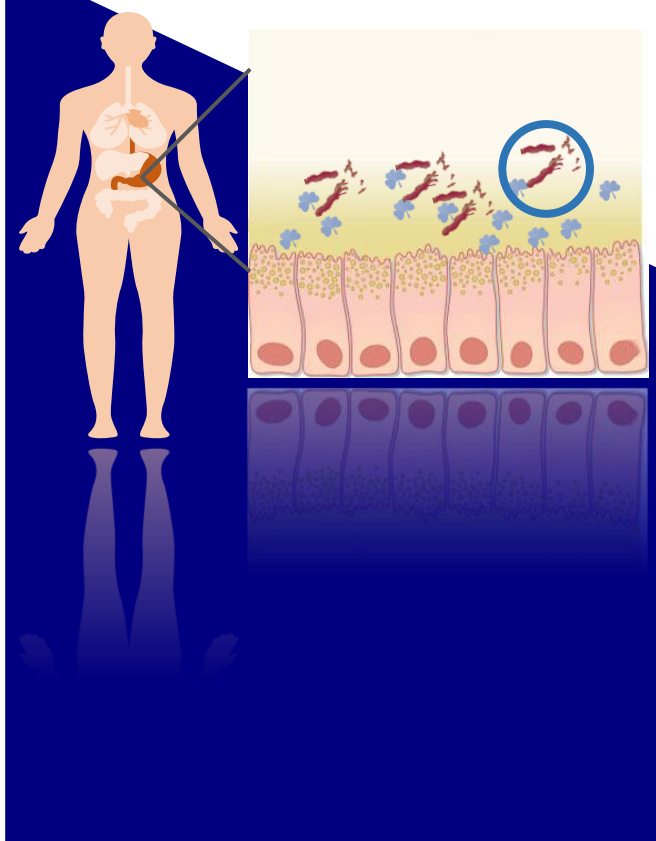
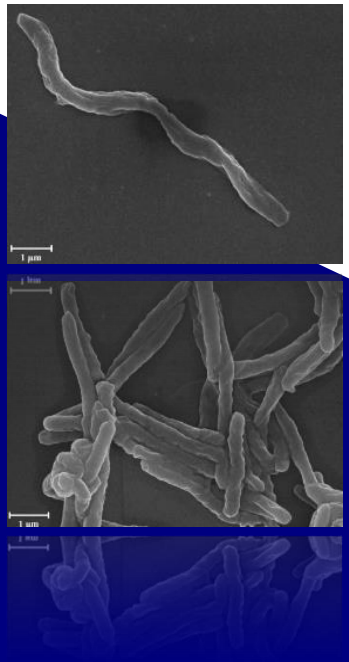
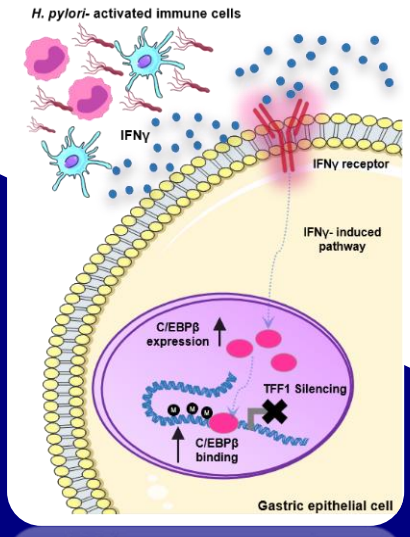
UNIVERSITÀ DEGLI STUDI DI SALERNO

PhD Program
in Drug Discovery and Development
XXXV Cycle

# *Molecular mechanisms of TFF1-Helicobacter cross-talk*








*Fatima Maria Mentucci*


UNIVERSITÀ DEGLI STUDI DI SALERNO


UNIVERSITÀ DEGLI STUDI DI SALERNO
Dipartimento di Farmacia

Ph.D. Program

in **Drug Discovery and Development**

XXXV Cycle

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## PhD Thesis in

# *Molecular mechanisms of TFF1-Helicobacter cross-talk*

Candidate

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*"No importa lo difícil que parezca, siempre se puede si la fuerza viene del corazón"*

# ABSTRACT

*Helicobacter pylori* is one of the most important pathogenic bacteria associated with various gastric diseases, including cancer. This pathogen induces an acute mucosal inflammatory response and, if not eradicated, it could lead to chronic infection and eventually, in a subset of individuals, to gastric cancer. In response to the infection, the gastric mucosa releases several protective factors, including TFF1, which binds *Helicobacter* in the mucus layer and reduces inflammation caused by the infection. TFF1 is a gastro-specific tumor suppressor gene upregulated in the acute phase of the *Helicobacter* infection but silenced during the chronic infection. This Ph.D. thesis project aimed to evaluate the role of TFF1 in the context of infection.

