, then centrifuged for 1 minute, second with WS buffer, then centrifuged for 1 minute. Again the column was centrifuged at full speed for 5 minutes. To collect the DNA the column was placed into a clean microcentrifuge tube, incubated for 2-3 minutes after the application of Elution Buffer and centrifuged for 2 minutes. The extracted DNA was stored in the fridge at 4°C.

2.2.2.6 Measurement of DNA concentration

For the measurement of DNA concentration in one solution, e.g. after DNA extraction, the NanoDrop was used. The machine is connected and controlled by a computer. The function “Nucleic acid” was activated and 1-1.5µl of water was loaded on a spot which is outmost to initialize the measurement. Then the spot had to be cleaned with a piece of

paper dipped in ethanol. To adjust the measurement a drop (1-1.5µl) of the liquid used for DNA dilution ("blank") was loaded on the spot. Thereafter, the samples could be loaded for the measurements, 1-1.5µl of each. Between every sample the spot had to be cleaned with ethanol.

2.2.2.7 Cleaning of PCR products

After the amplification of a DNA fragment, it has to be cleaned from buffer, enzymes and primers to continue on working with the products. To do that the kit "illustra™ GFX™ PCR DNA and Gel Band Purification Kit" was used.
500µl of Capture buffer type 3 was applied to up to 100µl sample. The mixture was put into a GFX MicroSpin™ column which was placed into a collection tube (both included in the kit). After 1 minute of centrifugation at 13000rpm, 500µl of the Washing buffer type I was added. After another minute of centrifugation and 1 minute of incubation of the column in 25µl of $T_{10}E_1$ the flow through with the DNA was collected in a microcentrifuge tube and stored at -20°C.

2.2.2.8 Digestion

To digest DNA fragments or plasmids, the DNA, restriction enzymes, the special buffer for those enzymes and distilled water were mixed together following instructions of the companies. In order to make them fit to each other, DNA-insert ends and vector sides were adapted by cutting them with the same pair of restriction enzymes, one for each side. The mixes were incubated at 37°C for 90 minutes.

2.2.2.9 Ligation

Using the enzyme T4 ligase + 10x buffer, the vector and the insert were mixed together with a relation of 100ng vector to 30ng insert. Water was filled in up to the total volume of 20µl. The ligation of vector and insert took place in 4°C overnight.

2.2.2.10 Amplification of plasmid DNA

Is the plasmid once created it had to be amplified by transforming it into bacteria (Top10). Two days after the transformation the bacteria cells were collected to perform extraction of plasmid DNA as described above. To control the right insertion via looking on the size of the created plasmid, it needed to be digested with one of the restriction enzymes used for its creation before running through an agarose gel. By doing this, the round plasmid DNA gets linearized.

2.2.2.11 DNA sequencing

The company to sequence the DNA strains in our work group is GATC Biotech.
The requirements for samples to sequence are the following: Plasmids 80-100 ng/µl and primers 5µM (5 pmol/µl). Both have to be sent in separate microcentrifuge tubes and each with an amount of 5µl. The difference between the sample amount and the final amount of 5µl was filled with distilled water.

2.2.2.12 Isolation of RNA

The RNA was isolated to make cDNA out of mRNA. As recommended in the kit from Promega “SV total RNA isolation system” at least $1.5x10^3$ to $5x10^6$ cells are needed. The already prepared pellet with $6x10^5$ Jurkat AEGG cells was induced with TNFα and incubated for 1 hour in 37°C, 5% CO_2. The extraction was done in an RNase-free spot. First, 175µl of RNA Lysis Buffer was added to homogenize the sample by mixing. After applicating of 350µl of RNA Dilution Buffer, the tube was mixed and heated for 3 minutes at 70°C. The tube was centrifuged for 10 minutes at up to 14000g at room temperature. The cleared lysate was then transferred to a fresh tube. 200µl of 95%-ethanol was added and mixed with the cleared lysate. The mixture was transferred to the Spin Basket Assembly and centrifuged for 1 minute. After discarding the flow-through, 600µl of RNA Wash Solution was added. Again, the tube was centrifuged for 1 minute and the flow-through discarded. 50µl of DNase was incubated for 15 minutes, then 200µl of DNase stop solution was applied to the spin column which was again centrifuged for 1 minute. The RNA was washed twice with 600µl and 250µl RNA Wash Solution and centrifuged for 1 and 2 minutes. The RNA was eluted into an Elution Tube by adding 100µl RNase-free water and centrifuging for 1 minute.

2.2.2.13 Cloning

Project cloning pEYFP-IRF7

In order to clone the sequence of IRF7 into the commercial plasmid pEYFP-C1, the IRF7 was first amplified with the primers LJ214 and LJ215 and pRenilla-IRF7 as template as described above.

The PCR product was cleaned from buffers and primers with the kit “illustra™ GFX™ PCR and Gel Band Purification”. Then the vector pEYFP-C1 and the cleaned PCR fragments were digested with the two restriction enzymes HindIII and KpnI in Tango buffer (10x).

The digested DNA sequences were load on agarose gel to be stained with methylene blue. After gel extraction with the kit “mi-Gel Extraction Kit” the components were ligated to each other with T4 ligase (10x) with 10x buffer. After the transformation in *E. coli* Top10 and selection on kanamycin plates, the plasmid pEYFP-IRF7 was isolated out of the bacteria cells. To test on the functionality of the plasmids, they were transfected to AGS and Hela cells and investigated under microscope concerning fluorescence. The IRF7 insert was sequenced with the primers GFP-C2-FP and GFP-C2-RP.

Project cloning GST-IRF7

In order to show interaction between IRF7 and CagY, the fusion protein was to be created to do pulldown experiments. The IRF7 sequence was amplified with LJ227 and LJ228 as primers and pRenilla-IRF7 or cDNA as template. The PCR products (1512 bp) were cleaned from buffer and enzyme with “illustra™ GFX™ PCR and Gel Band Purification”. To digest the vector pGEX-4T-3 (4968 bp) and the fragments the restriction enzymes BamHI and NotI with Tango (2x) buffer were used. After running in an agarose gel and staining with methylene blue the DNA fragments were isolated to be ligated to the vector overnight at 4°C with T4 ligase (relation of 100ng vector and 30ng insert). Then 10µl of the ligation was transformed to *E.coli* Top10. The bacteria solution was spread on an LB agar plate with selection of ampicillin resistance to be amplified.

Project repair of pGFP-RelA and cloning RelA in EYFP (EYFP-RelA)

In order to repair the wrong amino acids in the RelA sequence of the pGFP-RelA plasmid that was a gift from another laboratory the primer pair LJ216 and LJ217 were used to set up the PCR reaction. Before continuing, the reparation of the RelA sequence in the plasmid had to be verified. The PCR product was digested with DnpI to eliminate all template DNA. Using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit the PCR mixture was cleaned. Both ends of the PCR fragment were ligated with the enzyme T4 ligase. The plasmid was transformed into *E.coli* Top10 and incubated in SOC media. Then the bacteria were put on LB agar plates to be selected with kanamycin. The aim was to pick up single colonies to isolate only the plasmid which was corrected by the PCR reaction described above. To verify the repair region, sequencing was performed with primer GFP-C2-FP.

The sequence of RelA with a size of 1678 bp was amplified with PCR and cDNA was taken as template. The primers needed were LJ223 and LJ224. The following steps should have been cleaning the PCR-product from buffers and enzymes by using the kit "illustra™ GFX™ PCR and Gel Band Purification", digestion of the vector pEYFP-C1 and the PCR fragments with HindIII and KpnI, loading the isolated DNA on a 0.8%-agarose gel with big pockets to stain in methylene blue and destain in water, extraction of the DNA out of the gel with the kit "mi-Gel Extraction Kit", the ligation of vector and DNA sequence by T4-ligase with a 1:1 ratio (100ng vector and 30ng insert) overnight at 4°C, the transformation of the created plasmid into *E. coli* Top10, distribution on LB-plates with kanamycin, isolation of plasmid DNA out of bacteria, checking the correct insertion by sequencing the plasmid after having it linearized with a single-cutter enzyme.

Cloning of CagY into pET151

After having amplified CagY (5700 bp) with 2-step-PCR using the primers LJ136,132 for *H. pylori* strain P12 and LJ134, 132 for strain 26695*,* the PCR-product was cleaned from buffers, etc. Depending on the quality of the PCR product the following step was decided: If there was a clean band in the agarose gel stained with ethidiumbromid the PCR products could be prepared with "illustra™ GFX™ PCR and Gel Band Purification". If there was no single clear band, the PCR product was loaded on a 0.8%-gel with big pockets, cut and purificated from gel with the kit "mi-Gel Extraction Kit" as described above. The isolation out of the agarose gel was to make sure that the CagY that was going to be used had the right size. Using the kit Champion™ pET151 Direktionales TOPO™ Expressionskit mit BL21 Star™ (DE3) One Shot™ Chemisch kompetente E. coli (Invitrogen) the DNA fragments of the *cagY* gene were mixed with the salt solution included in the kit. After addition of the plasmid pET151, the whole mixture was incubated at 4°C for 30 minutes. Then the tube was put in ice until the plasmid pET151 with CagY was transformed into bacteria strain BL21 Star™ (DE3). Then 1ml of Brucella Broth media was added and the bacterial solution was incubated for 55 minutes at 37°C. Transformed bacteria were distributed on pre-treated ampicillin LB-plates with x-gal and incubated at 37°C (see 2.2.1.7).

In order to isolate the DNA out of the bacteria cells cracking buffer was used. To final the cracking buffer before use, 6x loading buffer and GEBS were added. The final buffer mix was mixed with a bacteria colony resuspended in P1 buffer with RNase (1mm colony+

20µl P1 buffer with RNase+ 5µl final cracking buffer). Then the DNA was electrophoresed in an agarose gel, stained in ethidium bromide and detected under UV-light. The analysis of the sequence was done after linearizing the plasmid with restriction enzyme NdeI and the corresponding buffer orange (10x).

2.2.2.14 siRNA

To transfect siRNA the cells were first splitted into three 6-well-plates. Like doing transfection of plasmid DNA, Optimem and RNAiMax (Lipofectamine-equivalent for RNA) were used. The cells were transfected with 6.25 µl and 9µl siRNA (stock with 16.6 pmol/µl) and harvested after 24h, 48h and 72h. AllStars Negative Control siRNA was taken as control siRNA.

2.2.3 Working with cells

2.2.3.1 Defrosting cells

The tube with the cells to defrost was first taken out of the -180°C freezer and was heated up in a 37°C water bath until the tube is partly defrosted. The cells were taken and transferred into 10ml of media in a falcon. The falcon was centrifuged at 900rpm, room temperature for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 2.5ml of media. After the cells were passed into a cell culture bottle, media was added up to a total volume of 14ml. Then the cells were incubated in 37°C until the bottle got full to be splitted. Penstrep (1:100) and G418 (1:100), if needed, were added with the first split.

2.2.3.2 Culture of cells

AGS cells:
AGS were cultured in 12ml of RPMI medium with 10% FCS at 37°C, 5% CO_2 and had to be splitted every second day or over the weekend. Since AGS cells are adherent cells, they had to be detached from the bottom of the tissue culture flask before splitting. The media inside the bottles was exhausted completely. The cells were washed with 10ml PBS. Then 2ml Trypsin-EDTA was added. The cells were then incubated at 37°C, 5% CO_2 for about 15 minutes to support the protease enzyme activity. The trypsin digested the surface proteins of the AGS cells that they were no longer adherent the bottle. After this period of time the cells were mostly detached. To have the last ones removed the bottles were hit slightly with the hand. To stop the enzyme reaction of the Trypsin-EDTA at least the same amount of media, in this case RPMI+ 10% FCS, was added. To culture the cells for the next two days the cells had to be splitted 1:8, and to culture them over the weekend they were splitted 1:10, in both cases to have a confluency of 80-100%.

HEK 293, HEK CC1, HEK CC5 cells:
HEK cells were cultured in 12ml of DMEM 1x with 15% FCS and 1% L-glutamine 200mM at 37°C, 5% CO_2 and had to be splitted every second day or over the weekend. HEK 293, HEK CC1 and HEK CC5 cells are semi-adherent cells. Before splitting the cells the old media was exhausted completely. To detach them, the media had to be pipetted against the bottom of the tissue culture flask with an electric pipette. G418 (1:100) was added to HEK CC1 and HEK CC5 cells after splitting. Like the AGS cells the HEK cells to be

cultured for the next two days had to be splitted 1:8, and to culture them over the weekend they were splitted 1:10, in both cases to have a confluency of 80-100%.

Jurkat cells and HeLa cells:
The cells were cultured in 12ml of RPMI medium with 10% FCS at 37°C, 5% CO_2 and had to be splitted every second day or over the weekend. Jurkat cells had a split ratio of 1:2 or 1:3. Since they are growing in suspension, no detach was necessary. HeLa cells had a split ratio of 1:4 and were detached using Trypsin- EDTA like for AGS cells (see above).

2.2.3.3 Freezing of cells

As pellet:
HEK 293, HEK CC1, HEK CC5 cells:
The cells were detached from the bottom of the tissue culture flasks as described above. The media with the detached cells were put into falcons and then centrifuged. The media was discarded. The cell pellet was dissolved in PBS buffer to distribute the cells into the microcentrifuge tube. The tube was again centrifuge at 4°C, 800rpm for 5 minutes to store only the pellet without liquid in -20°C.

AGS:
Using the cell detaching method for AGS cells as described above the cells were removed. To stop the enzyme reaction of the 2ml of Trypsin-EDTA the same amount of media, in this case RPMI+ 10% FCS was added. The whole liquid could be collected into a falcon or microcentrifuge tube to be centrifuged like the HEK cells.

In solution:
Before freezing AGS cells a 10% DMSO solution in serum was prepared. As described in 2.2.3.2 2ml of Trypsin-EDTA was added to the tissue culture flask with a confluency of about 70-80% on a surface of 75mm^2 to detach the AGS cells. Once 2ml of the 10% DMSO solution and 2ml of Trypsin-EDTA with detached AGS cells were gently mixed in one falcon, the aliquots of 1ml per tube were put in an ice box immediately. After the storage in -80°C overnight the tube were deplaced to the -180°C liquid nitrogen freezer.

2.2.3.4 Lysate

20µl of PBS* buffer was added to a tube containing a pellet of cells and put on ice immediately. Then 25µl of 2x SDS or 25µl of 2x SDS with mercaptoethanol was added. The mercaptoethanol is able to reduce cystein bridges in proteins. Afterwards, the tube was cooked in a water bath at 95°C for 10 minutes. The tube had to be put on ice right after the cooking. The lysates could be used or stored at -20°C.

2.2.3.5 Cell counting

The cells were counted with a cell counter machine from Casy. Therefore the cell solution was diluted with a relation of 1:200 by mixing 50µl of cells with 9950µl of Casy tone. Program 1 was to count AGS cells already set up by a lab member. After having cleaned the machine 3 times the falcon with the 10ml cell-Casy tone solution was entered the

machine. The result of the counting was given in number of cells per ml. To know the real amount of cells the dilution was to normalize.

2.2.3.6 Transfection

In these studies AGS cells, HEK 293, HEK CC1 and HEK CC5 cells were transfected. The cells were splitted into 6-well-plates, 12-well-plates or 24-well-plates before the transfection. To transfect cells in a 6-well-plate well with 2ml media, 9µl of Lipofectamine, 500µl of Optimem and maximal 500ng of DNA was needed per well. For a 12-well-plate with 1ml media in each well, the amount of each reagent was halved, and so on.
Two tubes were prepared because the Lipofectamine and the DNA had to be incubated in Optimem for 5 minutes. The first tube contained Lipofectamine and Optimem, the second tube contained DNA and Optimem. After 5 minutes both tubes were mixed and incubated for 20 minutes. Then the mixture was applied to the wells. After 45 minutes in the incubator the medium of AGS cells had to be changed. Otherwise the cells would die because of the Lipofectamine. Antibiotics (1:100 PensStrep) were added to AGS cells and the HEK cells. The plates were placed in the incubator overnight.

2.2.3.7 Creating a stable cell line

The plasmid pEYFP-IRF7 was transfected into AGS cells to test its functionality. In particular, the fluorescence of EYFP could be detected under the microscope. To be definitely sure about the right insertion of IRF7 the part between the two restriction enzymes HindIII and KpnI was confirmed by sequencing.
To start creating a stable cell line, the AGS cells were splitted into a 6-well-plate and transfected with pEYFP-IRF7. The plasmids of the bacterial clones #4 and #5 (number of the colonies grown on the agar plate after transformation of *E. coli*) worked the best so the following stable cell line was created with them. To select and keep the cells having the plasmid Geniticin or G418 was added to the media with a dilution of 1:50. After about 1 week the cells could be deplaced from the plates to the flasks. In order to sort the cells they had to be cultured up to a number of $20*10^6$ cells.
Five weeks after transfection, the sorting was done by a laboratory of the TU München. Therefore the cells had to be prepared following a certain protocol. First, the percentage of the fluorescent cells was counted by doing flow cytometry on the transfected cells: 5% for cells transfected with the plasmid of bacterial clone #5 but no result for the cells transfected with the plasmid of bacterial clone #4. Preparing the sorting process, 9 bottles, 3 of each, AGS cells, AGS EYFP-IRF7 #4 and AGS EYFP-IRF7 #5, were first washed twice with PBS. After about 10 minutes of incubation at 37°C, 10% CO_2 in 3ml of PBS with 2mM EDTA to detach the cells, 3ml of HBSS with 1% FCS was added. The cells of all three bottles with the same cell types were collected in one falcon. To know the amount of cells to sort, they were counted with the cell counter. After the cells were washed twice with 3ml HBSS with 1% FCS (centrifugation at 4°C, 700rpm), 7 ml of HBSS with 1% FCS and 7µl of PI to mark the cells were added. The cells stood in ice for 10 minutes until they were again washed twice. Resuspended in 1ml of HBSS with 1% FCS, the cells were transported in an ice box.

The 10000 sorted cells of AGS EYFP-IRF7 #4 were passed into a well of a 48-well-plate and the 25000 sorted cells of AGS EYFP-IRF7 #5 were passed into a well of a 24-well-plate. Penstrep was added to prevent contamination.
After some time the cells could be transferred to a tissue culture flask. PenStrep (1:100) and G418 (1:50) were always added. The sorted cells grew slowlier than the AGS cells so they were splitted 1:4 every second day and 1:5 over the weekend.

2.2.3.8 Infection with *H. pylori*

The infection of the cells by *H. pylori* was done in 12- and 24-well-plates. The day before the cells had been splitted that they could grow and cover ideally 100% of the well bottom.
Before infection the old media containing antibiotics had to be changed. The cells were infected with a MOI (**m**ultiplicity **o**f **i**nfection) of 60. The OD_{550} 0.1 was measured with a photospectrometer as described above. The volume of the bacteria-PBS-solution to add to the cells had to be calculated: V= (0.1/measured value) x 100 (because of the dilution 1:100). While bacteria were applied to the cells, the solution was pipetted onto the wall of the wells to avoid motion in the media. The cells were then incubated for 1 hour at 37°C, 5% CO_2. To stop/ decelerate the infection process the plates were put on ice to be continued to work on immediately.

2.2.3.9 Luciferase Assay

The HEK 293, HEK CC1 and HEK CC5 cells were splitted into 12-well-plates in the morning and transfected with two plasmids pairs in the evening, 25ng of pRLNull and 500ng of p125-luc or 25ng of pRLNulll and 500ng of pELAMluc each well.
The next day the media containing the antibiotics was replaced with new media without antibiotics. The cells were then infected with a MOI60 with different *H. pylori* strains. Each strain had a different deletion of one part of the *cag* Type IV secretion system: p12wt, p12ΔCagA, p12ΔCagY, p12ΔCagE, KG-HP2, 26695wt, 26695Δhp0547, 26695Δhp0527, 26695Δhp0544, 26695ΔPAI. After 4 hours of incubation in 37°C, 5% CO_2 the cells were harvested. Since the HEK cells are semi-adherent they were detached by pipetting the media against the bottom of the well. The content of each well was transferred to a microcentrifuge tube and put on ice. Then all tubes were centrifuged at 4°C for 10 minutes at 500g. The supernatant was kept to do ELISA on IFNβ and IL-8. The bacteria were lysed to do Western Blot with anti-CagA- and anti-CagY-antibodies in order to control the lacking of important proteins belonging to the T4SS.
To evaluate the induction of the IFN-promoter, the luciferase activity of the transfected cells was measured with the Clariostar using the Dual Luciferase Kit from Promega. The cell pellets were resuspended in 50µl of PLB (passive lysis buffer). Then 20µl of this solution was added to 100µl of LARII. The luciferase activity was read by the Clariostar. After having read the luciferase activity 100µl of the Stop and Glo® solution was added to measure the Renilla activity. The ratio of Firefly activity and Renilla activity, which is to normalize the data, was taken to evaluate the results.

2.2.3.10 Confocal microscopy of cells

The SP5 microscope was used to microscope the stained cells. Using the program LAS AF the microscope could be controlled and the conditions to take photos of the slides

could be set. The laser used corresponded to the wavelength of each color to stain the cells. The Argon laser (488nm), the 405 Diode, the DPSS 561 and the HeNe 633 were used for pEYFP-IRF7 (512nm), the anti-PanLamin antibody (555nm), the DAPI staining (405nm), the Phalloidin Texas Red (561nm), the pRFPLamin (558nm) and the Phalloidin Alexa 647 (647nm). The 63x objective with one drop of Type F immersion oil liquid for each slide was used to see the cells. An overview photo was taken of every slide with a format of 1024x1024 and a frequency of 400 Hz. Additionally, a zoomed in view was saved with a 2,5x magnification, a format of 1024x1024 and a frequency of 200 Hz.

2.2.3.11 Preparing microscope cover glasses

To have the surface of the microscope cover glasses rough in order to simplify the adhesion of the cells the microscope cover glasses needed a special treatment. They had to be cooked in boiling 20ml of 0,1 M HCl for 10 minutes. Then they were washed twice with 50ml 70% ethanol and dried at 37°C. Additionally, to avoid contamination of the cells the discs were dry-sterilized.

2.2.3.12 Staining protocol

2.2.3.12.1 Phalloidin Texas Red, DAPI

The cells were splitted with a dilution of 1:4 into a 24-well-plate with microscope cover glasses. The next day, the cells were transfected with the plasmid pEYFP-IRF7 and incubated at 37°C, 5% CO_2 overnight. The day after that, the media was changed and the cells were infected with MOI60. After incubation for one hour at 37°C, 5% CO_2 the cells were fixed with 2.5% PFA for 40 minutes at 37°C which had been defrosted at 60°C. The cover glasses with the adhered cells were fixed to a microscope slide afterwards to be examined under the microscope.

To stain the cells they were first washed 2 times with 1ml PBS. 0.5 ml of PBS+ Phalloidin Texas Red (1:2000) was added and incubated at 37°C for 45 minutes /one hour. The cells were washed 1/ 3 times with 1ml PBS. 0.5 ml of PBS with DAPI (1:10000) was added and incubated at room temperature for maximum 1 minute. After having washed the cells 2/ 3 times with 1ml PBS and 1 time with distilled water the plates were air-dried in the dark. While waiting the cover glasses to be ready the microscope slides were prepared by cleaning them with ethanol and labeling them with a pencil. The microscope cover glasses were fixed on the microscope slides with one drop of mounting media Prolong Gold each. These glasses were picked with tweezers out of the well, cleaned on the backside with a piece of paper and then put onto the mounting media with the cell side down. Then they were pushed to the microscope as tight as possible. The microscope slides were air-dried at room temperature and in the dark to protect the staining. It took 24 hours until the mounting media got solid.

2.2.3.12.2 Phalloidin Alexa 647, DAPI, anti-Lamin antibody

The cells were splitted with a dilution of 1:4 into a 24-well-plate with one microscope slides in each well. The next day, the cells were transfected with the plasmid pEYFP-IRF7 and incubated at 37°C overnight. Then media was changed and the cells were infected with a

MOI60 as described above. After the incubation for one hour at 37°C the cells were fixed by adding 2.5% PFA (Paraformaldehyd) which had been defrosted the tube at 60°C.

To stain the cells they were first washed twice with 1ml PBS. 0.5 ml of 1% Triton 100x in PBS (v/v= mix liquid with liquid meaning add volume to volume) was added and incubated at room temperature for 10 min. After having washed the cells 2 times with PBS, 0.5 ml of 10% FCS in PBS was added to each well to block and incubated at 37°C for one hour. Again the cells were washed twice. Then 250µl of PBS with antibody to PanLamin (1:100) was added to each well and incubated at 4°C overnight. After having washed 3 times with 1ml PBS, 250µl PBS with 2nd antibody (anti-mouse Alexa 555) (1:1000) and Phalloidin Alexa 647 (1:1000) were added and incubated at 37°C for one hour. The wells were washed 3 times with 1ml PBS. 0.5ml of DAPI (1:10000) in PBS was added to each well and incubated for maximum 1 minute at room temperature. The cells were washed twice with PBS and once with distilled water.

To optimize the staining results different modifications of the staining protocol were tried:

First modification:
Cell splitting and transfection were done at the same day (about 9 hours apart). 0.1% of Triton 100x in PBS was used to permeabilize the cells. The wells were blocked with 0.5 % BSA in PBS. Instead of a dilution of 1:100 for the anti-panLamin-antibody a dilution of 1:50 was set up. The concentration of Phalloidin Alexa 647 was increased to 1:500.

Second modification:
Since the staining with the antibody against PanLamin did not function, different steps in fixing, permeabilizing and staining the cells that might influence the antibody‘s function or effect were considered. Due to some papers publishing staining with the panLamin antibody, the following methods were investigated.

1. Instead of permeabilizing with Triton 100x the cells were permeabilized with icecold methanol (-20°C). Also different PFA concentrations were tried to fix the cells: 1%, 2.5%, 3%, 4% of PFA incubated for 30 minutes in 4°C.
2. The cells were fixed and additionally permeabilized by adding icecold methanol (-20°C) and incubating at 4°C for 10 minutes.

2.2.3.12.3 Phalloidin Alexa 647, DAPI, pRFPLamin

In the morning, the cells were splitted with a dilution of 1:4 into a 24-well-plate with one microscope cover glasses in each well. In the evening, they were transfected with the plasmids pEYFP-IRF7 (200ng/well), pRFPLamin (300ng/well). The next day, the cells were infected with a MOI60 and incubated for one hour at 37°C. By adding PFA (1:10) to the cells, they were fixed and incubated at 37°C for two hours. After having washed the cells twice with 1ml PBS per well they were stained with Phalloidin Alexa 647 in PBS with a dilution of 1:500 to be incubated at 37°C for one hour. After having washed the cells three times with 1ml PBS per well, 0.5 ml PBS+ DAPI (1:10000) was applied to the cells to be incubated at room temperature for maximum 1 minute. Then wells were then washed

twice with 1ml PBS and once with distilled water. Before fixing the class discs on microscope slides with mounting media the cells could air-dry in the dark.

Since the EYFP-IRF7 was always accumulated, even in the non-infected cells, the toxic character of IRF7 was considered. It might lead to accumulation of EYFP-IRF7 independently from infection with *H. pylori*. Suggesting the time between transfection and infection to be too long, it was shorten:
The cells were splitted into 24-well-plates and transfected right away in the morning. Then the cells were incubated for 7 hours at 37°C before infection with MOI60. After 1 hour of incubation at 37°C, 5% CO_2 the cells were fixed with PFA overnight at 4°C. The concentration of Phalloidin Alexa 647 was increased to 1:250.

2.2.4 ELISA

IL-8:
The ELISA is to evaluate the amount of proteins in a solution.
To coat the NUNC adsorbent 96-well plate 50µl of the antibody solution mix consisting of 5ml of coating buffer and 30µl of coating antibody was pipetted into each well. The plate was incubated overnight at 4°C. The next day, the plate was washed twice with 200µl of wash buffer per well. 100µl of PBS with 10% FCS was applied to each well to block the plate. After 2 hours of blocking at room temperature, the blocking solution was removed and the plate was again washed twice with 200µl of washing buffer per well. During blocking incubation, the standard solution was prepared: 990µl of media was added to 10µl standard stock with a concentration of 80 pg/µl to have a concentration of 800 pg/ml. In the following the standard was diluted to different concentrations: 800 pg/ml; 400 pg/ml; 200 pg/ml; 100 pg/ml; 50 pg/ml; 25 pg/ml; 12.5 pg/ml; 0 pg/ml. 100µl of standard solution and the samples with a final volume of 100µl per well were applied. Depending on the estimated IL-8 concentration of the sample different amounts of samples could be added. The difference to the final volume of 100µl was filled with the media the proteins were diluted in. The plate was covered with a transparency to be incubated overnight at 4 °C or for minimum 5 hours at 37°C. By washing the wells 6 times with 200µl wash buffer per well, the samples were removed. 100µl of 10µl of biotinylated IL-8 antibody in 10ml wash buffer with 10% FCS was applied to each well. After an incubation time of 45 minutes minimum at 37°C or 2 hours at room temperature, the wells were again washed 6 times. Then 100µl of the POX-Streptavidin complex solution (Vectastain ABC Kit) was added to each well and incubated at room temperature for 45 minutes. The POX-Streptavidin complex solution was prepared half an hour before by mixing 1.5µl of solution A with 1.5µl of solution B of the Vectastain ABC Kit in 200µl of ELISA buffer. The tube was vortexed briefly and incubated for 30 minutes at room temperature. Just before applying the complex solution to the wells it was diluted in 10ml of ELISA buffer. After 45 min of incubation the plates were washed 6 times. 5ml of Solution A and 5ml of Solution B from the kit TMB Substrate Reagent were mixed, then 100µl was applied to each well. When the solutions in the well turned blue the reaction had to be stopped by using 50µl of 1 M H_2SO_4 per well. The plate was then read in the ELISA plate reader.

IFNβ:
First, the 96-well ELISA plate was coated with 50µl of coating buffer (protocol 1) with 1 µg/ml anti-human IFNβ antibody. The plate was covered by a sealing foil and incubated at 4°C overnight. The next day the plate was washed 3 times with 200µl washing buffer into each well and then blocked with 1% milk powder in PBS at room temperature for 1 hour, covered by a sealing foil. While waiting, the standard solutions were prepared. The standard was diluted to a row of 7 dilutions: 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml, 0 pg/ml (This last one contained only media). After blocking the ELISA plate was washed 3 times with washing buffer. 100µl of the standard solution was added to the wells. 100µl of sample was pipetted into each well with a concentration of 50%, 25% and 12.5% diluted with media. The plate was incubated at room temperature for 2 hours. After having washed the plate 6 times, 100µl of 1% milk powder in PBS with the second antibody, the biotinylated polyclonal antibody to IFNβ, was added to the well. The stock of the second antibody with a concentration of 1 mg/ml was diluted to 0.5 µg/ml. After an incubation time of 1 hour at room temperature, the plates were washed 7 times with washing buffer. 100µl of the POX-Streptavidin complex solution (Vectastain ABC Kit) was added to each well and incubated at room temperature for 45 minutes. The POX-Streptavidin complex solution was prepared half an hour before by mixing 1.5 µl of solution A with 1.5 µl of solution B of the Vectastain ABC Kit in 200µl of ELISA buffer. The tube was vortexed briefly and incubated for 30 minutes at room temperature. Just before applying the complex solution to the wells it was diluted in 10ml of ELISA buffer. After 45 minutes of incubation the plates were washed 6 times. Then the TMB Substrate Reagent solution (mix of solution A and B) was added and incubated until the liquid in the wells to turn blue. Then the reaction was stopped with phosphoric acid. The plate was then read in the ELISA plate reader.

Since the protocol mentioned above did not work, IFNβ-ELISA was performed with the protocol used for IL-8. The coating antibody was set up with a concentration of 1 µg/ml / 2 µg/ml. The sample concentration was increased to 100%. And the following 3 options concerning the concentration of the antibodies were tried: first antibody doubled, second antibody doubled, both antibodies doubled.

2.2.5 Western blot

The Western Blots in this work were done with the single gel system. The proteins were all load onto 6%-protein acrylamide gels and run in an electrophoresis chamber from BioRad.

2.2.5.1 SDS-gel electrophoresis

To pour a protein acrylamide gel of 6% distilled water, Bis-Acrylamide, 2x single gel buffer, ammonium persulfate, TEMED were mixed together. For the amount of each ingredient see table 2. Because of the StainFree system used in this work, 50µl of trichlorethanol was added to each 5ml of gel mix. To balance the liquid amount 50µl of water had to be reduced. The whole mix was applied into the 1mm thick SDS-gel plate system until the space was completely filled. The comb was immediately introduced because the polymerization started right after the application of TEMED to the mixture. It took 10-15 minutes for the whole gel to be polymerized. The gel was put into a protein electrophoresis

chamber. The chamber was filled with SDS buffer and the gel could be loaded, with the ladder in the first pocket and the samples (cell or bacterial lysates) in the following pockets. The chamber was run at 90V for 10 minutes, then at 140V for 55 minutes.

6% gel									
	5ml	10ml	15ml	20ml	25ml	30ml	35ml	40ml	50ml
Water	1.3	2.6	3.9	5.2	6.5	7.8	9.1	10.4	13.0
Bis-Acrylamide 30%	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	10.0
2x Single-gel buffer	2.6	5.2	7.8	10.4	13.0	15.6	18.2	20.8	26.0
Ammonium Persulfate	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.50
TEMED	0.008	0.012	0.016	0.020	0.024	0.028	0.032	0.040	0.040

Table 2: Ingredients for 6% protein acrylamide gel
All amounts in ml.

2.2.5.2 Blotting

In order to use the StainFree system (BioRad) the gel had to be activated for 1 minute in the GelDoc before blotting. Therefore the gel was taken out of the spacer and put into the GelDoc. Distilled water was used to wet the surface before placing the gel on it. In the following, the blotting had to be prepared. While working, the gel had to be wet with distilled water from time to time to avoid exsiccation.
The PDVF-membrane had to be activated by placing it in methanol for 1 minute and transferring to the anode II buffer until using. Following the protocol of the semi-dry blotting system a column of Whatman paper was prepared. There were two types of papers having the same size of 6.5x 9cm but being different in their thickness. They were submerged into anode I buffer, anode II buffer and cathode buffer and placed over each other to form a column.

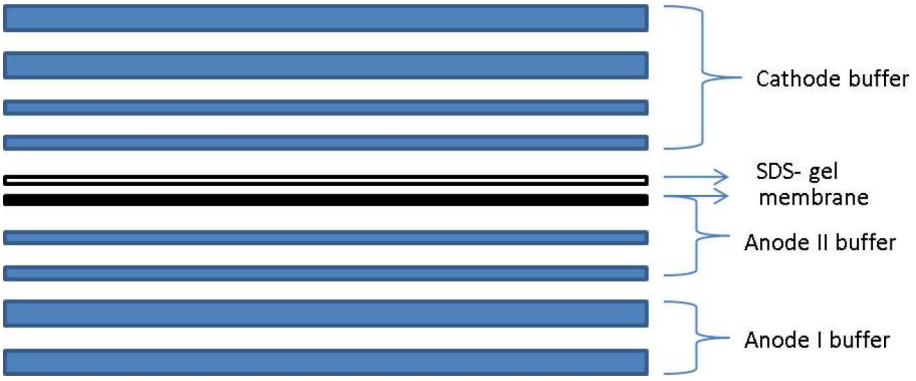


Figure 8: Organization of the papers to blot a SDS-protein gel
The scheme shows the organization of the papers to blot a SDS-protein gel. First the papers were dipped in the buffer indicated on the right side of the figure and put onto each other one after another.

The membrane was blotted in a blotting chamber which was connected to a voltage source. The process ran at 128 mA (1.2 mA per cm^2) or more depending on the number of membranes to blot. In one chamber run four membranes could be blotted at the same time. After 70 minutes of blotting the membrane was air-dried and then stored in 4°C.

2.2.5.3 Detection

The blotted membrane had to be activated with methanol. Therefore the membrane was placed into a 50ml falcon. 5ml of methanol was added and the falcon was rolled on the roll-machine for a couple of minutes. Then the methanol was discarded and 5ml of the blocking buffer was added instead. The falcon was rolled for 1 hour. There are different protocol variations depending on the protein to mark.

IRF7: Since the detection of IRF7 was not optimal, several protocol variations were tried:

1. 0.5µl of IRF7 antibody was added to the blocking buffer. After 1.5 hour rolling the membrane was washed 4 times with 5ml washing buffer every 15 minutes. Then 0.5 µl of anti-rabbit pox was added. After 45 minutes the membrane was washed 4 times every 15 minutes with 5ml washing buffer. Variation of anti-IRF7-antibody amount 0.3µl; 0.7µl.
2. 0.5µl of the antibody to IRF7 was added to the blocking buffer and rolled for 1 hour. The membrane was washed 4 times for 10 minutes each time. Then the second antibody was added for 45 minutes. Afterwards, the membrane again was washed 4 times for 10 minutes each time. Variation of anti-IRF7-antibody amount 0.7µl; 0.75µl.
3. 0.7µl of IRF7 antibody was incubated for 75 minutes. The membrane was then washed 4 times for 10 minutes/ 15 min. Then the second antibody was added for 45 minutes. Afterwards, the membrane again was washed 4 times for 10/ 15 minutes each time.
4. 0.9µl of IRF7 antibody was incubated for 75 minutes. The membrane was then washed 4 times for 15 min. Then the second antibody was added for 45 minutes. Afterwards, the membrane again was washed 4 times for 15 minutes each time.

CagY:
0.5µl of anti-CagY antibody (A273) was added to the blocking buffer. After one hour rolling the membrane was washed 4 times with 5ml washing buffer every 10 minutes. Then 0.5µl of anti-rabbit pox was added. After 45 minutes, the membrane was washed 4 times every 10 minutes with 5ml washing buffer.

CagA:
0.5µl of anti-CagA antibody (A257) was added to the blocking buffer. After one hour rolling the membrane was washed 4 times with 5ml washing buffer every 10 minutes. Then 0.5µl of anti-rabbit pox was added. After 45 minutes the membrane was washed 4 times every 10 minutes with 5ml washing buffer.

For all proteins:
The buffer was discarded. 1ml of each reagent in the kit Immobilon™ Western, Chemiluminescent HRP Substrate Peroxide Solution was added to the falcon. Right before protein detection in the GelDoc the reagents were distributed over the membrane inside the falcon and rolled.
The membranes could be used one more than one time to detect other proteins in the same samples. Therefore the already binded antibodies had to be washed away with 5ml stripping buffer that was added to the falcon with the detection solutions already discarded. The falcon was rolled for 1 hour before exchanging the buffer with blocking buffer. The following process was done as described above depending on the protein to be detected.

3 Results

3.1 Interaction of *H. pylori* T4SS with the Interferon type 1 pathway

The T4SS is an important virulence factor of *H. pylori*. This large protein complex consists of different parts. One of the most important proteins is the VirB10 homologue CagY. The sequence encoding this protein has two regions with repeating sequences. The two repeat regions are very variable and do not have any similarities to other already known proteins. However, there is one exception: The Late Membrane Protein (LMP1) of the Human Herpes Virus 4 (HHP4) or Epstein-Barr-Virus.
Previous studies in our workgroup have shown that the LMP1 protein, which is capable to induce the interferon pathway, and the repeat region I (RRI) of CagY have similarities (see 1.1.7). This indicated further studies on the interaction of the T4SS, in particular the CagY protein, and the interferon pathway.

3.1.1 T4SS-dependent induction of the IFNβ promoter in HEK 293, HEK CC1 and HEK CC5 cells

The investigation with the Luciferase Assay was performed with HEK 293 cells, HEK CC1 cells and HEK CC5 cells in order to examine in how far the IFN-pathway is upregulated after infection with *H. pylori*. Königer et. al. and Javaheri et. al. evidenced the importance of CEACAMs for *H. pylori*'s binding to the host cell. They also identified HopQ-CEACAM-interaction to be essential for CagA translocation. Since the HEK 293 cells were shown to not express CEACAMs [120] they were intended to be the negative control of the assay. Meanwhile the HEK CC1 and HEK CC5 cells expressing CEACAM 1 or CEACAM 5 were able to translocate CagA and induce the IFN pathway depending on the *H. pylori*-strains they were infected with, wildtype or mutants lacking components of the T4SS.
As already described in 2.2.3.9 the cells were transfected with the two plasmids p125luc and pRL-Null. The p125luc plasmid has an IFNβ-promoter followed by the sequence encoding the Firefly luciferase. The Firefly activity can give information about the induction of the interferon pathway because its activity can be only produced when the promoter is activated. The pRL-Null plasmid, encoding the Renilla luciferase, is the control plasmid for the success and the quality of the transfection process. Driven by the Herpes simplex virus thymidine kinase promoter, the Renilla luciferase is constantly produced. The luciferase activity of both plasmids was measured one after another after infection of the cells with *H. pylori* wildtype strains and mutants lacking different parts of the T4SS: P12wt, P12ΔcagA, P12ΔcagY, P12ΔcagE, KG-HP2 (P12ΔcagY), 26695wt, 26695Δhp0547, 26695Δhp0527, 26695Δhp0544, 26695ΔcagPAI.
With the bacteria added to induce the cells it was possible to find out which part of the T4SS exactly is important for an upregulation of IFN: A reduction of the IFN-pathway activity caused by the infection with a mutant allows the conclusion that the PAI-areas are important for the pathway.
To normalize the transfection effects of each assay the Firefly luciferase activity was divided by the Renilla luciferase activity (in figure 13 marked as ratio Firefly and Renilla).

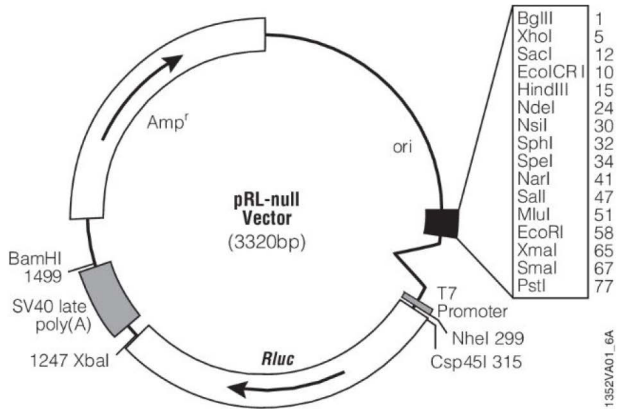


Figure 9: Plasmid pRL-null by Promega
The vector contains the sequence encoding the Renilla luciferase which was cloned from the Renilla reniformis.
Plasmid card from https://www.promega.de/products/luciferase-assays/genetic-reporter-vectors-and-cell-lines/prl-renilla-luciferase-control-reporter-vectors/?catNum=E2231, access date 24/10/2016.

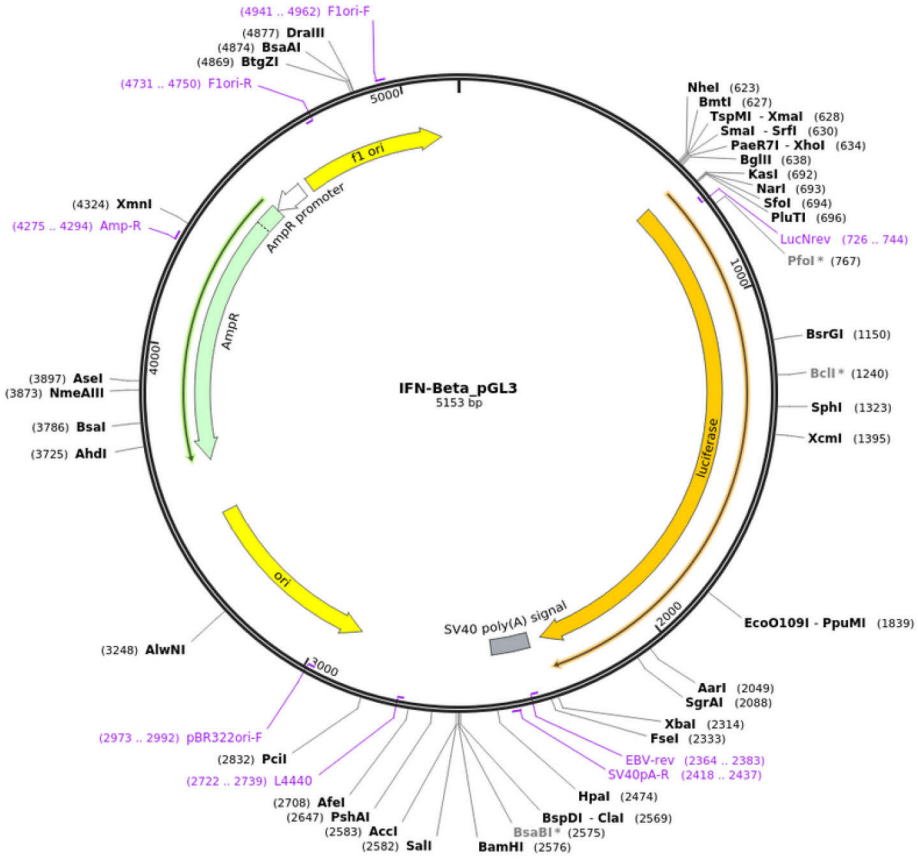


Figure 10: Plasmid p125luc (IFN-Beta_pGL3)
The promoter region of the human IFN-beta gene (−125 to +19) is in an upstream position of the Firefly luciferase gene in the pGL3-basic vector [137].
Plasmid card from https://www.addgene.org/102597/, access date 20/12/2018.