First, TFF1 was assayed for its effects on *Helicobacter* at molecular and morphological levels. The results showed that *H. pylori* is more prone to aggregate in a TFF1-concentration-dependent manner and as consequence its motility through a physiological mucus layer is compromised.

Since *Helicobacter* chronic infection promotes TFF1 silencing impairing the mucosal protection and contributing to the development of malignant transformation, the second aim of this thesis was to investigate the molecular pathways involved in this silencing. Our hypothesis was that the cytokines secreted by activated immune cells during *H. pylori* chronic infection would cause the silencing of TFF1 in gastric cells. Based on our results, IFNγ plays the major role in the TFF1 downregulation. Moreover, examining the TFF1 promoter, this phenotype seems to occur via C/EBPβ that works as a negative regulator during IFNγ stimulation. According to our evidence, IFNγ induces the expression of C/EBPβ and favors its binding to the TFF1 promoter, repressing its transcription. In addition, the involvement of DNA methylation in this process was evaluated, suggesting that this epigenetic mechanism is also involved in IFNγ-dependent TFF1 silencing.

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  *These authors equally contributed

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# CHAPTER 1

# INTRODUCTION

## 1.1. The stomach in health and disease

The stomach is considered the most sophisticated organ of the gastrointestinal system due to its essential physiologic and immunologic characteristics. This organ is composed of four main regions: cardia, fundus, corpus, and antrum. The stomach wall is made of the same four layers as most of the rest of the gastrointestinal canal, but with adaptations to the mucosa and muscularis for the unique functions of this organ. The inner wall of the stomach is lined by a mucous surface known as gastric mucosa (Figure 1.1). The latter shows a complex architecture building up from deep invaginations of the epithelium called gastric glands responsible for secreting gastric juice, whose structure varies between species. The acidic conditions are highest within the lumen and lower in the mucus layer and deep inside these glands (Burkitt *et al.*, 2017).

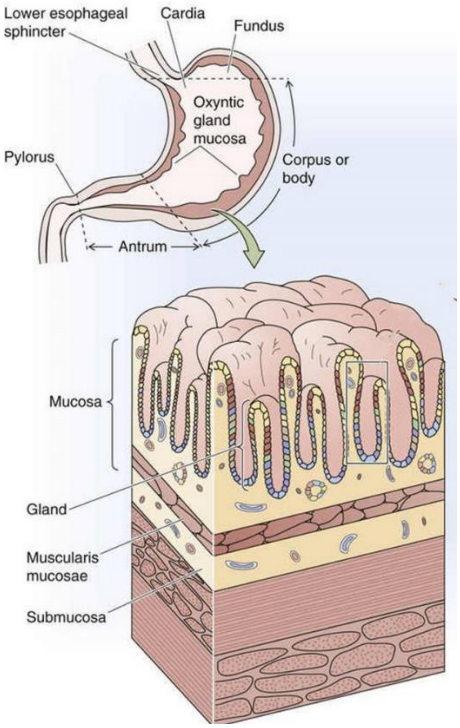


**Figure 1.1. Anatomy and Histology of the Stomach.** Illustration showing the regions of the stomach, the stomach wall with its layers. (Image adapted from doctorlib.info)

The human stomach possesses three glandular regions (fundus, corpus, and antrum), while the mouse stomach has glandular epithelium only in the antrum and corpus and replaces the fundus with a squamous epithelial compartment called forestomach (Burkitt *et al.*, 2017).

The glands contain distinct cell types whose distribution divides the gland into four regions: base, neck, isthmus, and pit (also known as foveola). The gland base mainly contains chief cells which secrete digestive pro-enzymes (pepsinogen) stored in intracellular granules. The neck and the pit are characterized by the mucus-secreting neck and foveolar cells, which secrete alkaline mucus.