While having the p125luc as the plasmid with the promoter to test, the plasmid pELAMluc was taken as positive control. This plasmid has an E-selectin promoter upstream of the firefly luciferase. The E-selectin promoter is known to be activated by proteins of the NF-κB pathway [134].

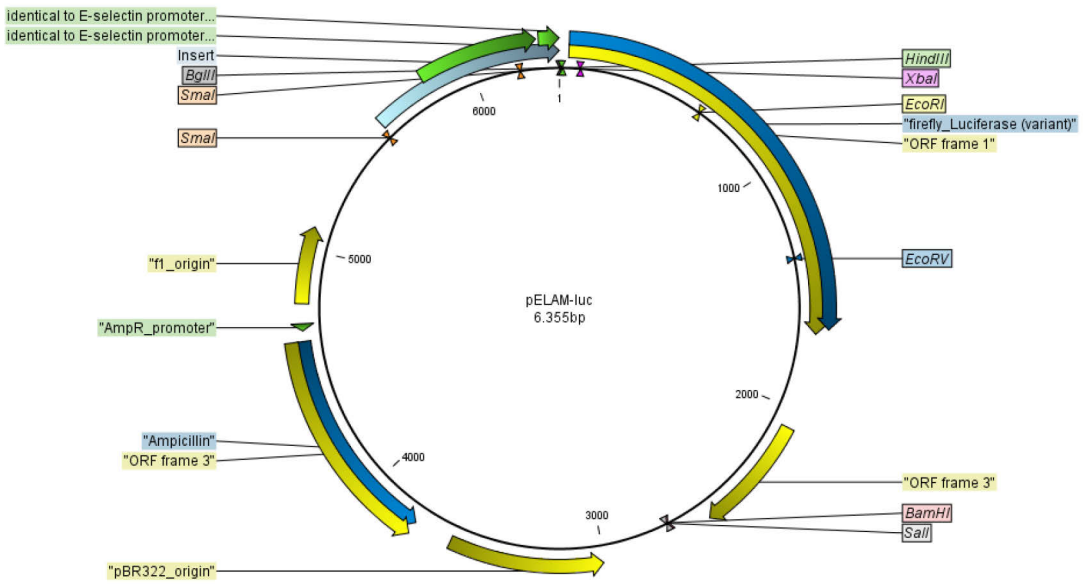


Figure 11: Plasmid pELAMluc
The plasmid pELAMluc contains the E-Selectin-promoter upstream of the Firefly luciferase in the base vector pGL3 reporter plasmid by Promega [133]. The E-selectin-promoter is activated by transcription factors of the NF-κB pathway.

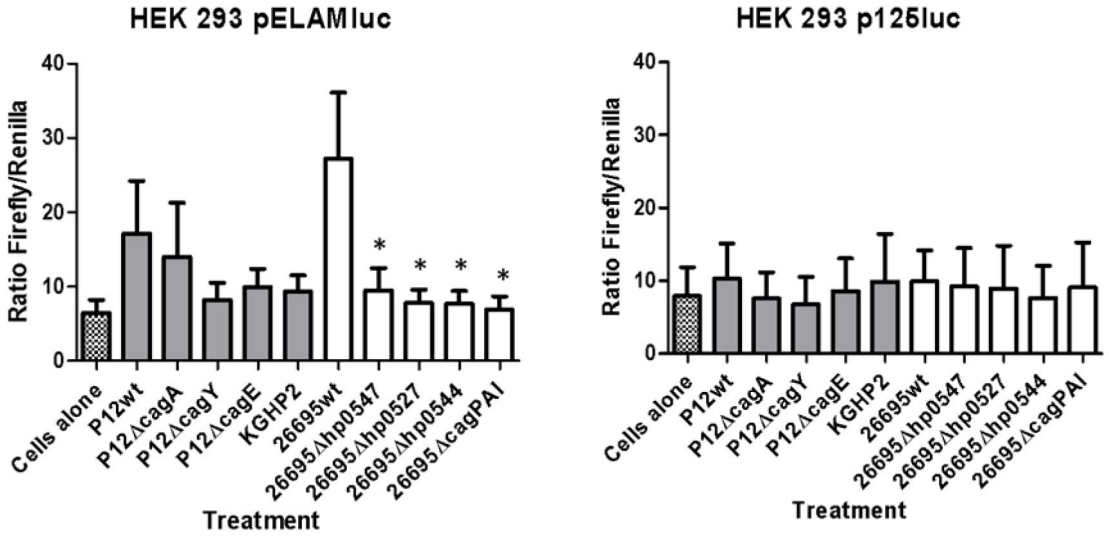
As described in 2.2.3.6 and 2.2.3.9 the plasmid pairs pELAMluc, pRL-Null or p125luc, pRL-Null were transfected into the cell lines HEK 293, HEK CC1 and HEK CC5. The following measurements of Luciferase activity of p125luc and pELAMluc were normalized to Renilla activity of pRL-Null.

Every transfected cell line showed a basic luciferase activation, which indicates a basic activation of the INFβ-promoter and E-selectin-promoter without any infection with *H. pylori*. Compared to these basic promoter activities, all promoter activities upon infection with *H. pylori* wildtype strains were higher, some significantly, some not.
For HEK 293 cells transfected with pELAMluc there was no significant difference between the NF-κB-induction by wildtype strain P12 and the basic activity. There were also no significant differences between the activity induced by P12wt and the corresponding mutants. The mutants' activities were reduced. The activity induced by mutants lacking CagY, P12ΔcagY, induced the lowest activity, close to the basic activity. Compared to P12wt, the E-selectin-activity upon infection with 26695 wildtype strain was higher. There was a significant difference between 26695wt induced activity and the basic activity. In opposite to the P12wt strain, the cells infected with the mutants of the bacterial strain 26695 caused a remarkable, significantly reduced activation of the E-selectin promoter as

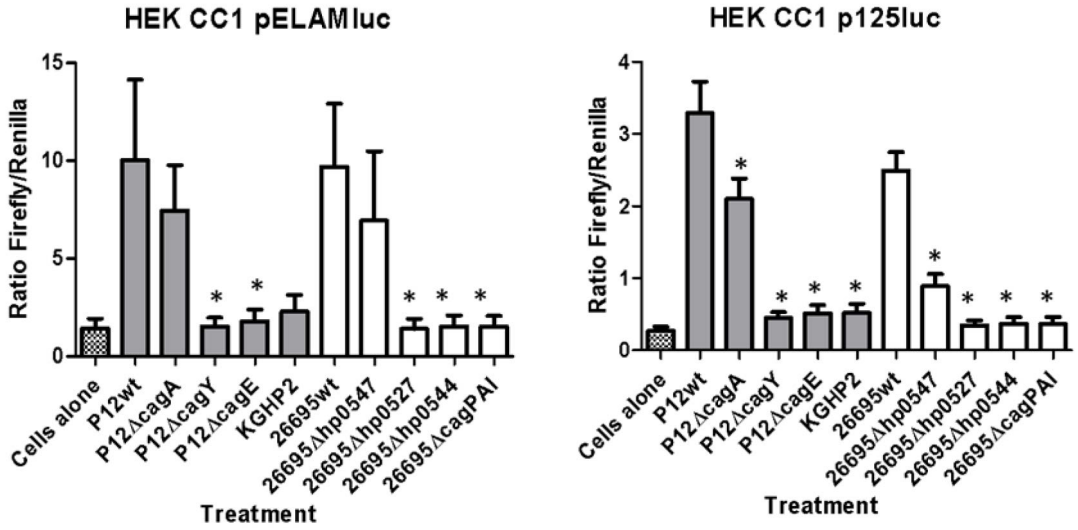
compared to the wildtype (figure 13A). The mutant lacking CagY and CagE induced nearly the same activity while the mutant lacking the whole PAI-region induced the lowest activity. The HEK 293 cells transfected with p125luc did not have any significant difference between the treatments, neither the wildtypes compared to the basic promoter activity, nor the mutants compared to their corresponding wildtypes. They all, infected with the wildtype strain or with a mutant, induced an IFNβ-promoter activity very similar to the basic activity.

The HEK CC1 cells transfected with pELAMluc and infected with the wildtype strains had a strong induction of the E-selectin-promoter. Luciferase activities in cells infected with P12wt and 26695wt were significantly higher than the basic activity. Except of the cells infected with KG-HP2, with P12 mutant lacking CagA and with 26695 mutant also lacking CagA, infections with the other mutants induced a significantly impaired luciferase activity and consequently a reduced promoter activity compared to wildtype strains. The 26695 mutant lacking CagY induced the lowest luciferase activation followed by P12ΔcagY, 26695Δhp0544 and 26695ΔcagPAI.
Transfected with p125luc, the Luciferase activity in HEK CC1 cells was significantly elevated by the infection with the *H. pylori* wildtype strains as compared to all mutants. (figure 13B). Also, infection with the wildtype strains significantly increased the Luciferase activity as compared to uninfected cells. Within every strain, the mutant lacking CagY induced the lowest luciferase activation, followed by P12ΔCagE and KG-HP2 or 26695Δhp0544 and 26695Δ*cag*PAI.

The HEK CC5 cells transfected with pELAMluc also had a strong induction of the E-selectin-promoter. Infection with the two wild type strains P12 and 26695 caused a significantly increased promoter activity compared to the basic activity. Among the P12 mutants, P12ΔcagY, P12ΔcagE and KG-HP2 induced a significantly reduced promoter activity as compared to their wildtype. The promoter activity of the two mutants lacking CagY and CagE were the lowest. All 26695 mutants caused a significantly reduced luciferase activity as compared to their wildtype strain. The lack of CagY had the weakest influence on the activity induction.
Thus, the HEK CC5 cells transfected with p125luc showed the same effect as the HEK CC5 cells transfected with pELAMluc. Luciferase activity in cells infected with wildtype strains was significantly higher than in uninfected cells. The wildtype strain P12 and the corresponding ΔcagA mutant induced the promoter quite well, while the other mutants caused a promoter activity which was significantly extenuated. The bacterial strain P12ΔcagY was the weakest promoter inductor. Compared to the effect on the promoter activity of the infection with the wildtype strain 26695, all corresponding mutants caused a significantly impaired effect. (figure 13C). Similar to the cases mentioned above, the ΔcagY mutant was the bacterium to induce the lowest IFNβ-promoter activity.


HEK 293 pELAMluc
Ratio Firefly/Renilla
0
10
20
30
40
Cells alone
P12wt
P12ΔcagA
P12ΔcagY
P12ΔcagE
KGHP2
26695wt
26695Δhp0547
26695Δhp0527
26695Δhp0544
26695ΔcagPAI
Treatment
HEK 293 p125luc
Ratio Firefly/Renilla
0
10
20
30
40
Cells alone
P12wt
P12ΔcagA
P12ΔcagY
P12ΔcagE
KGHP2
26695wt
26695Δhp0547
26695Δhp0527
26695Δhp0544
26695ΔcagPAI
Treatment

A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Ratio Firefly/Renilla
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5
10
15
Cells alone
P12wt
P12ΔcagA
P12ΔcagY
P12ΔcagE
KGHP2
26695wt
26695Δhp0547
26695Δhp0527
26695Δhp0544
26695ΔcagPAI
Treatment
HEK CC1 p125luc
Ratio Firefly/Renilla
0
1
2
3
4
Cells alone
P12wt
P12ΔcagA
P12ΔcagY
P12ΔcagE
KGHP2
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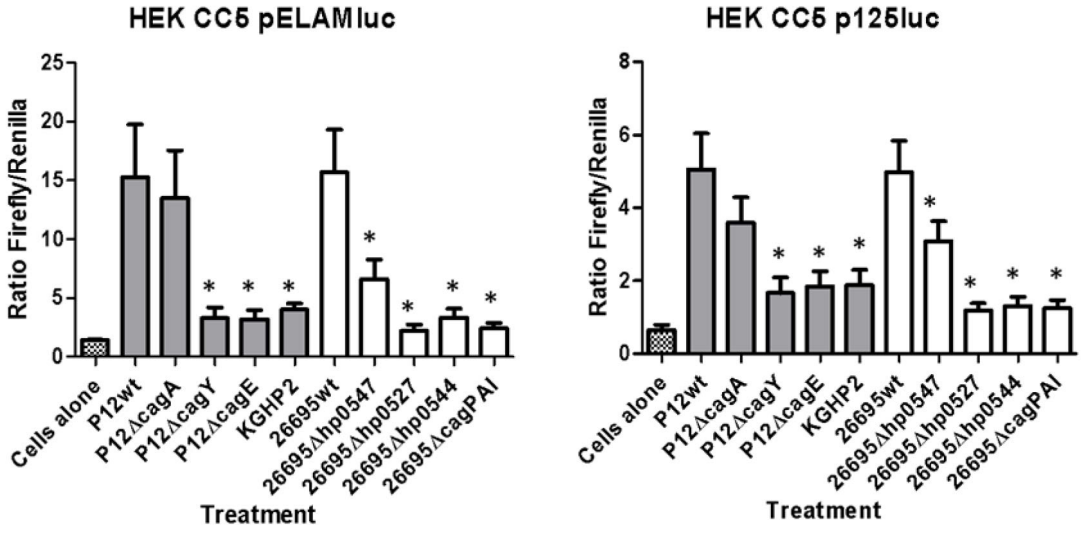
Treatment

B



C

Figure 12: Induction of the IFNβ-promoter and E-selectin promoter by *Helicobacter pylori*

To evaluate the effect of the infection with different *Helicobacter pylori* strains on the induction of the IFNβ promoter and E-selectin promoter, a bar graph was plotted with the ratio Firefly/ Renilla on the x-axis and the treatment of the cells, no infection and infections with different bacteria strains as well as mutants, on the y-axis.

In all graphs, even the uninfected cells (cells alone), had an induction of the IFNβ-promoter and E-selectin promoter, probably a basic metabolic rate. One-way ANOVA program and Tukey's Multiple Comparison Test was used to compare the Luciferase activities. Therefore “P12wt”-columns and the “26695wt”-columns were compared to “cells alone”-columns, and all “mutant”-columns were compared to the corresponding “wildtype” column. The significance level α was set at 0.05 (95% confidence interval). * marks mutant strains causing significant reduced luciferase activity compared to the corresponding wildtype strains.

The mean values of ratios Firefly/ Renilla are generated of at least 3 independent experiments.

A HEK 293 transfected with pELAMluc (4 independent experiments):
Cells alone: 6.50; P12wt: 17.16; P12ΔcagA: 14.00; P12ΔcagY: 8.21; P12ΔcagE: 9.93; KG-HP2: 9.33; 26695wt: 27.19; 26695Δhp0547: 9.49; 26695Δhp0527: 7.77; 26695Δhp0544: 7.70; 26695ΔPAI: 6.88
HEK 293 transfected with p125luc (3 independent experiments):
Cells alone: 7.91; P12wt: 10.30; P12ΔcagA: 7.55; P12ΔcagY: 6.77; P12ΔcagE: 8.58; KG-HP2: 9.88; 26695wt: 9.92; 26695Δhp0547: 9.21; 26695Δhp0527: 8.93; 26695Δhp0544: 7.66; 26695ΔPAI: 9.07
B HEK CC1 transfected with pELAMluc (3 independent experiments):
Cells alone: 1.43; P12wt: 10.04; P12ΔcagA: 7.46; P12ΔcagY: 1.53; P12ΔcagE: 1.79; KG-HP2: 2.29; 26695wt: 9.70; 26695Δhp0547: 6.95; 26695Δhp0527: 1.41; 26695Δhp0544: 1.53; 26695ΔPAI: 1.53
HEK CC1 transfected with p125luc (4 independent experiments):
Cells alone: 0.27; P12wt: 3.29; P12ΔcagA: 2.10; P12ΔcagY: 0.45; P12ΔcagE: 0.51; KG-HP2: 0.52; 26695wt: 2.49; 26695Δhp0547: 0.89; 26695Δhp0527: 0.35; 26695Δhp0544: 0.37; 26695ΔPAI: 0.37
C HEK CC5 transfected with pELAMluc (3 independent experiments):
Cells alone: 1.40; P12wt: 15.25; P12ΔcagA: 13.50; P12ΔcagY: 3.28; P12ΔcagE: 3.20; KG-HP2: 4.01; 26695wt: 15.67; 26695Δhp0547: 6.59; 26695Δhp0527: 2.21; 26695Δhp0544: 3.32; 26695ΔPAI: 2.40
HEK CC5 transfected with p125luc (5 independent experiments):
Cells alone: 0.65; P12wt: 5.06; P12ΔcagA: 3.60; P12ΔcagY: 1.66; P12ΔcagE: 1.85; KG-HP2: 1.88; 26695wt: 4.98; 26695Δhp0547: 3.09; 26695Δhp0527: 1.18; 26695Δhp0544: 1.31; 26695ΔPAI: 1.25

The IFNβ-promoter activity in HEK CC1 and HEK CC5 cells transfected with pELAMluc was always much higher than the promoter activity of HEK CC1 and HEK CC5 cells

transfected with p125luc. This indicated that *H. pylori* activates more intensively the NF-κB pathway in comparison to the Interferonβ-pathway.

Attendant to the Luciferase Assay of the cells, Western Blots detecting the proteins CagA and CagY were done in order to make sure that the bacteria strains, which were used to infect the cells, were the right ones. As shown in figure 14 the mutants lacking CagA did not show any bands after protein detection with the anti-CagA antibody. Also, mutants lacking CagY did not show any band after the protein detection with the anti-CagY antibody.

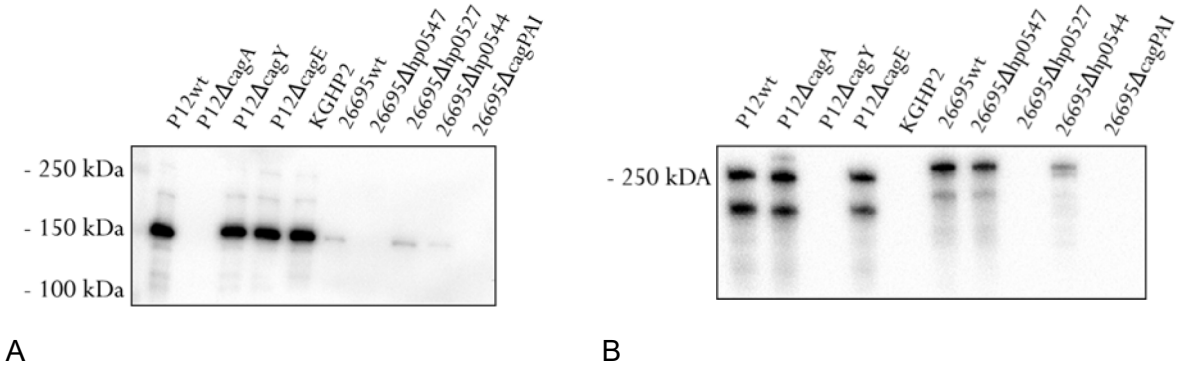


Figure 13: Detection of the proteins CagA and CagY in bacterial lysates to control the mutant strains
Two exemplary blots are shown. The proteins CagA and CagY were detected by Western Blot.
A To detect CagA with a size of 120-145 kDa the antibody anti-CagA 257 was used.
B To detect CagY with a size of 220 kDa the antibody anti-CagY 273 was used.

3.1.2 T4SS-dependent induction of the IFNβ-promoter in AGS cells

Based on the results of Dr. Luisa Jimenez-Soto doing the Luciferase Assay on AGS cells the suggestion was made that the AGS cells do not produce the protein IRF7 (unpublished). For this assay, the AGS cells were treated like the HEK cell lines as described above. Although AGS cells allow CagA translocation, there was not any significant reduction of the IFNβ-promoter activity upon infection with *H. pylori* mutants lacking different parts of the T4SS, compared to the corresponding wildtype strains. The growth conditions of the two cell types were similar, therefore the reason for the different behavior was suggested to be somewhere else. Differences of compartments in the interferon pathway were considered. Since IRF7 is a transcription factor binding to the promoter to activate the IFNβ-promoter, the absence of IRF7 in AGS cells could be the reason why the IFNβ-promoter activation was independent from *H. pylori* strains and mutants that were used to infect the cells.
To prove this idea and to show an interaction between the transcription factor IRF7 and the T4SS, Luciferase Assays with transient transfected AGS cells were initialized. To substitute the considered absence of IRF7 in AGS cells, the cells were transfected with the plasmid pHDN1/ pEYFP-IRF7 (see figure 15), as described in 2.2.3.6 and incubated overnight to be infected with *H. pylori* wildtype strains P12 and 26695 and with the corresponding mutants the next day. However, the cells seemed to have difficulties accepting the transfected plasmid. Although they were treated like other cell lines, a lot of

them died. Those still alive looked strange, not as usual as an oval formed cell adherent to the surface of the bottom of the plate, but more round and not stably adherent. Thus, the planned Luciferase Assay could not be done with the transfected AGS cells as planned before.

3.1.3 Attempt to create the stable cell line AGS pEYFP-IRF7

Since the Luciferase Assay could not be done with transient transfected cells, a stable cell line of AGS cells producing the protein IRF7 was created. Investigation with this cell line should prove the interaction of T4SS and the IFNβ-promoter and the interaction of T4SS and the IRF7.Therefore, the plasmid pHDN1/ pEYFP-IRF7 was transfected into AGS cells. (figure 14). The transfected cells were then selected by adding G418 (1:50). After a cultivation time of 3 weeks until the culture had reached a size of $20*10^6$ cells they were sorted by flow cytometry. At the beginning, in order to enhance the success rate of the stable cell line, AGS cells were transfected with pEYFP-IRF7 isolated of two different *E. coli* clones #4 and #5 leading to two cell line candidates AGS pEYFP-IRF7 #4 and pEYFP-IRF7 #5. For the preparation and the cell sorting process see 2.2.3.7.

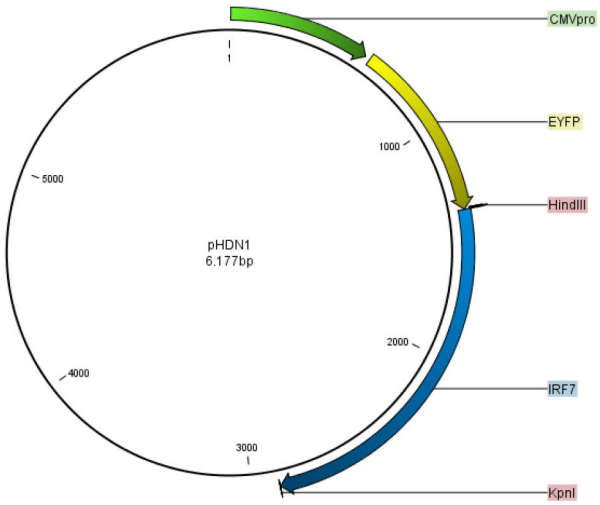
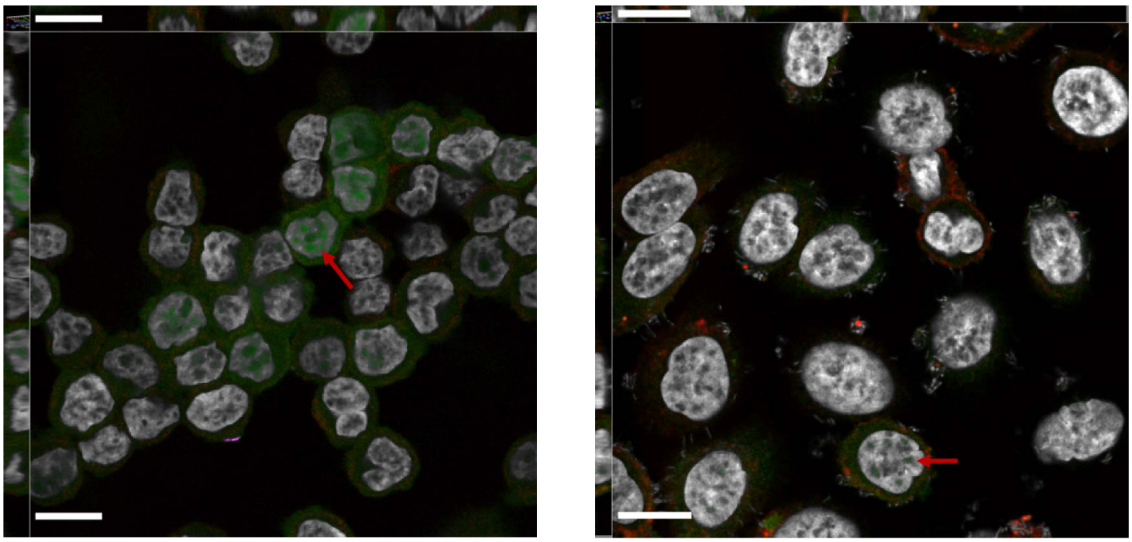


Figure 14: Plasmid card pHDN1/ pEYFP-IRF7
The plasmid map shows the IRF7 gene inserted into the commercial vector pEYFP-C1 (for details see 2.2.2.13). The CMV-promoter is constitutively activated and the fusion protein is therefore always produced.

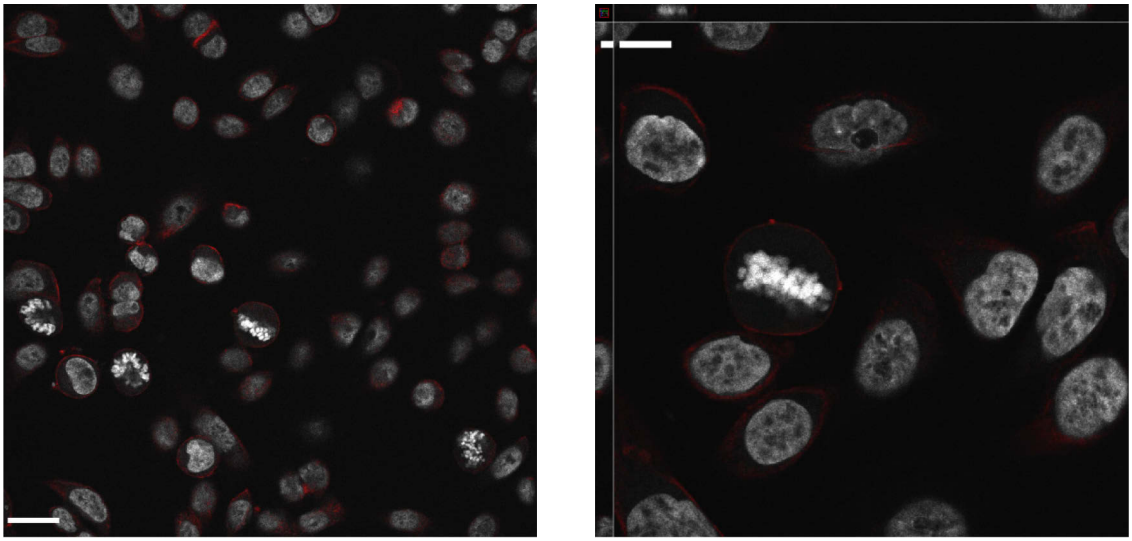
The cells containing the plasmid pHDN1/ pEYFP-IRF7 produced the fusion protein EYFP-IRF7. Since IRF7 is a transcription factor activating the IFN-promoter, this protein should translocate into the nucleus upon infection with bacteria. Because of the fluorescent protein fused to the transcription factor, its translocation was visible: After the infection with a *H. pylori* wildtype strain the fluorescence signal should move from the cytoplasm to the nucleus while nothing should happen after non-infection and after infection with bacterial mutants. Following the ideas from earlier publications [135, 136] as guide who had already done the confocal microscopy with the plasmid pGFP-RelA, infection experiments were done with the new created cells. The cells were infected with *H. pylori*, then fixed and stained (see 2.2.3.11) to be examined under the confocal microscope (figure 15, 16). The

interaction of IRF7 with the T4SS should be proven and also the function of the fusion protein demonstrated, in order to continue with the Luciferase Assay with AGS cells as explained above. An initial test of the cells was done after sorting. The AGS EYFP-IRF7 #5 cells barely showed some fluorescence, which only was detectable by the microscope, but could not be captured by the computer taking the images (figure 16). Facing this problem, these cells were not used further. The AGS EYFP-IRF7 #4 cells had a strong fluorescence and were intended to be used for further investigation. But after short time of cultivation also those cells lost their function.



A B

Figure 15: AGS pEYFP-IRF7 #4
Staining: grey= DNA; green= pEYFP; red= cytoskeleton
A AGS pEYFP-IRF7 #4 cells not infected show the EYFP protein (green) regularly distributed in the cytoplasm of most of the cells. There is one cell with translocated IRF7 (red arrow), even though the cells are not infected. 2.5 zoom; scale 13µm.
B AGS pEYFP-IRF7 #4 cells are infected with P12wt. But there is no translocation of IRF7 into the nucleus, except for one cell (red arrow). 2.5 zoom; scale 14µm.



A B

Figure 16: AGS pEYPF-IRF7 #5
Staining: grey= DNA; green= EYFP; red= cytoskeleton
A AGS pEYFP IRF7 #5 cells uninfected do not show any EYFP protein. The image was taken to get an overview of the cells.
B AGS pEYFP-IRF7 #5 cells uninfected in a 2.5 zoom shot. As already confirmed in the overview image (figure 16A), there is no production of the fusion protein EYFP-IRF7 neither in the cytoplasm nor in the nucleus.

Apparently, the EYFP-part of the plasmid functioned in pEYFP-IRF7 #4 cells as expected and therefore also the CMV promoter in the plasmid pEYFP-IRF7. However, the translocation of IRF7 was not observed as expected, but only partly, or even in non-infected cells. Further consideration led to the idea that the IRF7 protein might be the reason why there was no stable function of the fusion protein. A possible mutation in the IRF7 sequence could be a protection strategy of AGS cells, since this cell line reacted with cell death to the transient transfection of probable toxic plasmid pHDN1/ pEYFP-IRF7. Suggesting that the IRF7 part got damaged or mutated, this part was sequenced to identify a possible mutation responsible for the previous described dysfunction. As plasmid DNA normally is integrated into the chromosomal DNA, chromosomal DNA was extracted from transfected AGS cells before and after having been sorted in order to compare the sequences to the original plasmid that was used to establish the stable cell lines and to eventually set limits to the time point of the mutation. Initial transient transfection has shown the plasmid's functionality, meaning fluorescence and nuclear translocation upon bacterial infection. Its loss of function was assumed to be in the *IRF7* sequence. The possible mutation in the transfected cells should be found out by comparing the sequenced *IRF7* of the extracted chromosomal DNA to the original plasmid pHDN1/ pEYFP-IRF7 that was used to establish the stable AGS cell line. Chromosomal DNA of AGS cells was also sequenced like the ones of the transfected AGS cells to search for the DNA region coding for IRF7 and probably to verify the absence of the IRF7 protein in this cell line. For sequencing, the gene *IRF7* was amplified with the primers used to amplify IRF7 before cloning into the vector pEYFP-C1 taking chromosomal DNA from AGS cells and transfected AGS cells as samples and the plasmid pRenilla-IRF7 as positive control.

The amplification was checked in agarose gels showing clear IRF7-bands in all samples except for AGS chromosomal DNA (see figure 17).

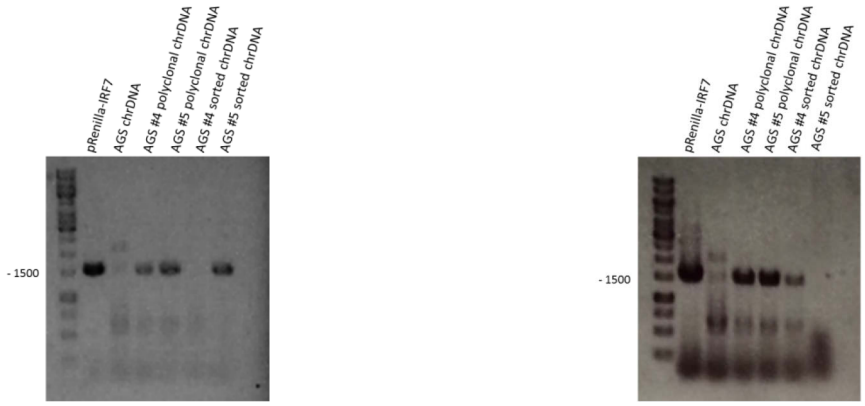
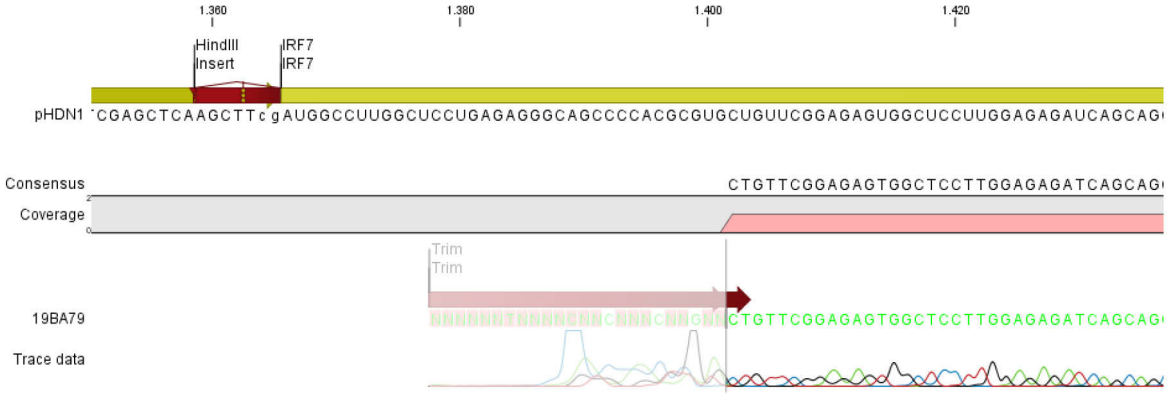
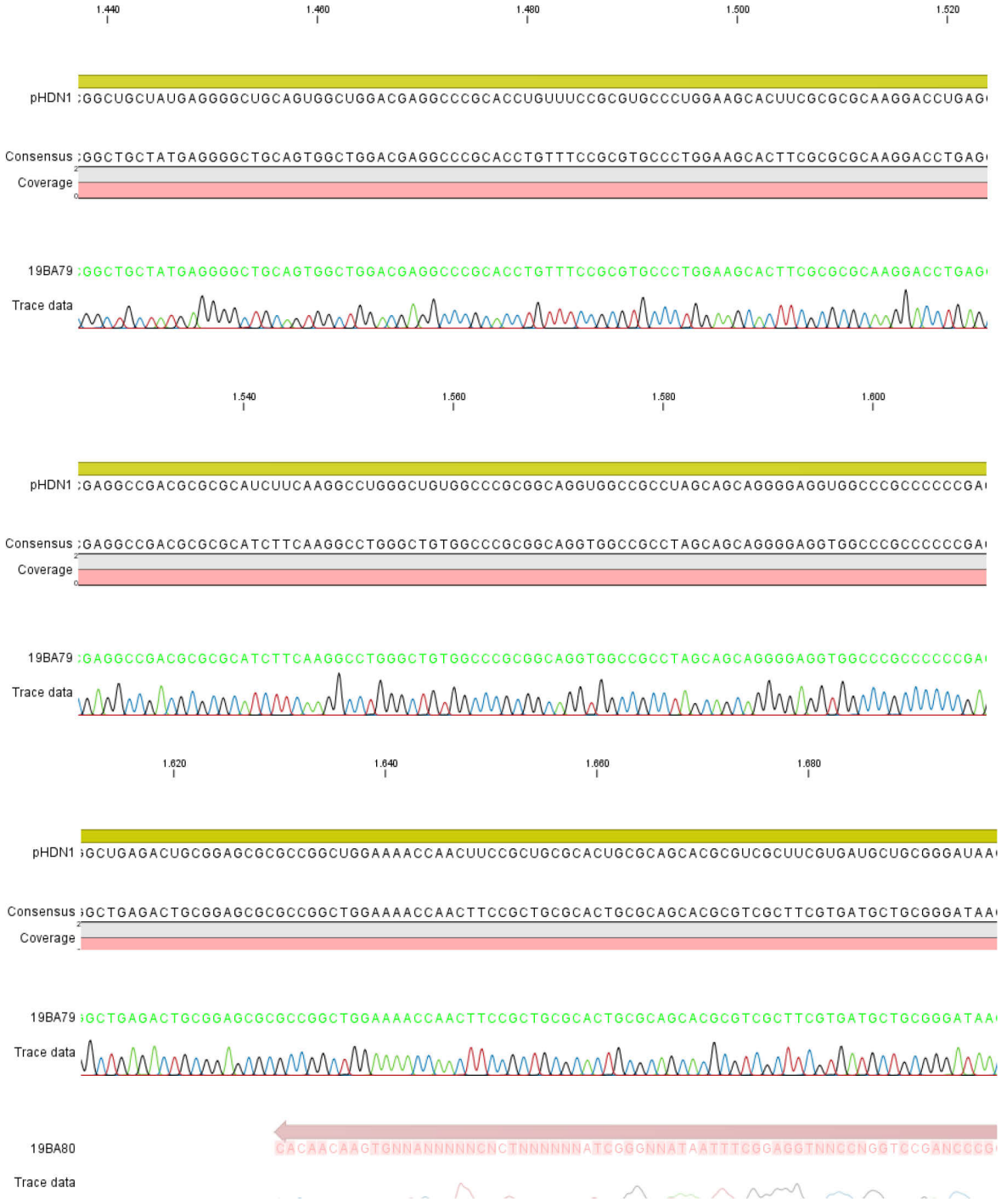


Figure 17: PCR of IRF7 sequences from chromosomal DNA
Both gels, left and right, show bands of the IRF7 gene amplified in two different cyclings but under identical conditions. Since the first cycling (left gel) could not amplify the IRF7 gene from chromosomal DNA of AGS#4 sorted, the amplification was repeated. The second cycling (right gel), on the other side could not amplify the IRF7 gene in AGS#5 sorted. In order to continue with the sequencing, chromosomal DNA of AGS#5 sorted was amplified successfully in particular (here not shown).
The IRF7 sequence with a size of 1512 bp formed clear bands. Except for chromosomal DNA from non-transfected AGS cells, every cell clone had a successful amplification of IRF7.
Polyclonal= transfected cells before sorting
Sorted= transfected cells after sorting