The mucins are also stored in granules, and once secreted, they form a thick barrier that protects the epithelium from toxic compounds and microorganisms. The neck

region also contains parietal cells, which produce HCl and intrinsic factors. HCl is responsible for the high acidity (pH 1.5 to 3.5) of the stomach contents. The isthmus contains granule-free undifferentiated cells, identified as the progenitor of all lineages, and acid-producing parietal cells. The hormone-producing enteroendocrine cells are scattered throughout the gland, and they regulate the secretion of acid and digestive pro-enzymes by sensing the presence of food. While the pit and the isthmus are relatively similar in all glandular gastric units throughout the stomach, the corpus and the antrum possess substantial differences in the cellular composition of the neck and the base that reflects the specific functions of these regions (Figure 1.2A).

In the diseased stomach, histological aberrations are observed (Figure 1.2B) characterized by modification of the proportion of cell types within the gland. Specific alterations of the gland architecture are associated with a higher risk of cancer development (Zagami *et al.*, 2022).

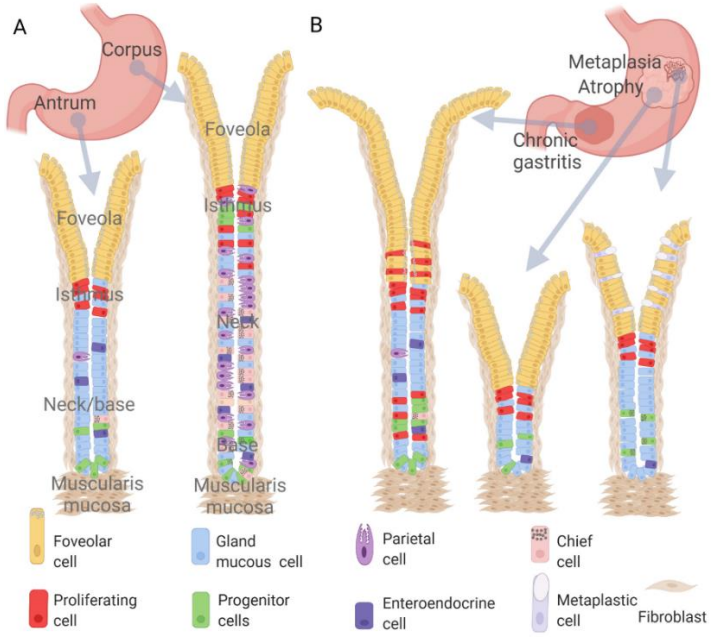


**Figure 1.2. Composition and Distribution of stomach gland cells in health and disease.** (A) Schematic illustration of the gastric glands from the distal and proximal part of the healthy human stomach. (B)

Morphological changes of stomach glands in consecutive pre-cancerous conditions of the Correas' cascade: chronic gastritis, atrophic gastritis, and intestinal metaplasia (Zagami *et al.*, 2022).

### 1.2. The Gastric Mucosa: an important Barrier

The stomach is protected by the mucosal barrier, described as a viscous, elastic, adherent, and transparent gel produced and secreted by mucus-producing cells of gastric glands. The stomach mucus lining is continuous and can be divided into two layers: a loosely adherent layer removable by suction and a layer firmly attached to the mucosa. The mucus is composed primarily of water (about 90-95%), salts, lipids such as fatty acids, phospholipids, cholesterol, proteins with a defensive action such as lysozyme, immunoglobulins, growth factors, and trefoil factors (Ichikawa *et al.*, 2011; Bansil *et al.*, 2006). However, the major constituent of mucus are mucins, extracellular glycoproteins biosynthesized and secreted by the mucus-producing cells. Mucins can be classified into two types: transmembrane (e.g., MUC1, MUC4, MUC16) and secreted (e.g., MUC2, MUC5AC, MUC5B, MUC6, MUC9). Their expression is cell- and tissue-specific. The primary gastric mucins are: MUC1, a membrane-bound expressed mucin, and MUC5AC and MUC6, two secreted mucins. The gastric glands are characterized by two types of mucus-producing cells, the surface mucus and the glands mucus cell. The secreted mucin composition differs between them: MUC5AC is mainly expressed in the surface mucus cells, while MUC6 in the gland-type mucus cells (Figure 1.3).