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1.520
pHDN1
Consensus
Coverage
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Trace data
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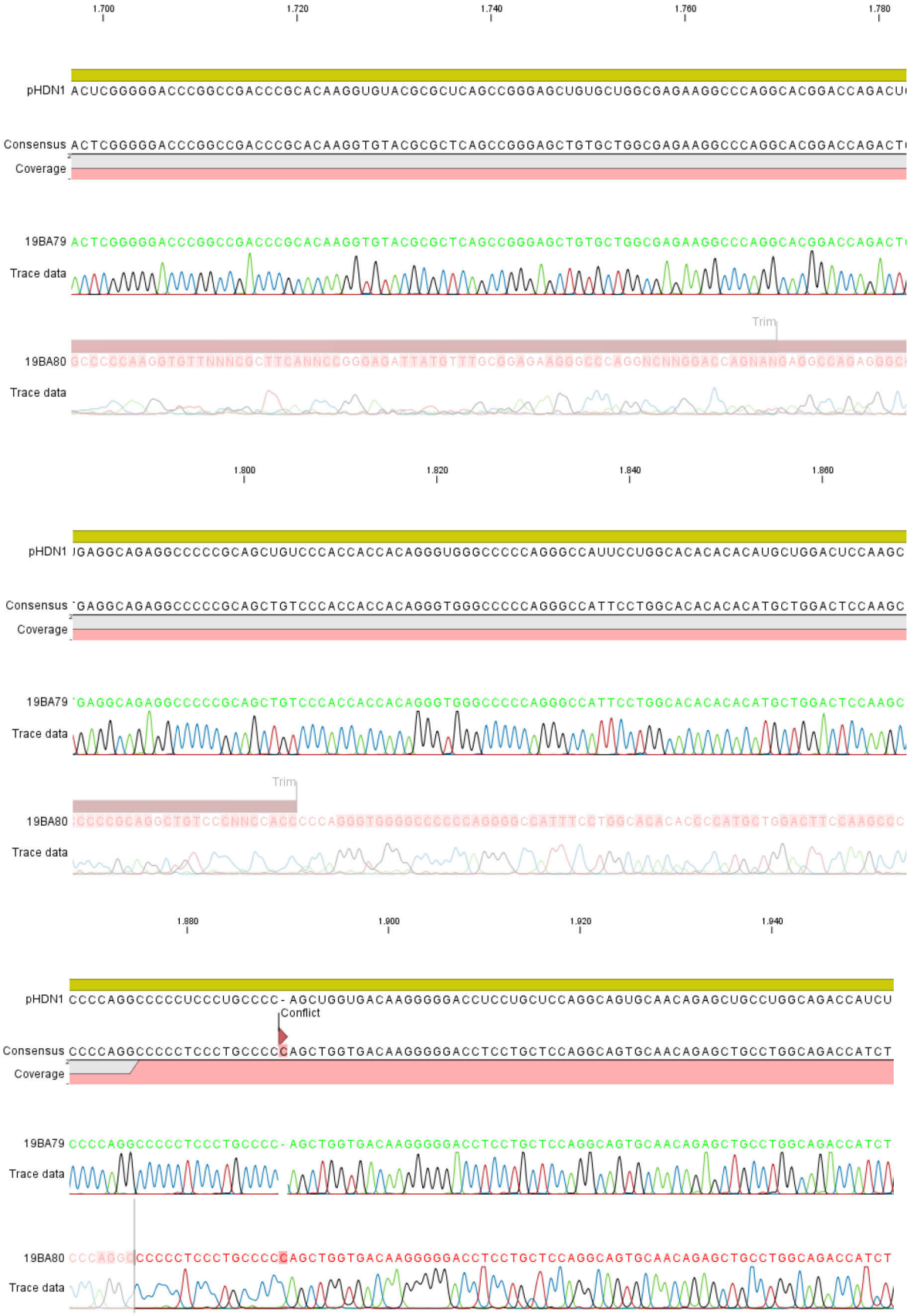
19BA80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Coverage
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Trace data
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Coverage
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Trim
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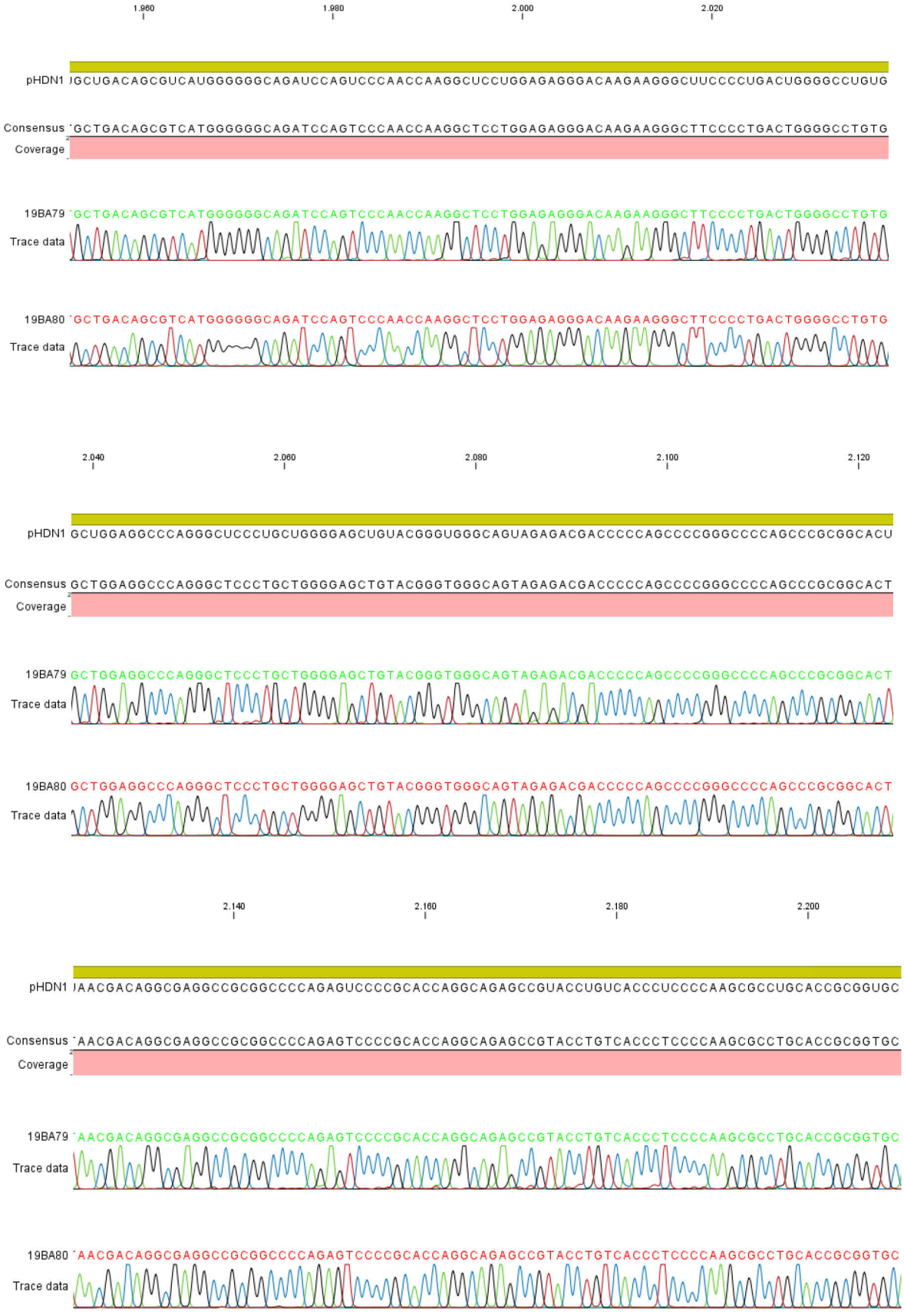
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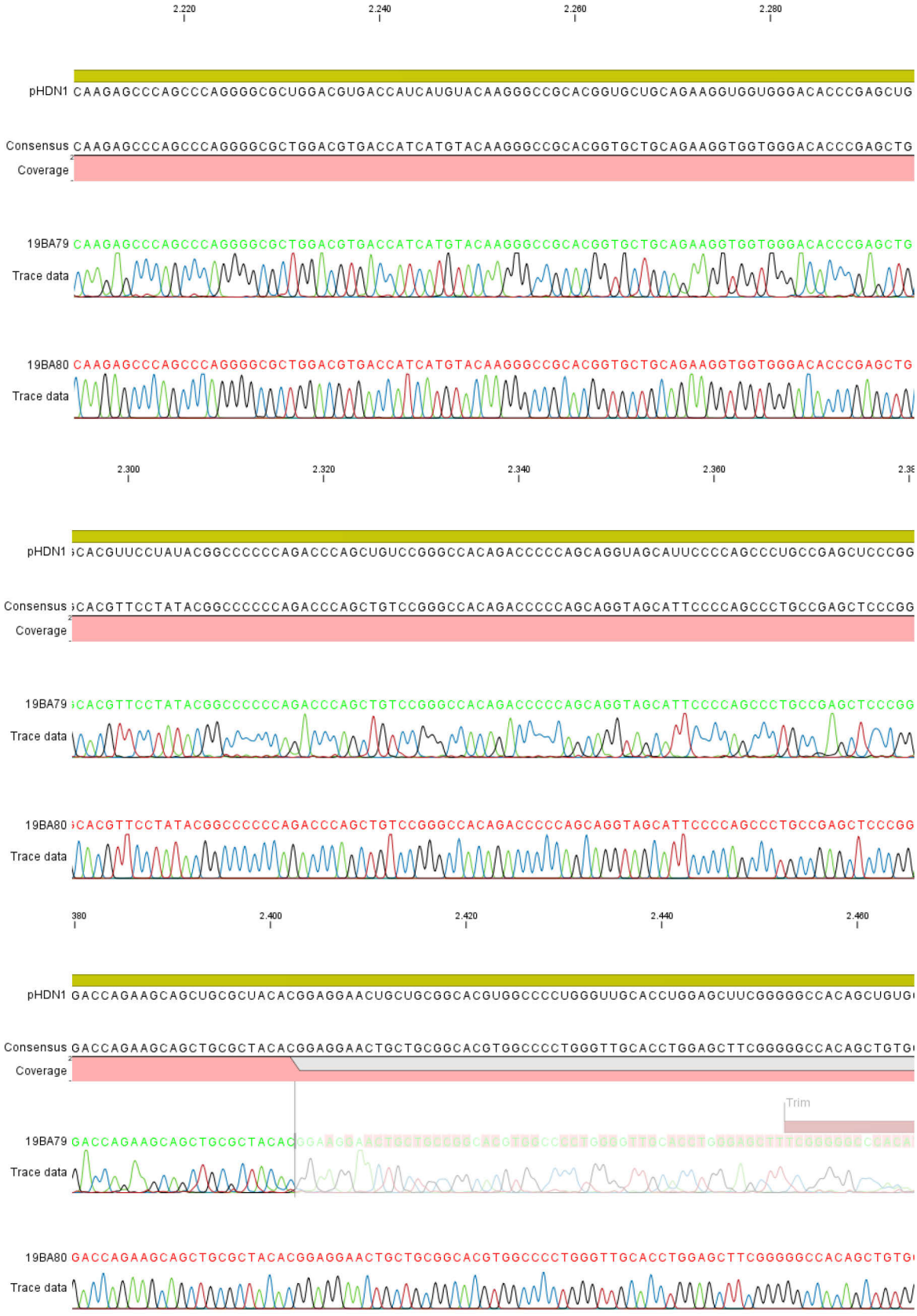
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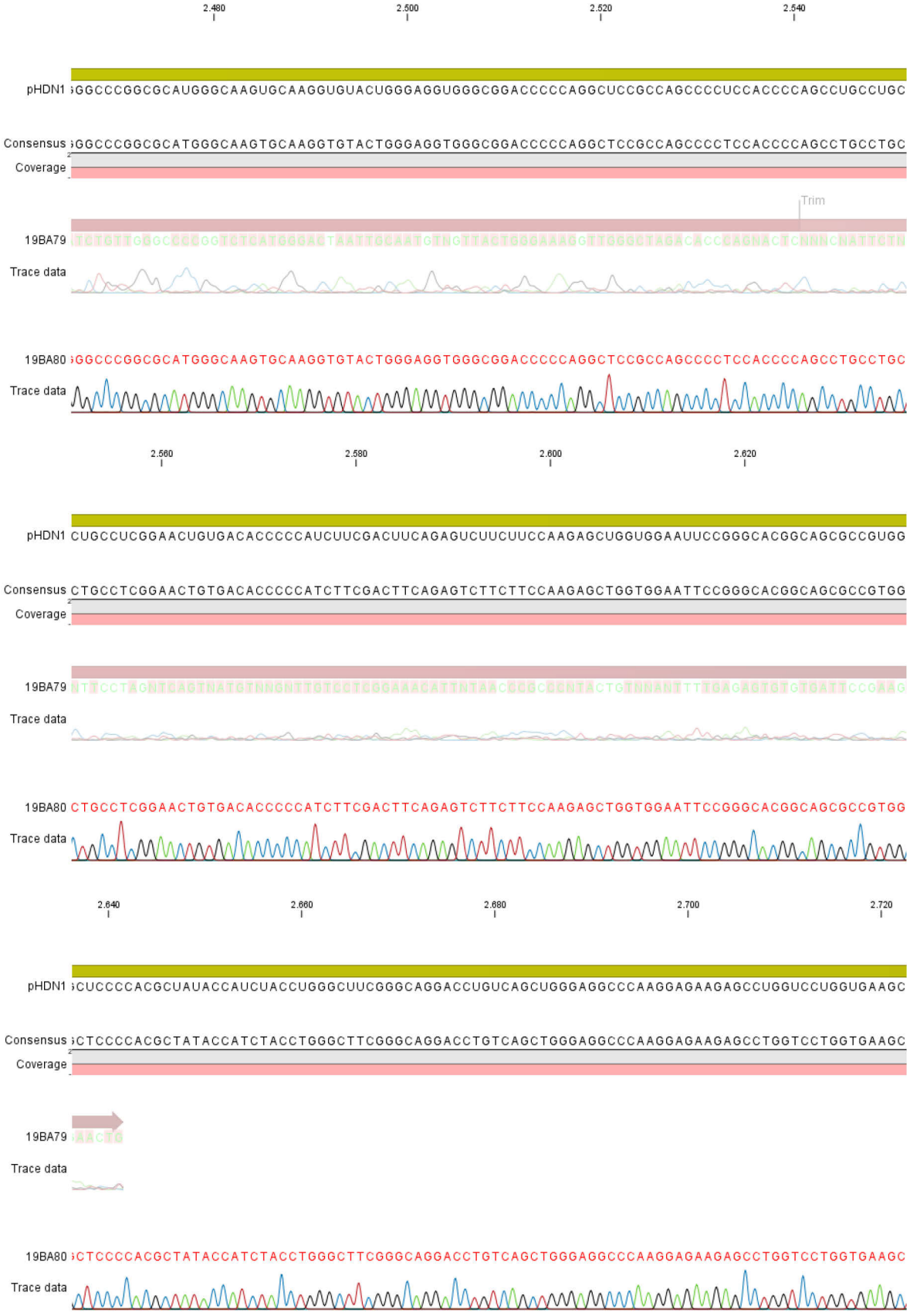
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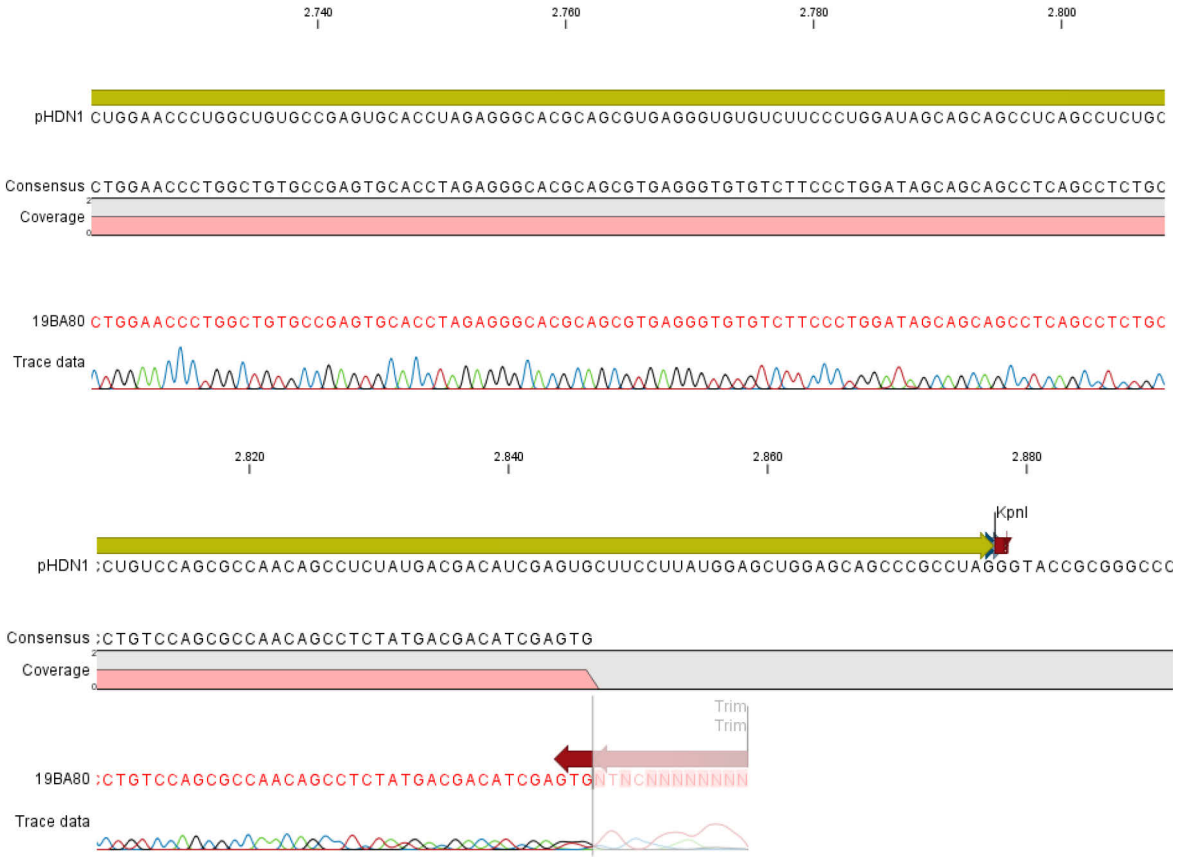


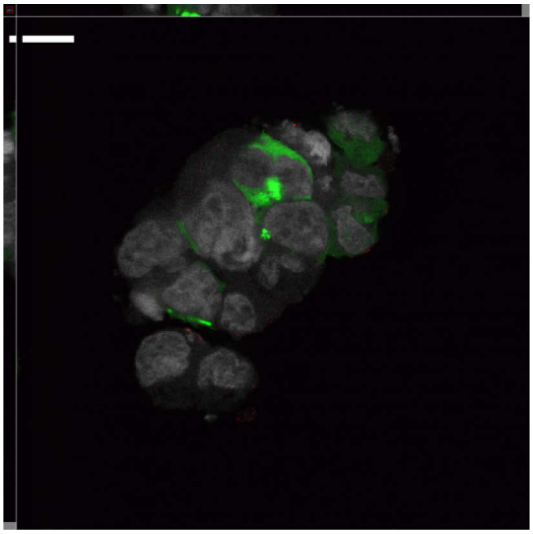
Figure 18: Alignment between the sequenced IRF7 and the original plasmid
Alignment between pHDN1 and the sequence result whose DNA was extracted from AGS pEYFP-IRF7 #5 sorted. Open reading frame of IRF7 between the two restriction enzymes HindIII and KpnI at the positions 1366 and 2877.
pHDN1: original plasmid; consensus/ coverage: consensus of the compared sequences (pHDN1 and IRF7); 19BA79: IRF7 sequence amplified with primer LJ 214; 19BA80: IRF7 sequence amplified with primer LJ 215; trace data: curve shows reliability of sequencing result.

The sequencing of the *IRF7* in AGS cells and in all transfected cells was not successful, except of AGS pEYFP-IRF7 #5 sorted (figure 18). This sequence was aligned to the original plasmid to find out mutations (figure 18). Since the initial transfection of AGS cells with this plasmid showed fluorescence, the reason about the loss of fluorescence should be identified.
Due to fluctuations the first and the last bases of a sequencing result cannot allow explicit conclusions. Therefore, the initial bases and the expiring ending bases of the sequenced IRF7 gene within the extracted chromosomal DNA could not be evaluated. Nevertheless, the main sequence part in the middle could clearly been sequenced and did not show any hints for mutation as it fits perfectly to the sequence of the original plasmid.

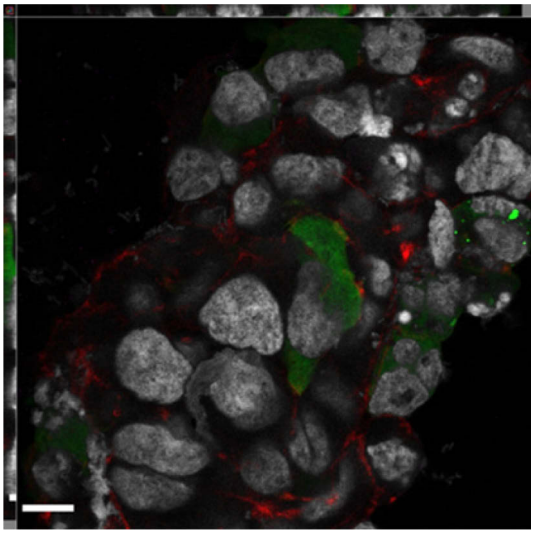
3.1.4 Translocation of IRF7 into the nucleus upon infection with *H. pylori*

This study has already shown that the IFN pathway is T4SS-dependently activated upon infection with *H. pylori* (see 3.1.1). Following this result, a more detailed investigation was planned. The aim was to focus on the T4SS-part that is necessary for IFN induction. Since

the CagY protein was observed to have similarities to the protein LMP1 from Human Herpes Virus 4 or Epstein-Barr-Virus, which interacts with transcription factor IRF7, it was planned to demonstrate a direct interaction between CagY and IRF7 (for details see 1.1.7). In order to visualize the CagY-induced translocation of the IRF7 into the nucleus, the plasmid pEYFP-IRF7 was created (for details see 2.2.2.13). The produced fusion protein EYFP-IRF7 was detectable due to the fluorescent EYFP-part. The first idea was to apply the plasmid into the cell with a microinjection technique by life cell microscopy. Upon stimulation, the fusion protein EYFP-IRF7 and CagY produced by the corresponding injected plasmids should bind and translocate into the nucleus. The whole process should have been imaged with a live cell imaging program. However, HEK CC5 cells were difficult to punctate with the injection needle. Since the NF-κB pathway was shown to be induced upon *H. pylori* infection, pGFP-RelA was chosen as positive control (see 3.1.5). RelA is an important transcription factor of the NF-κB pathway and involved in the LMP1-triggered induction of this pathway (figure 6). However, there was no effect on translocation of the transfected plasmid pGFP-RelA after stimulation with TNFα (data not shown).
After this pilot experiment to test the conditions, the live cell imaging was replaced by confocal microscopy. With this technique no cell puncture was necessary and longer incubation time could be set up. The restricted incubation time while doing the live cell imaging was suggested to be the reason why the injected plasmid did not cause the expected effects.
The HEK 293, HEK CC1 and HEK CC5 cells were transfected with the plasmid pEYFP-IRF7. After the infection with *H. pylori* wildtype and mutant strains, the cells were fixed and stained as described in 2.2.3.11. Using the SP5 confocal microscope, images were taken of chosen cell accumulations. The evaluation was like reading a CT scan by scrolling through the layers of the images taken. Only the IRF7 that obviously was inside the grey-marked area of the DNA was estimated as translocated protein. The IRF7 accumulations that were close to the DNA but not clearly inside were estimated as unclear status.
Corresponding to the results from the Luciferase Assay (see 3.1.1) that show the importance of CEACAM proteins for CagA translocation and therefore also for the induction of the IFN-pathway upon *H. pylori* infection, confocal microscopy was performed on HEK CC1 and HEK CC5 cells, in parallel to the HEK 293 cells. A confirmation of the dependency of IRF7-translocation on the presence or absence of CEACAM proteins was expected: While IRF7-translocation in HEK 293 cells upon *H. pylori* infection should not happen, it should be observed regularly in HEK CC1 and HEK CC5 cells infected with *H. pylori* wildtype strains. The following figures show the results obtained.



A



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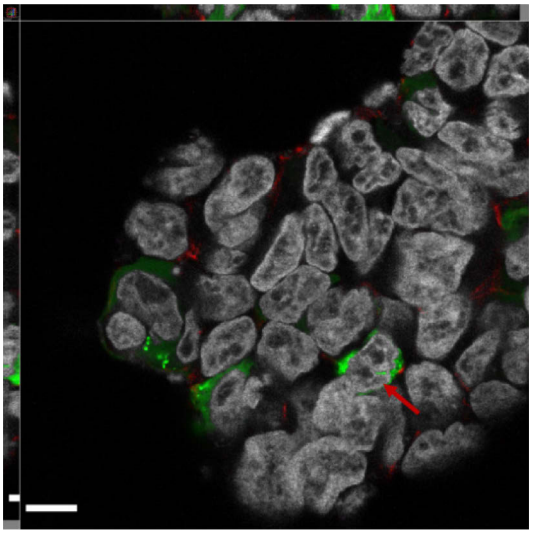

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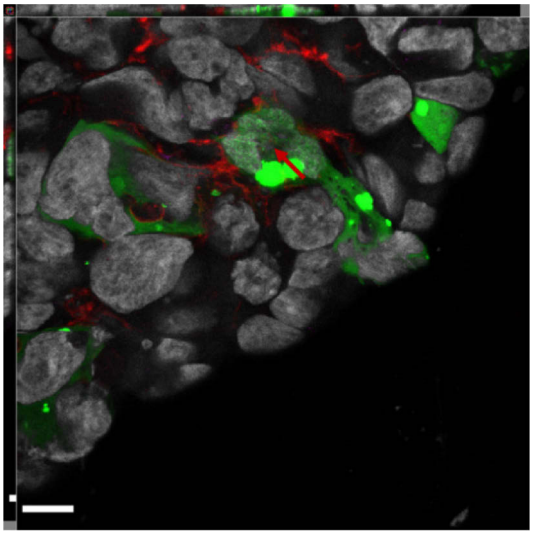

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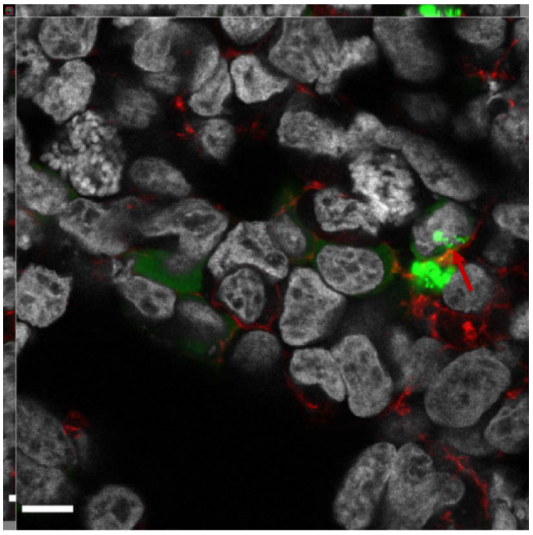
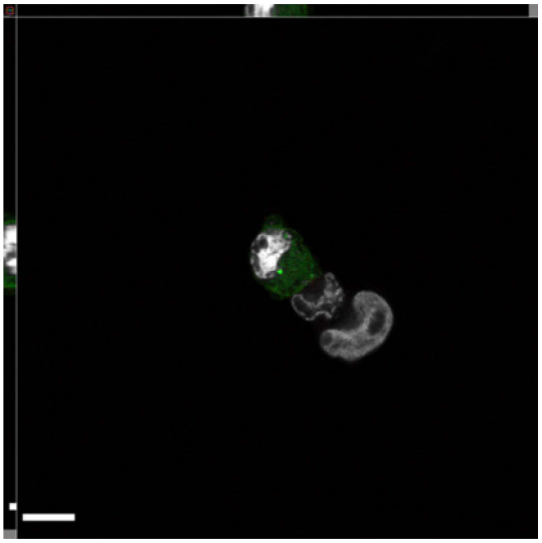


E

Figure 19: HEK 293 transfected with pEYFP-IRF7
Staining: grey= DNA; green= EYFP-IRF7; red= cytoskeleton; red arrow marks the translocated IRF7
A HEK 293 cells uninfected. 2.5 zoom, scale 10µm.
B HEK 293 cells infected with P12wt. 2.5 zoom, scale 10µm. The IRF7 is not translocated to the nucleus. But in the same slide focusing another cell accumulation, there was IRF7 translocated to the nucleus of one cell (not shown).
C HEK 293 infected with P12ΔcagY. 2.5 zoom, scale 10µm. IRF7 is translocated into the nucleus of some cells. And there are also cells where IRF7 seems to be in the nucleus but it was not very clear to see.
D HEK 293 infected with 26695wt. 2.5 zoom, scale 10µm. It is not clearly to see whether the IRF7 is translocated to the nucleus or not.
E HEK 293 infected with 26695Δhp0527. 2.5 zoom, scale 10µm. Probably IRF7 is not translocated to the nucleus.

The non-infected HEK 293 cells showed regular distributed EYFP-IRF7 proteins in the cytoplasm. Infected HEK 293 cells showed partly regular distributed EYFP-IRF7 proteins, partly accumulations of EYFP-IRF7 proteins in the cytoplasm. The translocation of IRF7 was not always clear to evaluate. There was no distinct difference between the cells infected with wildtype strain bacteria and the mutants. HEK 293 cells infected with P12wt had the IRF7 translocated in some cells, while IRF7 was not translocated in other cells. The cells infected with the corresponding mutant lacking CagY were more difficult to evaluate: some cells showed translocation of IRF7, the other cells could not be evaluated, neither with nor without protein translocation. Infection with 26695wt caused accumulation of IRF7 that was close to the nucleus but not clearly translocated. The cells infected with 26695Δhp0527 did not cause translocation of IRF7.
The results do not correspond to the initial expectations. Since HEK 293 cells do not express CEACAM proteins on their cell surfaces, CagA translocation and therefore no activation of the IFN-pathway should be possible. However, translocation of IRF7 could be observed.



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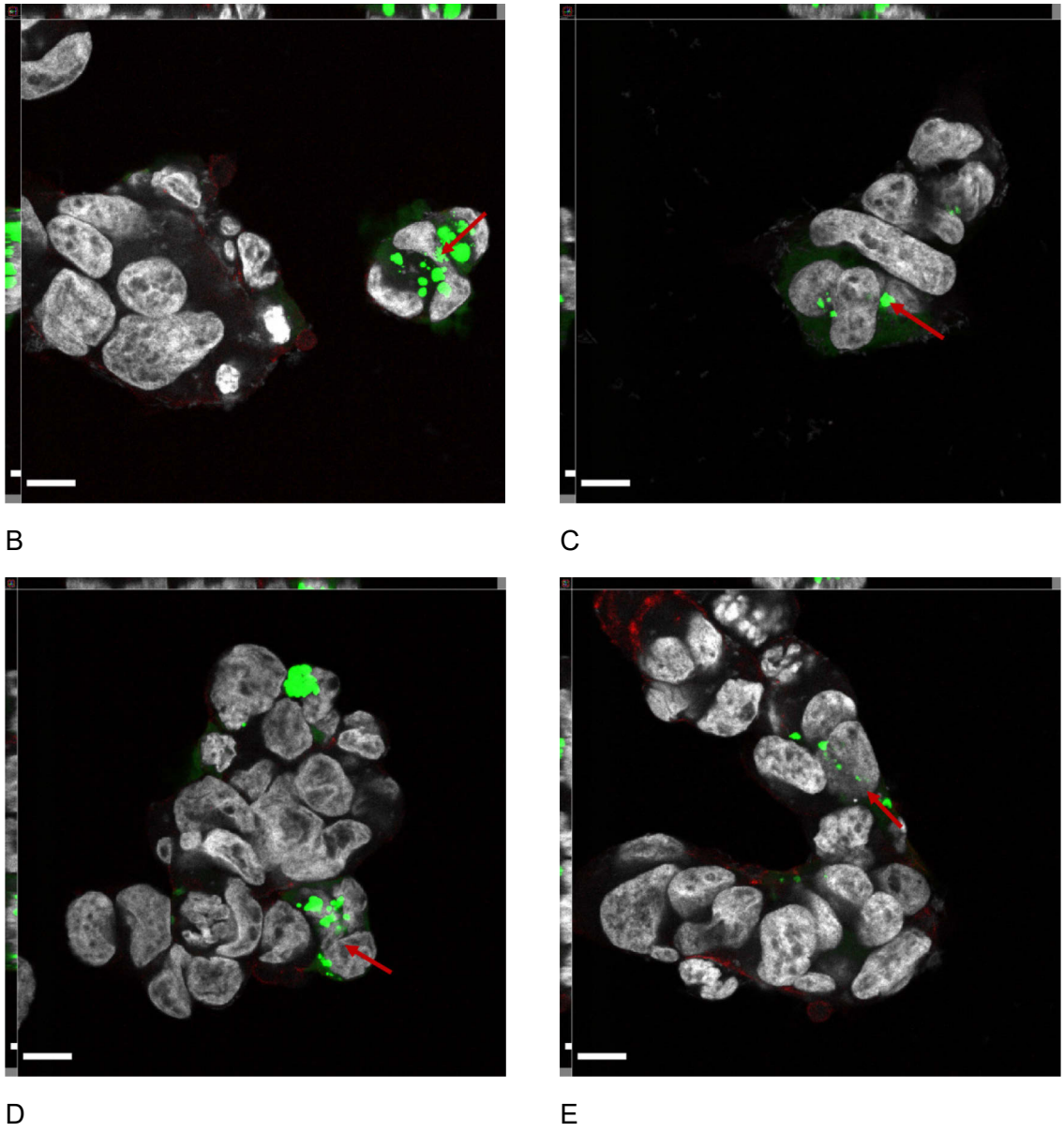
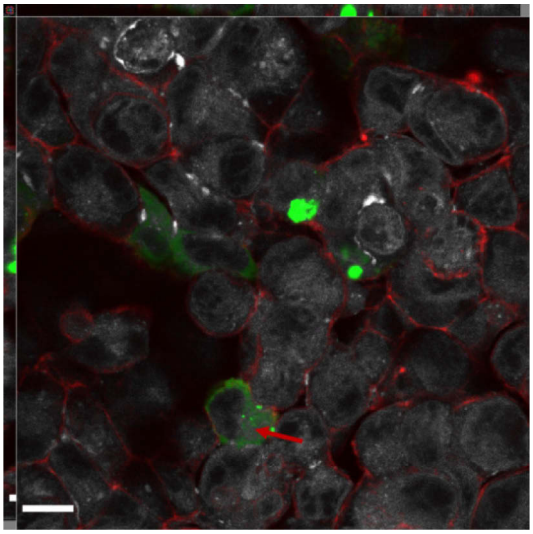
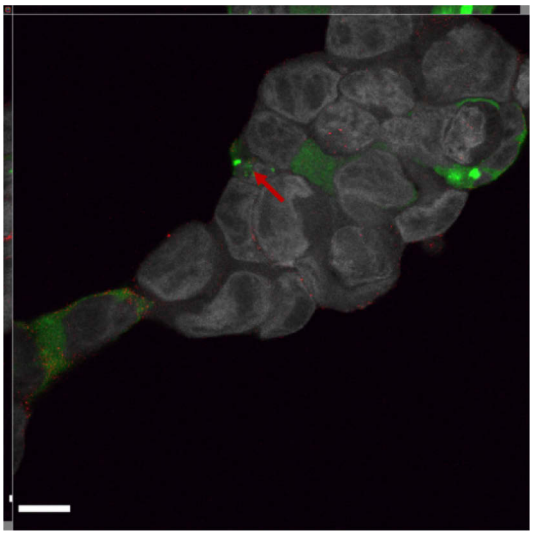


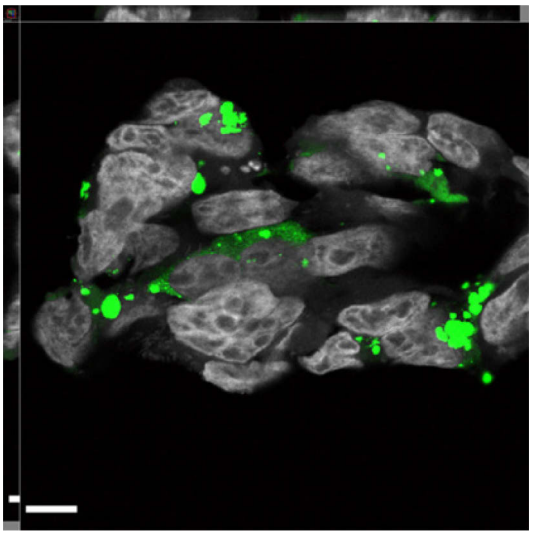
Figure 20: HEK CC1 cells transfected with pEYFP-IRF7
Staining: grey= DNA; green= EYFP-IRF7; red= cytoskeleton; red arrow marks the translocated IRF7
A HEK CC1 cells uninfected. 2.5 zoom, scale 10µm.
B HEK CC1 cells infected with P12wt. 2.5 zoom, scale 10µm. IRF7 is definitely translocated to the nucleus.
C HEK CC1 cells infected with P12ΔcagY. 2.5 zoom, scale 10µm. IRF7 is translocated to the nucleus.
D HEK CC1 cells infected with 26695wt. 2.5 zoom, scale 10µm. IRF7 is in the nucleus.
E HEK CC1 infected with 29965Δhp0527. 2.5 zoom, scale 10µm. The IRF7 is very close to the nucleus, but not for sure inside.



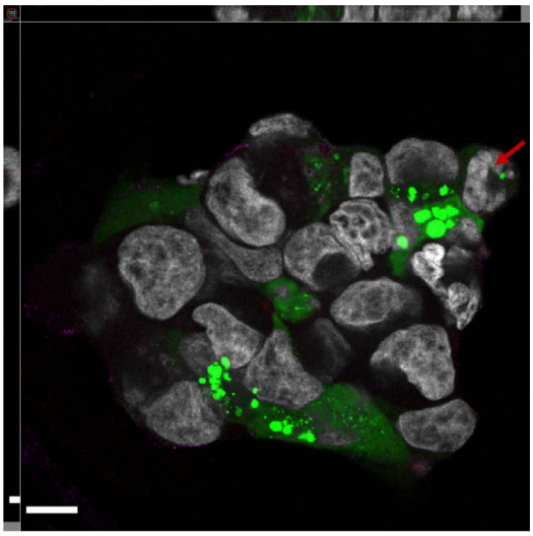
A



B



C



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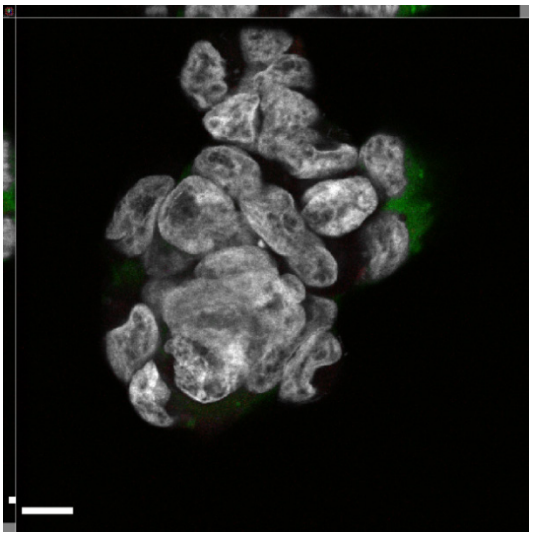


E

Figure 21: HEK CC5 cells transfected with pEYFP-IRF7
Staining: grey= DNA; green= EYFP-IRF7; red= cytoskeleton; red arrow marks the translocated IRF7
A HEK CC5 cells uninfected. 2.5 zoom, scale 10µm. In all cells but one there is no IRF7 in the nucleus.
B HEK CC5 cells infected with P12wt. 2.5 zoom, scale 10µm. IRF7 is translocated into the nucleus.
C HEK CC5 cells infected with P12ΔcagY. 2.5 zoom, scale 10µm. It is not clearly to see whether the IRF7 is in the nucleus or not.
D HEK CC5 cells infected with 26695wt. 2.5 zoom, scale 10µm. In one cell, there was a translocation of IRF7 into the nucleus.
E HEK CC5 infected with 26695Δhp0527. 2.5 zoom, scale 10µm. Nothing has gone into the nucleus.

The HEK CC1 and HEK CC5 cells showed regular distributed EYFP-IRF7 proteins and also accumulations of EYFP-IRF7 proteins in the cytoplasm. In contrary to the HEK 293 cells, even the non-infected HEK CC1 and HEK CC5 cells had accumulated EYFP-IRF7 fusion proteins in their cytoplasm. There was no difference in protein grouping or IRF7-translocation between the non-infected and infected cells, with both wildtype and mutant bacteria strains. Uninfected HEK CC1 cells showed protein accumulation but no translocation. The ones infected with P12wt, P12ΔCagY, 26695wt showed translocation of the protein IRF7. HEK CC1 cells infected with 26695Δhp0527 had protein accumulation of IRF7 that was very close to the nucleus but not clearly to see whether this complex was translocated or not. In HEK CC5 cells, there was IRF7-translocation even in uninfected cells. Translocation was also observed upon infection with P12wt and 26695wt. HEK CC5 cells infected with P12ΔCagY showed an unclear status of translocation. The corresponding mutant lacking CagY of 26695wt did not cause translocation of IRF7.

In sum, no regularity or pattern in the nuclear translocation of IRF7 was identifiable. After some test series a terminal conclusion about the necessity of CagY cannot be made, but the results are more suggestive of a CagY-independent IRF7 nuclear translocation. Additionally, the presence or absence of CEACAM proteins does not seem to have influence on IRF7 translocation upon *H. pylori* infection.
Furthermore, accumulation of IRF7 could be observed in infected HEK 293 cells and in uninfected and infected HEK CC1 and HEK CC5 cells. Probably CEACAM proteins have direct impact on IRF7. The presence of this protein together with infection with bacteria might have brought stress to the cells which led to accumulation of IRF7.

3.1.5 pGFP-RelA and TNFα as positive control

RelA is an important transcription factor of the NF-κB pathway and involved in the LMP1-triggered induction of this pathway (see 1.2.2, figure 6). Since the NF-κB pathway was shown to be induced upon *H. pylori* infection, pGFP-RelA was chosen as positive control (see 3.1.5). In previous studies [135, 136] the plasmid pGFP-RelA was shown to work for microscopy experiments.
The plasmid pGFP-RelA was a gift from another laboratory and did not work as expected. Comparing the RelA sequence in the given plasmid to the one from gene bank of NCBI, additional base pairs (494-502) were found (figure 21). Multiple efforts to correct the RelA sequence in the plasmid pGFP-RelA by PCR and to create a new RelA plasmid with pEYFP-C1 were not successful (see 3.4.2).

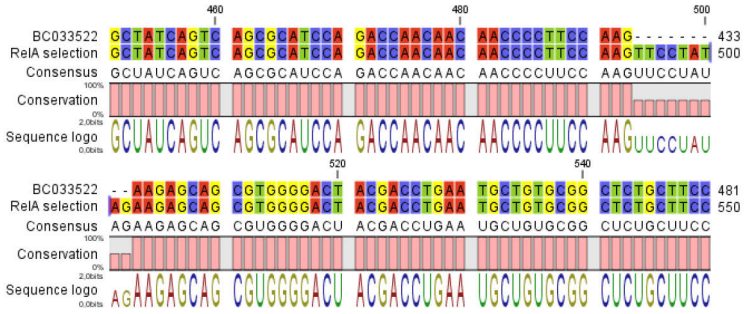


Figure 22: Alignment of RelA sequences
The alignment of the RelA sequence in the plasmid pGFP-RelA and RelA sequence taken from gene bank of NCBI show an insertion of 9 base pairs, equivalent to 3 amino acids at position 494 to 502.

Next, TNFα was considered to be the positive control. Following the paper of Li, S.D. et al. [38] the amount of 50 ng/ml TNFα was added to the cells. However, even after increasing the concentration of this cytokine no effect on the cells could be marked out.

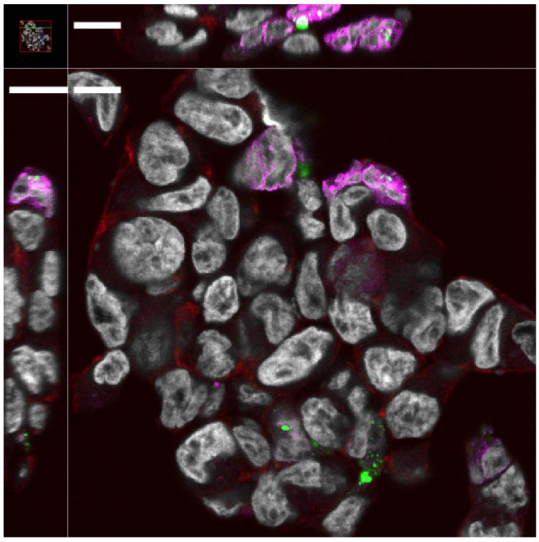


Figure 23: TNFα applied to the cells as an inducer of the IFN pathway
Staining: grey= DNA; green= EYFP-IRF7; red= cytoplasm; violet= nuclear membrane; 2.5 zoom, scale 10µm
TNFα was applied to HEK CC1 cells. But the expected effect of translocation of the IRF7 protein did not happen.

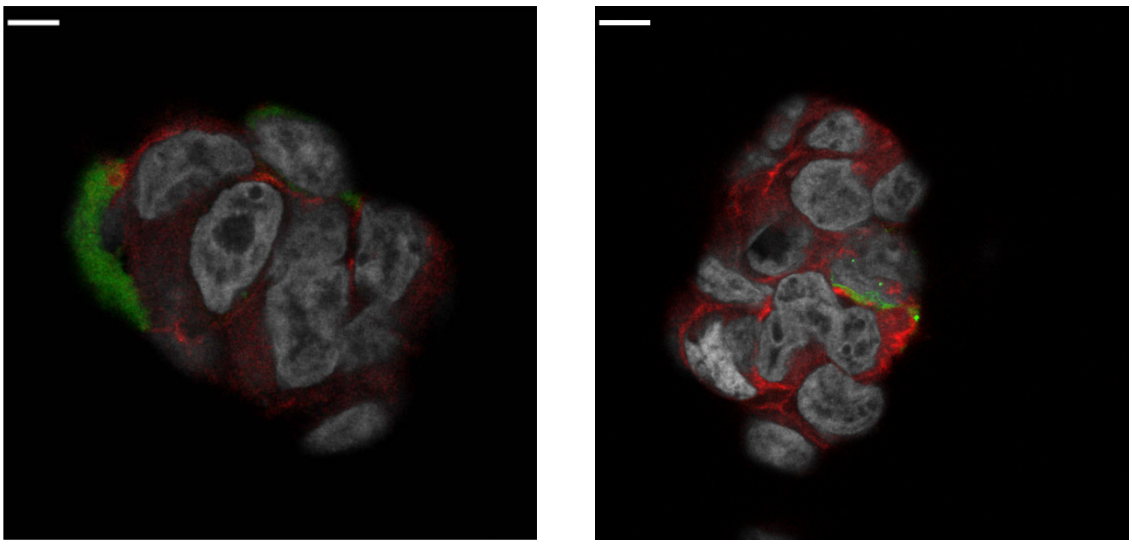
3.2 Establishment of a staining method for a better evaluation of IRF7-translocation into the nucleus

The evaluation of the microscopy experiments was difficult because of the demarcation of the nucleus. The DAPI staining only marked the DNA, but not the nuclear membrane. So it could not be determined for sure whether the EYFP-IRF7 was translocated into the nucleus or not. There was no computer program to measure the position of IRF7, but the evaluation of each image was done manually one by one. Since the images were shot in a planigraphy way, the principle of evaluation was like reading a CT scan. Parallel to the

attempt to establish a working positive control for the cell investigation with confocal microscopy, different staining methods of the nuclear membrane were tried in order to make the evaluation better and more reliable. For a detailed staining description see 2.2.3.11.

3.2.1 DAPI, Phalloidin Texas Red, pEYFP-IRF7

The first stainings were done with DAPI to stain the DNA and Phalloidin Texas Red to stain the cytoplasm.



A B

Figure 24: Cell staining with DAPI and Phalloidin Texas Red
Staining: grey= DNA; red= cytoskeleton; green= EYFP-IRF7, closeup; scale 8µm
A Snapshot of overlay HEK 293 cells transfected with pEYFP-IRF7. Scale 8µm.
B Snapshot of overlay HEK 293 cells transfected with pEYFP-IRF7. Scale 11µm.

This staining method marked DNA and cytoskeleton very good. Also the fluorescent fusion protein EYFP-IRF7 was clearly to see. As shown in figure 24, the EYFP-IRF7 was in the cytoplasm since the fluorescence was regularly spread in the cell. However, in figure 24B there are accumulations of EYFP-IRF7 which are localized very close to the DNA stained in grey. Still, no clear statement could be made concerning the translocation of IRF7. So the nuclear membrane needed to be stained as well.

3.2.2 DAPI, Phalloidin Alexa 647, anti-panLamin antibody, pEYFP-IRF7

Modifying the protocol of [138] an antibody against the protein Lamin, a compartment of the nuclear membrane, was used to visualize the nuclear membrane. Additionally, the cytoplasm staining Phalloidin Texas Red was replaced by the staining Phalloidin Alexa 647 to have better detection under the microscope using another laser. For detailed protocol see 2.2.3.12.2.

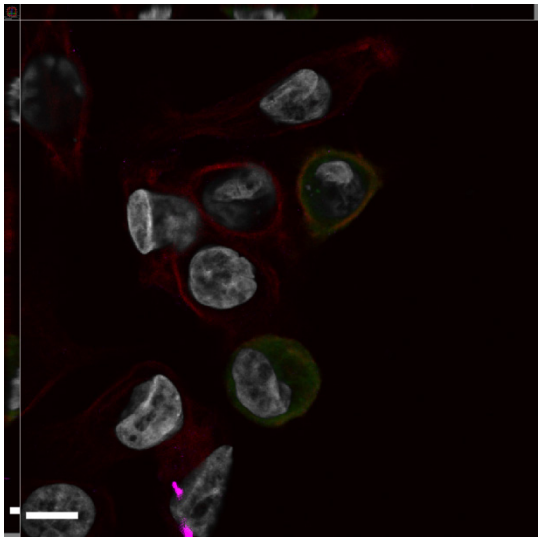


Figure 25: Cell staining with anti-Lamin antibody, DAPI and Phalloidin Alexa 647
Staining: grey= DNA; red= cytoskeleton; green= EYFP-IRF7; violet= nuclear membrane, 2.5 zoom; scale 10µm
The AGS cells were not infected. 2.5% of PFA was used to fix and 0.1% Triton 100x was used to permeabilize the cells.