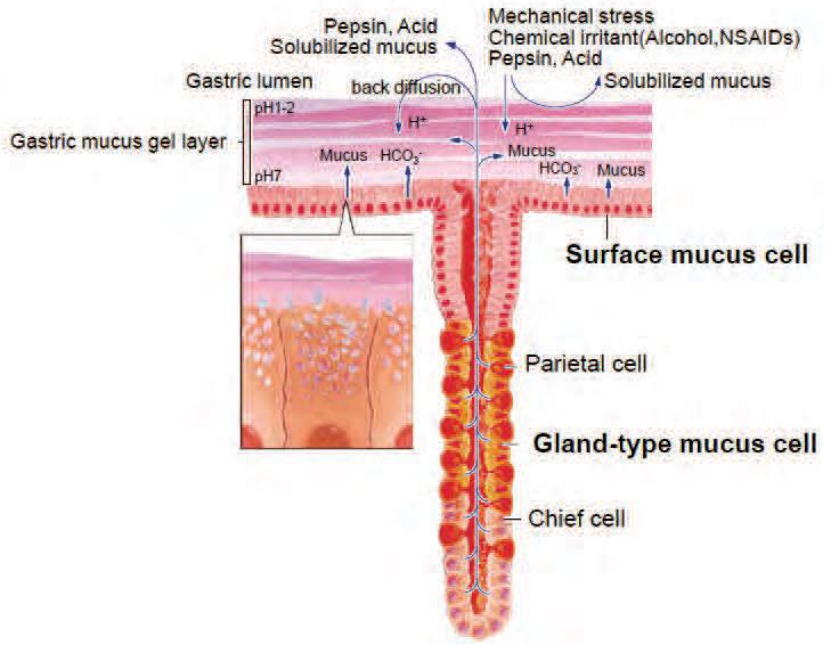


**Figure 1.3. Characteristic of the gastric mucus gel layer.** Surface mucus and gland mucus cells composing the mammalian gastric mucosa. Respective mucins differ in their peptide sequences and chemical composition of the carbohydrate moieties (Ichikawa *et al.*, 2011).

The protective functions consist in providing a stable unstirred layer supporting surface neutralization of luminal acid by mucosal bicarbonate secretion. In this way, it maintains a pH gradient from acid in the lumen to near-neutral at the mucosal surface. In addition, the mucus layer has been shown to act as a physical barrier and prevent access of luminal pepsin to the underlying mucosal surface (Atuma *et al.*, 2001). The mucus also performs other actions such as maintaining lubrication of the mucosal surface, covering ingested foods to mix them, helping digestion, and protecting the surface epithelium from harmful agents, including bacteria, for which a defensive physical barrier is required. The efficacy of protective properties of the mucus barrier depends not only on the gel structure and composition but also on the thickness of the layer covering the mucosal surface. Changes in the composition and

structural features of the gastric mucosal barrier have been identified in GI pathologies.

### 1.3. The Gastric Mucosa in disease: Gastritis

Gastritis is characterized by excessive inflammation of gastric mucosa, which can lead to peptic ulcers and eventually to gastric cancer. Several factors can lead to inflammation, such as excessive intake of drugs, alcohol, and non-steroidal anti-inflammatory drugs, including aspirin and ibuprofen, unbalanced diets where the stomach is damaged by its own gastric acid, long-term stress due to the population rhythm of life (Jaroenlapnopparat *et al*., 2022). However, one of the most important causes of gastritis is the infection by *Helicobacter pylori* which colonizes the gastric mucosa of at least half of the world's population. The inflammation following *Helicobacter* infection may occur as a short episode (acute gastritis) or develop gradually, persisting for a long time (chronic gastritis). Acute inflammation is the immune system's defense mechanism primarily driven by myeloid phagocytic cells (e.g., macrophages). The activation and response of macrophages are controlled by subsets of differently polarized CD4+ T lymphocytes (Th1, Th2, and Th17 cells), each secreting signature cytokines and expressing a lineage-specifying transcription factor (Neurath *et al*., 2002; Sallusto, 2016; Ruterbusch *et al*., 2020). The type of immune response after injury or infection depends on the specific pathogen/danger-associated molecular patterns (PAMPs/DAMPs) and is characteristic of different pathogens (e.g., extracellular or intracellular bacteria, parasitic helminths, fungi, and viruses) (Sallusto *et al*., 2016; Eletto *et al*., 2022). These PAMPs and DAMPs, as well as ROS, are sensed by pattern-recognition receptors (PRRs), which are activators of the inflammasome and also direct triggers of (acute) inflammatory as well as regenerative processes

(reparative inflammation) (Karin *et al*., 2016). Furthermore, inflammation underlies many chronic and degenerative diseases. Of special note, most, but not all, chronic inflammatory diseases increase the risk of cancer (Grivennikov *et al*., 2010; Karin *et al*., 2016). Thus, cytokines produced by activated immune cells are an important link between inflammation and cancer (Lin *et al*., 2007; West *et al*., 2015).