Even after experiment set-up changes (first modification), the staining of the nuclear membrane failed to mark the nuclear membrane, as shown in figure 25. So the staining protocol with the anti-Lamin antibody was modified based on papers dealing with experiments with the same antibody against Lamin [139-141] in order to test in how far the function of the anti-panLamin antibody was influenced by different fixing and permeabilization techniques.

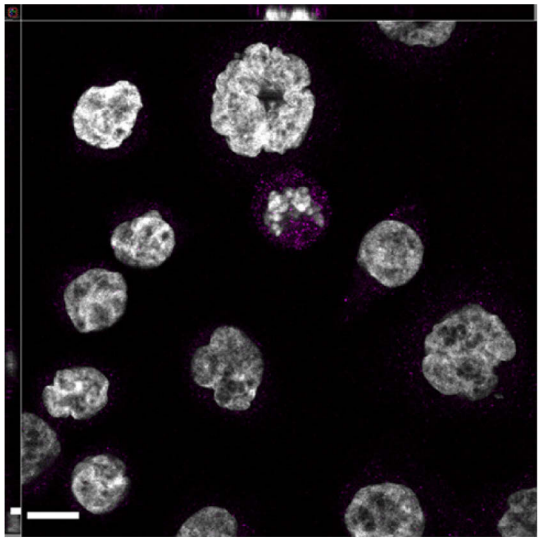


Figure 26: Modified staining with anti-Lamin antibody, DAPI and Phalloidin Alexa 647
Staining: grey= DNA; violet= nuclear membrane; 2.5 zoom; scale 10µm
The AGS cells were uninfected, fixed with 1 % PFA and permeabilized with methanol.

The cells were first fixed with PFA in different concentrations (1%, 3%, 4%) and then permeabilized with methanol. The cells did not show any staining with Phalloidin Alexa

647. There might be an interaction with methanol especially because Phalloidin Alexa 647 contains methanol. It looked like the anti-panLamin antibody had stained the cytoplasm instead of Phalloidin Alexa 647.

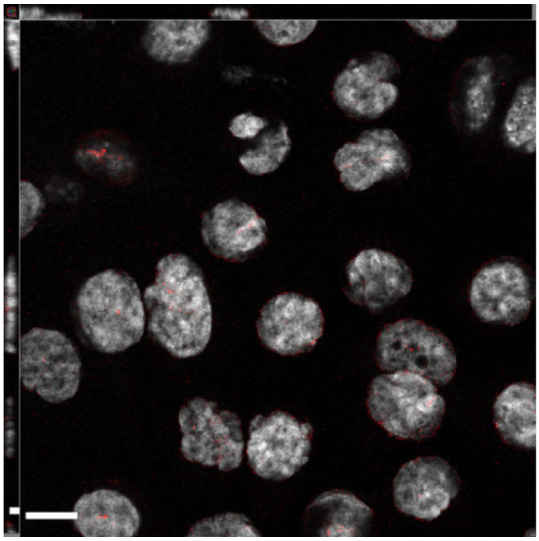
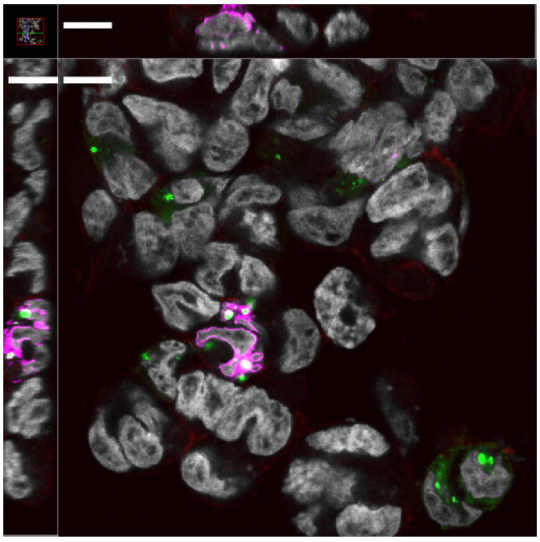


Figure 27: Modified staining with anti-Lamin antibody, DAPI and Phalloidin Alexa 647
Staining: grey= DNA; red= nuclear membrane; scale 10µm
The AGS cells were not infected, fixed and permeabilized with methanol.

The modification shown in figure 27 had the best staining of the nuclear membrane. The anti-panLamin antibody marked an area around the DNA which was the stained nuclear membrane. However, now there was no staining of the cytoplasm with Phalloidin Alexa 647. Not seeing the cytoplasm, and therefore the whole cell, was also not advantageous for the evaluation of the microscopy data. In addition the staining was very time-consuming, since the anti-panLamin antibody has an incubation time of 16 hours.

3.2.3 DAPI, Phalloidin Alexa 647, pRFPLamin, pEYFP-IRF7

Since the nuclear staining with methanol worked but was not beneficial, neither for the evaluation processes nor for the evaluation of the microscopy data, the ‘marker’-plasmid pRFLamin was used to stain the nuclear membrane. The plasmid was a kind gift from the work group of Dr. Sewald in the Virology Department of the Max-von-Pettenkofer-Institut, LMU München. The plasmid transfected into the cells encode marked proteins binding Lamin. Since Lamin staining was obtained by the production and binding of the plasmid-encoded proteins, no staining of the nuclear membrane in particular was necessary any longer.



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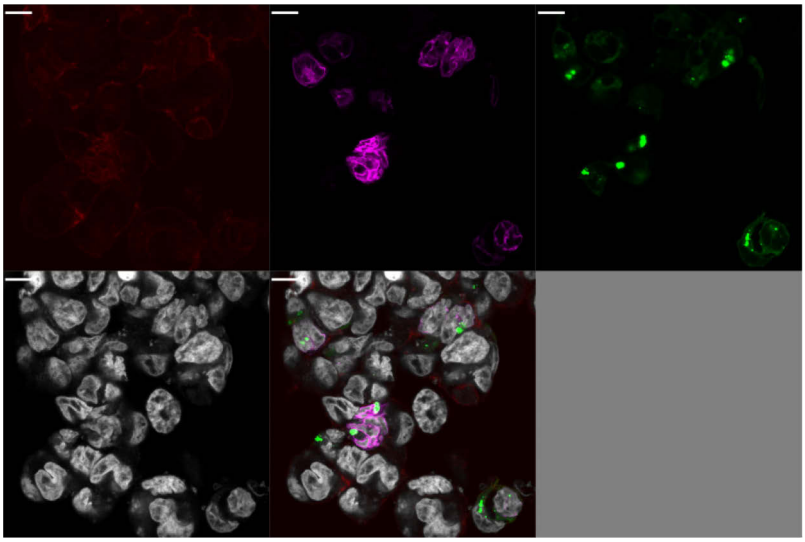

B

Figure 28: Cell staining with DAPI, Phalloidin Alexa 647 and pRFPLamin
Staining: grey= DNA; green= EYFP-IRF7; red= cytoplasm; violet= nuclear membrane; 2.5 zoom; scale 10µm
A HEK CC1 cells were transfected with pEYFP IRF7.
B HEK CC1 cells transfected with pEYFP-IRF7 shown in splitted staining layers. The nuclear membrane is clearly marked.

As shown in figure 28, this staining combination offers a good staining of all important cell compartments: DNA via DAPI, cytoplasm via Phalloidin 647, nuclear membrane via plasmid pRFLamin and the fluorescent fusion protein EYFP-IRF7. This staining technique can be used for further studies on nuclear translocation with the confocal microscope and offers a good basis to visualize the localization of the transcription factor IRF7.

3.3 Interaction of CagY with IRF7 protein

To prove the direct interaction of IRF7 and CagY, their DNA sequences had to be cloned into plasmids for their expression as fusion proteins. Following pulldown assays and their detection via Western Blot should show the direct binding of the two proteins. Therefore different steps had to be prepared simultaneously, e.g. creating the fusion proteins and establishing a protocol for IRF7-Western Blot.

To create the CagY fusion protein, it was planned to insert the gene into the commercial plasmid pET151 (see 2.2.2.13). The amplification of the sequence encoding CagY was very difficult, due to the highly repetitive structure of the gene sequence. The PCR reactions with chromosomal DNA from *H. pylori* strains P12 and 26695 succeeded only two times from multiple attempts. To get a clean PCR product the samples were load on agarose gel to be extracted. The clean DNA fragment was inserted into the vector pET151 and the completed plasmid was then transformed into *E. coli* strain BL21 Star™ (DE3). The fragments got lost during the work process. Afterwards, even after lots of cycles, the DNA amplification did not work anymore. Nor the primary cycling protocol, neither the modified protocol with another annealing temperature led to successful PCR reactions.

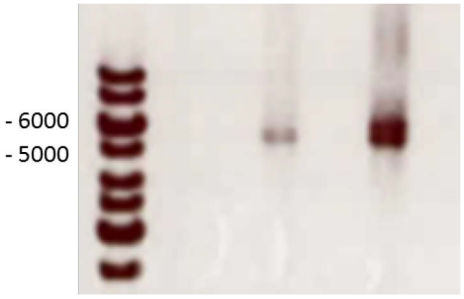

Figure 29: Amplified CagY in 0.8% agarose gel
The first band shows the CagY amplified from chromosomal DNA of P12, the second one shows the CagY from 26695.

The sequence of IRF7 was cloned into the vector pGEX-4T-3 to be expressed as fusion protein GST-IRF7. This fusion protein should have been mixed with the CagY to enable the two proteins' binding. In the following, after co-precipitation (pulldown), the recombinant proteins of CagY and IRF7 should be detected by Western Blot with antibodies against CagY and IRF7. The bands of the Western Blot would provide the size of the co-precipitations, which hence, would allow the conclusion of the binding between CagY and IRF7. The cloning was not successful due to problems of the plasmid preparation and of the transformation process. Therefore, no plasmid could be extracted.

To detect the pulldowns of CagY and IRF7 recombinant proteins, Western Blots with anti-CagY antibody and anti-IRF7 antibody were necessary. Since the IRF7 antibody for Western Blot was completely new in the work group and therefore no protocol available, the experimental conditions for the use of the anti-IRF7 antibody had to be established first. After numerous tries with different variations of the detection process (see 2.2.5.3)

there was no protocol found giving really satisfying results. The blots did not show explicit bands marking the searched protein with the size of about 56 kDa. The data sheet announced an observed band size of 50.6 kDa compared to the expected band size of 54 kDa. But the blots showed bands of both sizes. It was also difficult to evaluate the absence of an IRF7-band in one sample as not existent protein or not proper working antibody. As demonstrated in figure 30, several bands were visible, the blot itself was impure and showed washy spots.

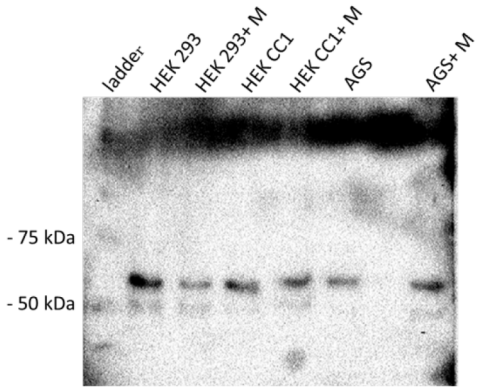

Figure 30: Western Blot detecting IRF7
0.9µl anti-IRF7 antibody for 1 hour 15 min, 4x15 min of washing.
This blot is an exemplary blot showing the protein detection with the anti-IRF7 antibody. It represents one of the best blots. M= lysates prepared with SDS+Mercaptoethanol

3.4 Interaction of *H. pylori* T4SS with the NF-κB pathway

3.4.1 T4SS-dependent induction of a NF-κB pathway promoter in HEK 293, HEK CC1 and HEK CC5 cells

The induction of the NF-κB pathway upon infection with *H. pylori* is T4SS-dependent and results in the secretion of the chemokine IL-8 (see 1.1.2).
To measure the T4SS-dependent induction of the NF-κB pathway the Luciferase Assay was performed with the plasmid pELAMluc proving E-selectin promoter activation upon infection with *H. pylori* strains with intact *cag*PAI (see 3.1.1). Since the T4SS-dependent pathway induction was already known, this plasmid was taken to reevaluate the T4SS-dependency, but also as positive control for the plasmid p125luc, whose function had to be tested. At the same time, the two pathways were compared to each other to find out the difference of the induction intensity. For more details see 3.1.1.

3.4.2 Translocation of GFP-RelA into the nucleus upon induction

RelA is a transcription factor of the NF-κB pathway. It is translocated into the nucleus upon infection with *H. pylori* (1.2.2).
In a pilot experiment based on [135] with Dr. Jimenez-Soto the cell lines AGS, HEK CC1 and HEK CC5 were transfected with the plasmid pGFP-RelA, which was a gift from another laboratory. For the life cell imaging the cells were placed in the chamber at 37°C

with 5% CO_2 during video recording and they were induced with TNFα. The fusion protein is expected to enter the nucleus after being phosphorylated due to the induction with TNFα. Accordingly, the nucleus in the images taken should turn to green-fluorescence. Since the experiment did not work as published in several papers, the sequence of the plasmid pGFP-RelA was checked with the primers pEGFP-FP, pEGFP_C2-FP, pEGFP_C2-RP to get the complete sequence of RelA. Since the primer pEGFP-FP seemed to be not really reliable, this part of the plasmid was resequenced with primer CMV-FP. There were 3 amino acids (position 494 to 502) inserted in comparison to the plasmid sequence of RelA in the gene bank of NCBI. With the intention to remove the 3 amino acids, the sequence of RelA was amplified with the primers LJ216 and LJ217 annealing close to the region to be corrected. The PCR results were checked by sequencing with the primer CMV-F to investigate the concerned part by comparison with the GFP-RelA sequence of the gene bank of NCBI. Unfortunately, the sequence of RelA could not be repaired by PCR. Thus, the production of cDNA from mRNA was planned to get the correct sequence of RelA. Therefore, mRNA was purified from Jurkat AEGG cells induced by TNFα. But the isolated RNA was not enough (4 ng/µl) to proceed to the first-strand cDNA. Several further attempts to get the correct RelA sequence were not successful, so that the project could not be finalized.

3.5 Cytokine secretion of cells upon infection with *H. pylori*

3.5.1 Interferon β secretion

The Luciferase Assay has already shown the T4SS-dependent activation of the IFNβ promoter (see Fig. 12). Next, an IFNβ-ELISA should be established to show the influence of the T4SS on secretion of the cytokine IFNβ responsible for the recruitment of immune cells and, probably, chronic inflammation (see 1.2.1). While doing the Luciferase Assay (see 3.1.1), the media from the infected HEK 293, HEK CC1 and HEK CC5 cells were collected to be used as sample for the ELISA assays.
The ELISA was done as described in 2.2.4. In first attempts the concentration of the standard was considered to be too low and was therefore increased. At the end it turned out that the antibody for the Interferon β-ELISA kit used in our work group was only appropriate for higher concentrations of the searched proteins in the samples. The supernatants to analyze seemed to have a very low concentration of IFNβ, which was undetectable with the first used standard. Because of a non-analyzable regression curve the evaluation of the results was not possible.

3.5.2 IL-8 secretion

The samples for IL-8 ELISA were the same as for the IFNβ-ELISA, supernatant collected from the Luciferase-Assay-experiments with HEK 293, HEK CC1, HEK CC5 cells. The assay has already shown that the activation of the NF-κB-promoter is dependent on T4SS. Therefore the secretion of IL-8, a cytokine closely dependent on the NF-kB pathway, should be investigated to demonstrate its dependence on the T4SS. To measure the amount of IL-8, ELISA assays were performed with the mentioned samples. Unfortunately the assay did not work, even after several attempts with different sample concentrations.

Since the priority was put on the analysis of the IFN pathway no further attempts were initialized due to time restrictions.

4 Discussion

H. pylori colonizes half of the world's population. This bacterium is an important cause for diseases like gastritis, gastric ulcer, development of gastric cancer and other extra-gastrointestinal diseases. An important virulence factor is the *cagPAI*-region with its encoded proteins. Among them, CagY is an essential compartment of the T4SS apparatus responsible for protein injection into human host cells. This protein has a remarkable characteristic in its gene sequence encoding a large protein with extensive repeat regions. The repeat regions have no similarities to other proteins except for LMP1. Former studies focused on these repeat regions, while current studies focus on the interaction of this protein with receptors and on its role within the T4SS apparatus.
This study investigates the interaction of the *cag*PAI and the CagY protein with pathways of the human immune system, in particular pathway components like IRF7 from the IFNβ-pathway and RelA from NF-κB-pathway. Furthermore, a staining method was established to investigate the impact of *cag*PAI and CagY on IRF7, a transcription factor of the IFN pathway.

4.1 *cag*PAI- and CagY-dependent activation of pathway promoters

4.1.1 IFNβ-promoter of Interferon type 1 pathway

Starting with the investigation on the role of CagY in IFN-pathway induction, its function in the human immune system upon *H. pylori* infection should be determined. Therefore, reporter gene experiments were performed on not infected and infected cells. On the opposite to HEK 293 cells that were shown not to express CEACAMs [120], HEK 293 cells with stable expression of CEACAM 1 and CEACAM 5, termed HEK CC1 and HEK CC5 cells, are able to translocate CagA, which means the ability of the induction of the IFN pathway.
As expected, HEK 293 cells transfected with p125luc did not show any (significant) reduction of the Luciferase activity, a scale for IFNβ-promoter induction, upon infection with *H. pylori* mutant strains as compared to wildtype strains. This emphasizes the importance of CEACAMs to enable *H. pylori* to induce the IFN pathway and indirectly the importance of CagA for IFN-induction [23]. The promoter activations observed have the same level as the non-infected cells, indicating a basic constitutive weak production of IFNβ [93]. In contrast, HEK CC1 cells transfected with p125luc show significantly reduced IFNβ-promoter induction upon infection with *H. pylori* mutant strains. So all deleted *cag*PAI-proteins, including CagA, CagY and CagE, with the PAI-region itself seem to be essential for IFN induction. The cells infected with ΔCagA-mutants of both strains have the weakest impact on promoter induction, since the activity reduction is the lowest. Interestingly, the promotor activity is strongly reduced in cells infected with ΔCagY- and ΔPAI-mutants, the former even the most. This proves *cag*PAI- and CagY-dependent activation of the IFN type I pathway. The strongest reduction of Luciferase activity with *H. pylori* mutants lacking CagY shows CagY's essential role in IFNβ-promoter activation upon infection with this bacterium and is a hint for CagY's importance, not only for the IFN

pathway, but also downstream, for cytokine release and immune response of the host. HEK CC5 cells behave quite similarly to HEK CC1 cells upon infection with *H. pylori*, except of the fact that P12ΔCagA does not cause a significant reduction of the Luciferase activity. These results from HEK CC1 cells (significant but weak reduction of promoter activity) and from HEK CC5 cells (insignificant reduction of promoter activity) hypothesize a mostly CagA-independent induction of the IFNβ-promoter in HEK CC5 cells. The aspect of different functions of the CEACAM proteins could be a subject for further studies. *H. pylori* strains also seem to have diverse effects on host cells. The reason for the difference between the *H. pylori* strains P12 and 26695 infecting HEK cells still remains to be investigated. They might have different factors influencing several regulation mechanisms. Or even regulation on other levels, e.g. post translational modifications [142].

Comparing the results of this study to other publications and ranging them into current state of research, some peculiarities can be pointed out.
In comparison to HEK CC1 and HEK CC5 cells the HEK 293 cells cannot translocate CagA, which can explain the big difference between the luciferase activities upon infection in HEK 293 and HEK CC1/ CC5 cells. However, former publications have shown that the *cag*PAI is not definitely necessary for IFN type 1 activation [82, 86, 87]. So why is there such a huge difference between those cell lines? HEK 293 cells cannot translocate CagA since there are no CEACAM proteins on their surface. The still observable IFN promoter activity might be caused by a CagA- and *cag*PAI-independent process, or probably by a basic production level. Therefore, there are no significant differences in promoter activation upon infection with wildtype or mutant strains. Since HEK CC1 and HEK CC5 expressing CEACAM induce the IFN pathway CagA and CagPAI-dependently, there is a significant reduction of promoter activity upon infection with mutant strains. However, there should be an overlap of PAI-dependent and PAI-independent activation of IFN in those cells. Apparently, the results show the opposite case. Probably, there are factors to influence this effect. Further investigation on IFN-activation pathways, PAI-dependent and PAI-independent, can be made to find out about possible interactions. On the other hand, there is a characteristic of both activation ways to be similar: Infection with ΔCagY-strain led to the strongest downregulation of promoter induction. This marks CagY as very important in IFN activation, PAI-dependent and PAI-independent. Regarding the results in this study, an interaction of CagY and other *cag*PAI proteins [57] is more likely, since luciferase activity is still influenced when infected with other mutants with lacking PAI-region.
Another interesting point is that the ratio of luciferase and renilla activity, which stands for the activity of the IFNβ promoter, has a high range of about 10 in HEK 293 cells while the CEACAM expressing cells have a ratio of luciferase/renilla activity at a range of 6 (see figure 12). Actually, a higher range of activity is to expect due to the combined PAI-dependent and PAI-independent activation. On the opposite, the activity of the cells expected to have a higher activity due to combination of PAI-dependent and PAI-independent IFN-promotor activity has even a lower activity. Apparently, together with the PAI-dependent activation, the PAI-independent IFN-activation is not functional. This observation can be deeper examined concerning interaction of both IFN-activation pathways. The pathways might disable each other.

4.1.2 E-selectin-promoter of NF-κB pathway

E-selectin-promoter activity in HEK 293 cells transfected with pELAM-luc was significantly reduced upon infection with 26695 mutants, while promoter activity in HEK 293 cells infected with P12 mutants was not, suggesting a difference between the two bacterial strains that is able to compensate the absence of CEACAMs. Since CEACAMs are needed for CagA translocation into the host cell and NF-κB-pathway activation is known to be CagA-dependent, this observation is surprising. Inferential, 26695 strain seems to activate NF-κB-pathway independently from CagA. HEK CC1 cells infected with *H. pylori* mutants show significantly reduced luciferase activity except of the ones infected with ΔCagA-mutants. This result additionally underlines CagA-independent NF-κB-activation upon *H. pylori* infection. Infected HEK CC5 cells have significantly reduced luciferase activity upon infection with *H. pylori* mutants, again, except of P12ΔCagA. Interestingly, infected HEK CC1 cells show no significant reduction of the luciferase activity also with strain 26695 lacking CagA. Probably there is a difference between the CEACAM proteins equivalent to the cell line concerned. They both might be essential for *H. pylori* to bind to host cells in manifold ways.
As already seen in the IFN-promoter activation, the range of NF-κB-promoter activation in HEK 293 is higher than in HEK CC1 or HEK CC5 cells (figure 12) which is unexpected since this pathway is known to be CagA-dependent.

4.1.3 Difference between Interferon pathway and NF-κB pathway

The luciferase activity in HEK 293, HEK CC1 and HEK CC5 cells transfected with pELAM-luc and infected with *H. pylori* wildtype and mutant strains had mostly the same behavior as the one in cells transfected with p125-luc. These similarities indicate possible parallels in the activation of both pathways, IFN and NF-κB, upon *H. pylori* infection. Nevertheless, there are still some differences.

In HEK 293 cells only *H. pylori* 26695-mutants reduced significantly NF-κB-promoter activation, while IFN-promoter activity was not significantly affected by any *H. pylori* mutant. The impact of *H. pylori* on different immune reaction pathways seems to differ and to be dependent on the strain, but also on the pathway targeted. Thus, also in HEK CC1 cells NF-κB-promotor activation is not significantly reduced upon ΔCagA-mutant infection, while IFN-promoter activation is completely dependent on all Cag-proteins tested. The promoter activity of both pathways in HEK CC5 cells shows a similar reduction. To sum up, different cell lines (especially focused on CEACAM proteins that are expressed) and different *H. pylori* strains have in combination different influence on immune pathways and apparently different requirements for their induction. Further investigation might focus on each of them separately to distinguish the relation between them.

The comparison of promoter activity of IFN- and NF-κB-pathway outlines a difference in the activity range. The promoter activity of the IFNβ-promoter is much weaker than the one of the E-selectin-promoter. Thinking of the reason why the range of the NF-κB-promoter activity is much higher, some suggestions might explain the results. The basic promoter activity of the NF-κB-pathway has already a higher range, so upon bacterial infection the

activity rises and is naturally higher than the one of the IFNβ-pathway. Another reason could be that the cell line might be an influencing factor leading to the unexpected activation strength: HEK cells are derived from human kidney cells, while *H. pylori* infection targets gastric cells, so the immune reaction generated by these cells might be different. To investigate this hypothesis the same experiment design should be performed on AGS cells. The importance of IFN in generation of immune response upon *H. pylori* infection could be a cooperation of different cells in the stomach, a sum of small activities.

4.2 Relevance of CagY for Interferon β- and IL-8 secretion

The interferon pathway is best described in immune processes caused by virus infection. pDCs (plasmacytoid dendritic cells) are seen as main producers of IFN type I [79]. Bacterial infections also do so. But until now, there is less known on the bacterial host defense as compared to the viral host defense with IFN [74]. This work focuses on the IFN pathway of immune response upon bacterial infection. *cag*PAI-dependent and especially CagY-dependent promoter activation after infection with *H. pylori* has already been shown with the luciferase reporter assay. So the next step was to examine the impact of the promoter activation on the secretion of corresponding cytokines and whether it is also CagY-dependent, IFNβ downstream of the IFNβ-promoter and IL-8 downstream of the E-selectin-promoter in the NF-κB-pathway.
The results could not be evaluated successfully due to different technical problems. The protocols for ELISA assays detecting IL-8 and IFNβ did not work. For IL-8, it was quite peculiar, since the protocol is often used in the work group. But even several attempts and sample amount modifications could not turn the examinations successful. IL-8 was already shown to play a major role and to be released after activation of NF-κB upon *H. pylori* infection (for details see introduction). Therefore, it was initially intended as positive control of the experiment setting. The protocol for IFNβ ELISA assay was not established before, but was set up based on the protocol of the IL-8 ELISA assay. The evaluation resulted in a not exploitable regression curve. Speculations that the concentrations of the IFNβ standard might be to low were confirmed after increasing them for test. The antibodies available in the laboratory were for samples with higher cytokine concentration. Searching and testing of other antibodies to establish a new protocol would have gone beyond the scope of this thesis.
Nevertheless, the findings still give a hint on the impact of the IFNβ-promoter activation on their downstream process: The low cytokine concentration corresponds with the clear but lower promoter activity of the IFNβ-promoter compared to the E-selectin promoter. Upon bacterial infections, NOD alone is supposed to be not enough to induce IFNβ. NOD also activates IFN type 1 via NF-κB, which on its side cooperates with other transcription factors to be able to induce IFNβ [68]. Probably, the same happens in the experiment setting of this study. *H. pylori* infection activates IFN via NOD1. However, since NOD is not enough to activate the pathway, promoter activity and cytokine secretion are, in the following, also weakly activated. Nevertheless, here the question arises as to the reason why there might be no cooperation with other transcription factors, leading to a strong IFNβ secretion.

Of course, there are a lot of other immune responses to a *H. pylori* infection in addition to the described pathways [85, 143]. However, CagY-dependent cytokine secretion has still to be deeper illuminated in the future due to its potential medical value. Since cytokines are the factors to directly cause immune responses in humans, targeting them and the factors they depend on might have influence on inflammation and disease development caused by *H. pylori*.

4.3 Interaction between CagY and IRF7

It is known that Epstein-Barr-Virus LMP1 and transcription factor IRF7 interact with each other. Since previous work in the lab group has shown similarities between the LMP1 and the RRI of the CagY protein, a part of this work aimed to find out a possible interaction between CagY and IRF7. The interaction should be demonstrated on protein-level and visualized accordingly.
First, life cell imaging should show the assumed interaction between CagY and IRF7. It was planned to create fusion proteins of CagY's repeat regions to microinject them into cells. Upon stimulation or infection the fusion proteins could bind to the transcription factors, e.g. IRF7 or RelA, before translocating into the nucleus. Due to technical problems, i.e. not injectable cells and no reaction of the transfected transcription factor upon stimulation and especially the incubation time after stimulation assumed to be too short to enable reaction, the visual method was changed into confocal microscopy. The first experiments showed multiple accumulations of the EYFP-IRF7 fusion-protein after transfection of the cells. The accumulations were independent of infection by *H. pylori*, wildtype and mutant strains, and also were observed in non-infected cells. The translocation was difficult to see, but there were still distinctive cases of IRF7-translocation into the nucleus, indicated by fluorescence in the middle of the DNA. As for the accumulation, the IRF7-translocation did not follow any regularity and made no difference between uninfected and infected cells, or wildtype and mutant bacterial strains. Facing these results, CagY seems not to be interacting with IRF7. On the other hand, regarding the supposed lower importance of IRF7 in the IFN pathway upon *H. pylori* infection, interaction of CagY with IRF7 might be redundant. Besides, CagY is also able to vary its size due to multiple repeat regions in the proteins sequence [55], which might alter interaction effects with other proteins. After having found the accumulations that were hardly to explain, several tests were done. The incubation time after transfection was shortened assuming the accumulations to be a kind of cell reaction to IRF7's toxicity. The accumulations first gave an idea of cell toxicity of the fusion proteins that have to be degraded in phagosomes. So the EYFP-IRF7 was assumed to be spread in the cell cytoplasm in an earlier time point before activation of the phagosomes. However, a reduction of accumulation was not really observed. Therefore, a timeline test was started to find out an optimal time to see IRF7-translocation before its accumulation. AGS cells were transiently transfected with pEYFP-IRF7 and the cells AGS pEYFP-IRF7 were used. The first accumulations started after 4-6 hours transfection with no meantime spread in the cytoplasm and without any infection or stimulation. This means that once produced, the fusion proteins accumulated directly. Accumulation in AGS pEYFP-IRF7 cells started a little later. This is also a factor showing the dysfunction of those cells. Since every single

component during the transfection may cause accumulations, as sign for irritation or overtaxing of the host cell, every single component was tested. To distinguish the reason, cells were transfected with pEYFP-C1 or pEYFP-IRF7 and incubated overnight. None but the IRF7 protein caused accumulations, even the raw plasmid without insertion pEYFP-C1 did not, confirming the accumulation comes from the IRF7 protein. The test got contaminated, so it does not have high informative value, but it can be concluded that even upon stimulation by bacteria, only pEYFP-IRF7 caused accumulations. These results suggest to consider that IRF7 might be modified after the translation process [94] or that the IRF7 is repressed in gastric cancer cells, so the cell is not used to its production [144]. Another possible explanation might be cross-reactions with other proteins and factors in the cell. As example, IRF3 overexpression inhibits IRF7 translocation [101]. Alternatively, this might be characteristic for EYFP-IRF7. Regarding former translocation studies with fusion proteins, e.g GFP-RelA, they are distributed in the cytoplasm [135, 145, 146].

Pulldown experiments and Western Blot should have shown direct binding of CagY and IRF7. Therefore plasmids, each containing sequences of repeat region 1, repeat region 2, the whole sequence of CagY or IRF7, should be created. The fusion proteins were then to be expressed in *E. coli*. In pulldown assays the fusion proteins should be mixed. IRF7 might bind directly to CagY, which would be seen on Western Blots detecting IRF7 and CagY or CagY components. Due to the extraordinary DNA sequence of *cagY*, amplification of its sequence and further preparation for cloning was hardly possible. During the filtration process the PCR product got lost. Further attempt to amplify *cagY* failed. Besides, a satisfying protocol for the Western Blot with anti-IRF7 antibody could not be established. On the blot, there were several bands near the predicted size. None of the blots were like in the manufacturer's instruction and no positive control was available. Former studies have shown different splicing variants of IRF7, which can have different functionality [98]. This is probably a reason why multiple bands are marked in the blot. But, a wider range of IRF7- functionality upon *H. pylori* infection can also not be excluded, i.e. that IRF7 might be able to change its own function by influencing the splice process.

4.3.1 Staining method

As already mentioned above, life cell imaging was not performed due to technical problems. The cells used were hardly to punctuate, so the plasmids were barely to inject into the cells. Injectable cells showed no reaction of the transcription factor upon stimulation. The incubation time after stimulation was intended to be too short for generation of a reaction. Therefore, in the following microscopy experiments all infections were performed with a longer incubation time. Comparing to former publications, life cell imaging was done with a stable cell line expressing GFP-RelA [145, 146]. In this study, on the other hand, transient transfected cells were used. In addition, translocation of the transcription factor into the nucleus was already described to happen 1 hour after infection or induction [147]. The discrepancy to the already published findings might come from the transient transfected cells. Probably they did not have enough time for protein production, or to get used to the new overexpressed protein.
Next, the visual method to show interaction between CagY and IRF7 was changed to confocal microscopy. The most difficult factor in evaluation of the images was to

distinguish a nuclear translocation from a protein location near the nucleus. With the first staining methods, it was not possible to define the fluorescent protein's localization. Different staining methods and their combinations were tested. The best one found was the staining of the nuclear membrane with the fusions protein produced by the plasmid pRFPLamin that is transfected into the cells. The fusion protein marks Lamin proteins, which are components of the nuclear membrane. Technically, this staining method was the most favorable, due to uncomplicated steps and short staining time because of no necessity to stain the nuclear membrane in a particular step. The staining result was also the most satisfying one. The stained nuclear membrane facilitated the evaluation considerably. Anyway, facing advantages of this staining method and also of the images taken by the confocal microscope, there are, as in every issue, disadvantages to consider. In contrast to life cell imaging, taking images with the confocal microscope only catches the cells and the location of IRF7 at a certain time point. IRF7 is highly expressed in early or late phase, on the other hand, levels of IRF7 expression also depends on the cell type [99]. IRF7 is reported to have a quite short half-life [103]. The instability of this protein might make it harder to fix the right moment for confocal microscopy, but also for doing Western Blot. Taking multiple image slices under the confocal microscope enabled a 3D-inspection on the cells. The evaluation of the 3D-images took long, because they were read by scrolling them up and down like reading a CT-scan. This type of evaluation is a bit influenced subjectively. Since only a part of the cells on one slide gets analyzed, there is quantification neither of the translocation nor of the transfection efficiency. In future investigation, a faster and more objective way of evaluation, such as a structured scan program might improve the process. Despite the good staining results and facilitated evaluation conditions, somehow, the nuclear staining was not like in former studies describing pRFPLamin with a clear marked nuclear membrane as shown in Wang et. al. [148].

4.3.2 Positive control pGFP-RelA

pGFP-RelA was first intended to be the positive control of the microscope assays. Since the first pilot experiments did not show expected results and a sequence anomaly within the plasmid was detected, but was not correctable, TNFα was chosen as positive control. After all, TNFα was already used as positive control for NF-κB before [62] and was shown to induce IRF7 [149]. However, even cell induction with TNFα did not cause IRF7-translocation into the nucleus.

4.4 IRF7 is probably not necessary for Interferon β activation by *H. pylori*

To find out whether the cell line influences pathway induction via promoter activity, the Luciferase Assay was performed on AGS cells. The results were not as expected. Promoter activities were not significantly reduced upon infection with *H. pylori* mutants. In the following, AGS cells were suggested not to express IRF7 and a new investigation series was started, transfecting the plasmid pEYFP-IRF7 into AGS cells and performing the Luciferase Assay on them. Western Blot detecting IRF7 was not really reliable due to

bad quality of the blots. The protocol for the new anti-IRF7-antibody could not be satisfyingly established, and therefore the absence or presence of IRF7 protein in AGS cells could be neither identified nor excluded. Actually, any compartment of the IFN pathway could be responsible. If IRF7 might not be existent in every cell line, other proteins of the IFN pathway might not be as well. These experiments targeted the aim to find out the role of IRF7 in IFN pathway upon infection with *H. pylori* and the interaction of IRF7 with CagY. Surprisingly, the results were not evaluable because of a high range of cell death after transfection. Since AGS were often used in the labgroup for diverse transfection experiments, IRF7 was suggested to be eventually toxic to the AGS cells. This in turn enhanced the assumption of the absence of IRF7 in AGS cells.
Since transient infection did not work as planned, creation of a stable cell line expressing IRF7 was attempted. Before creating the stable cell line, transient transfection was performed first to test effects. A pilot experiment with confocal microscopy investigating fluorescence due to the EYFP-part of the fusion protein EYFP-IRF7 detected regularly distributed IRF7 in the cytoplasm of non-infected cells and translocation of IRF7 upon infection with *H. pylori* P12. Thus, an AGS cell line expressing IRF7 was created. But in the following experiments with the cells, also the cells alone without any infection or stimulation led to accumulation of the fusion protein. The functionality of transfected cells was not tested before the first sorting, but evaluated with the microscope due to their fluorescence. However, fluorescence in the transfected AGS cells was much stronger than after sorting, which is surprising, because sorting is a process of collecting and accumulating transfected cells. In order sort cells, they had to be cultured up to a certain amount with a certain percentage transfected. Before sorting, AGS pEYFP-IRF7 cells had 5-10% of fluorescent cells estimated by microscopy and 0- 5% roughly measured by flow cytometry. Probably, the plasmid sequence might have mutated in the AGS cells leading to loss-of-function of the fusion-protein. To check out this hypothesis, chromosomal DNA from the transfected cells frozen at different dates was isolated, amplified and sequenced. PCR amplification of the IRF7 sequence from chromosomal DNA in AGS pEYFP-IRF7 cells worked without problems, while PCR from chromosomal DNA of AGS cells did not work well. Again, this situation might be a hint for the absence of IRF7 in AGS cells. As always, the sequencing result on both ends could not be analyzed due to unreliability of the results. In the middle part of the gene sequence, there was not any mutation visible. Thus, on the basis of the DNA sequence there was hardly to deduce a mutation. Possibly, there are other modifications on different levels involved, e.g. genetic imprinting, posttranslational parameters, etc.
Originally, the transfected cells should have been sorted several times to isolate more and more exactly the correct cells. To create the stable cell line, a single cell or up to 4 cells were aimed to pick. But the cells showed dysfunction already in the first steps of cell line generation.

Since the complete absence of IRF7 in AGS cells could not be investigated by Western Blot, the relevance of IRF7 for the IFN pathway could not be proven, either. Based on the method/ idea of Watanabe et. al. [63], IRF7-siRNA was used in order to examine the activity of the IFNβ-promoter, which is probably not only PAI-dependent, but also IRF7-dependent. IRF7-siRNA was transfected to HEK CC1 cells together with p125-luc+

pRenilla to show the disappearance of IFN-promoter activity because of the decrease/ knockdown of IRF7-expression. To control the function of the new ordered siRNA, but also to have reliable probes on the absence of IRF7, Western Blot on lysates transfected with IRF7-siRNA was performed. But still the blot with the new anti-IRF7-antibody was not satisfying and the functionality of IRF7-siRNA as well as the experimental setup could not be monitored and not confirmed.

Investigation on translocation of IRF7 into the nucleus upon infection with *H. pylori* was to determine the role of IRF7 in IFN pathway. Using wildtype and mutant strain was to mark out essential PAI-regions. Experiments with the confocal microscopy provided unclear results concerning IRF7 translocation upon *H. pylori* infection. In this study, IRF7 showed accumulation even in non-infected cells, which was unexpected. But taking the NF-κB pathway as parallel, RelA was already shown by publication to do both, accumulation [150] and no accumulation [135, 147] after stimulation in cells. And Keates et. al. has shown NF-κB to be slightly positive even in uninfected cells [113]. Probably, there are some interactions or parallels between the pathways. Probably, IRF7 might not matter for IFNβ-promoter induction.

After all, a clear conclusion concerning the role of IRF7 in the IFN pathway upon infection with *H. pylori* cannot be made. But facing the inconclusive results, IRF7 probably is not necessary for Interferonβ activation by *H. pylori* in HEK cells and AGS cells.
Studies have already shown that not all cells require IRF7 to activate the IFNβ pathway. It is possible to induce IFNβ despite the lacking of IRFs like IRF3, IRF7 and IRF5 [151]. Correlation with the cells might be an important reason, since lymphatic cells, for example, were observed to produce IRF7 constituently [101]. Moreover, different endogenous levels of IRF7 depending on the cell type have been observed [97-99]. On the contrary, Taniguchi et. al. has proved IRF7's production to be dependent on IFN pathway [104] and Rad et. al. was able to show an increased production of IRF7 in DCs upon infection with *H. pylori* [85]. Divergent results in this study compared to former publications might be because of the different cell lines used. While former results based on immune or lymphoma cells, HEK and AGS cells were utilized in this work. Primarily, IRF7 is expressed in spleen, thymus and peripheral blood leukocytes [95]. So probably in gastric cells IRF7 is not important for the IFN pathway upon *H. pylori* infection. Further investigation could focus on other compartments of the IFN pathway and even on posttranslational mechanisms.
Further studies on IRF7 can lead into multiple directions. IRF7 has got multifaceted interactions, wide impact and also seems to be multifunctional. There has been proven a competition with IRF1 to bind to ISRE (interferon-stimulated response element) [95]. IRF1 is known to also activate the IFN type 1 pathway. Furthermore, there are cross-reactions with other proteins in the cell. As example, IRF3 overexpression inhibits IRF7 translocation [101]. The exact conditions leading and enabling the different functions of the IRF7 transcription factor remain to be further investigated.

4.5 Outlook and Conclusion

Immune signaling pathways are good targets for medical treatment. Inhibiting single components of them leads to interruption or downregulation. Substitution of components strengthens them, which also can lead to alleviation of symptoms. Several biologicals already belong to medical treatment schemes, such as Infliximab for interrupting signaling pathways or Interferons α, β and γ for supporting signaling pathways.
Upon *H. pylori* infection, immune response leads to gastric and duodenal inflammation causing gastritis and ulcer. Since inflammation is tied to malignancy [110] the risk of developing gastric cancer is also enhanced. Therefore, inflammation needs to be treated. Current therapy for *H. pylori* infection is quite restricted to the eradication with medical schemes mostly containing proton-pump inhibitors and antibiotics (see German S2k guideline) or treatment of clinical outcomes. It was observed that after eradication treatment, cytokines such as IL-8 and TNFα decreased, but the chronic infiltration did not disappear [116]. Possibility of eradication failure is due to bacterial internalization into host cell to escape the immune system and antibiotics [152]. Current therapy guidelines are under evolution to reach better eradication, as new drugs are implemented, like Vonoprazan instead of PPI for patients with clarithromycin resistance in Asia [153]. Knowing interactions of *H. pylori* with the human immune response and understanding of disease dynamics could offer more possibilities of treatment that might be more effective, not only for the infection with *H. pylori* itself, but also for following diseases like gastritis, ulcer and is therefore stomach cancer prevention.
NF-κB is known to be important for *H. pylori* virulence, since IL-8 secretion is linked to virulence factor and oncogene CagA. Effect of bacteria on NF-κB pathway [154] and misregulated NF-κB activity lead to various diseases [109]. Since it is present in different chronic inflammatory diseases, it could be used as target for anti-inflammatory treatment [106]. Probably NF-κB also plays an important role in regulation of inflammation in tissue, which is antithetical to what is known: Components of the NF-κB signaling pathway were observed to have reductive function on inflammation [105]. Those factors also provide starting points for medical treatment.
This study investigated the role of T4SS-component CagY as well as the role of IFN pathway upon *H. pylori* infection, taking the NF-κB pathway as parallel. The focus was put on possible interactions between transcription factor IRF7 and CagY and the downstream produced cytokine IFNβ. The aim was to find out whether CagY is important for *H. pylori*'s virulence and generation of diseases. Following the results, transcription factor IRF7 might not interact with CagY and might not be important for IFN signaling upon *H. pylori* infection, even though there is promoter activation proven that is CagY-dependent. CagY might be a candidate for biological targets, shows potential and is worth further examination and tests in future research. It seems to have a deeper role in IFN activation, but only being part of the T4SS.
Interestingly, IFN was described to have protective effects on host cells upon *H. pylori* infection. The relevant factors should be found out and investigated. They could be targets for biologicals but in the opposite way as CagY. Those factors might be supported by upregulation or substitution.

In literature, there exists a suggestion that *H. pylori* lives in co-evolution with humans and is seen as human pathogen only because it was discovered in a pathogenic context. On the contrary, *H. pylori* also should protect from other diseases [155].
In the end, whether to treat and how to treat a *H. pylori* infection remains an individual decision depending on the patient. It is also interesting that patients seem to react differently upon infection with *H. pylori*. There are patients developing ulcer, while some do not, despite being infected with this pathogen [31]. Trigger factor causing these effects remain to be further investigated in order to develop better treatment criteria. Immune reactions could be imitated by medicaments to prevent progression of inflammation consequences in more vulnerable patients, but they also could be downregulated or inhibited in case of overexpression of immune cells. Finding an antigen that is unique to *H. pylori,* but common for all *H. pylori* strains, or at least the pathogenic strains, could enable generation of vaccines as it is already tried [156].
In general, sound knowledge about development of diseases caused by *H. pylori* infections could help finding adequate prevention and treatment strategies. Future investigation might focus on more and more virulence factors of this bacterium, their interaction with the human immune response, but also on the human immune response with multiple interactions between the signaling pathways itself.

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Lebenslauf