### 1.4. Gastric Cancer

According to GLOBOCAN 2020, gastric cancer is the fifth most common cancer and the fourth most lethal cancer in the world population; although its incidence has declined over the past century thank to a reduced incidence of *H. pylori* infection, but also to progresses in food preservation and reduction in the consumption of salted and smoked foods. This decrease mainly concerns distal GC, while the incidence of proximal GC is constantly increasing, probably due to an increase in obesity and gastroesophageal reflux disease, which are considered the main risk factors (Petryszyn *et al*., 2020).

90% of stomach tumors are adenocarcinomas, followed by gastric lymphoma. Gastric adenocarcinomas are primarily classified as cardia and non-cardia based on their anatomic site. Cancers of gastric cardia arise in the region adjoining the esophageal-gastric junction, and non-cardia cancer, also known as distal stomach cancer, arises in the lower portion of the stomach (Rawla *et al*., 2019). Gastric cancer also can be histologically differentiated between diffuse or intestinal types. The pathogenesis of the diffuse-type carcinoma has been frequently associated with the loss of expression of the cell adhesion molecule and tumor suppressor E-cadherin (CDH1), characterized by the loss of cellular adhesions and invasion of the adjacent tissue. Contrary to the diffuse type, the intestinal type of gastric cancer, mainly caused

by *H. pylori* infection, is characterized by a series of sequential processes known as Correa's cascade (Figure 1.4). According to this model, gastritis may progress to atrophic gastritis, then intestinal metaplasia, and finally to dysplasia and adenocarcinoma (Figure 1.5) (Correa *et al.*, 2007).

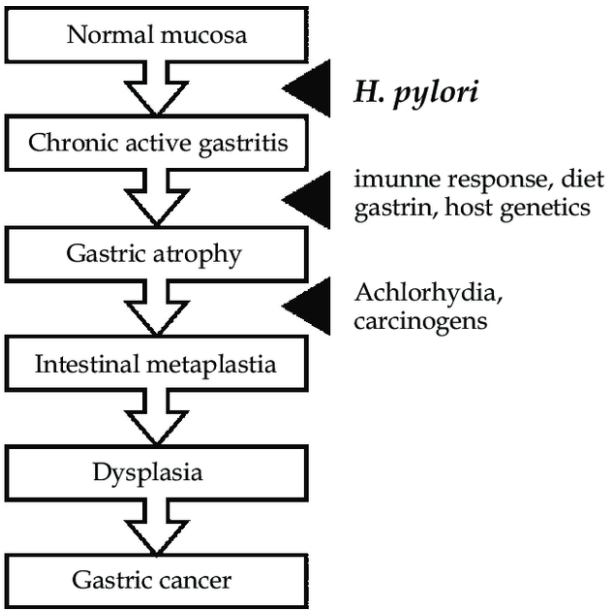


**Figure 1.4. Model of the linear progression towards gastric cancer development as proposed by Correa.** In the presence of *H. pylori* infection, the inflammatory response causes the accumulation of cytokines within the normal gastric mucosa resulting in the development of chronic active gastritis that may progress to gastric atrophy, metaplasia, dysplasia and Gastric cancer (Kargar *et al.*, 2011).

Recently, gastric cancer, according to Modified Laurén Classification (MLC), which includes histology and anatomical location of GC, can be divided into three subtypes: proximal non-diffuse (PND), distal non-diffuse (DND), and diffuse (D) types (Moore *et al.*, 2022). The development of Gastric cancer is the outcome of a complex interaction between environmental, host genetic, and microbial factors (Jaroenlapnopparat *et al*., 2022). Several environmental factors have been identified

as risk factors for gastric cancer, such as salt-preserved foods, dietary nitrite, smoking, alcohol, GERD, *H. pylori* infection, *Epstein-Barr* virus infection, etc.

The progression of gastric cancer is characterized by several stages:

- the subthreshold neoplastic state is the initial stage in which microorganisms or chemical carcinogens attack normal tissues and cause permanent DNA alteration.
- the promotion is the second stimulus on the initiation sites when irritation or inflammation occurs. This dual-step stimulation promotes cell proliferation, recruits immune cells, and produces more reactive oxygen species to further damage DNA and reduce DNA repair mechanisms.
- Tumor development: the growth control system fails to function normally, and inflammation never subsides (Mackenzie *et al*., 1940; Rous *et al*., 1940). Inflammatory mediators attract immune cells to the site, communicate with resident and tumor cells, and induce an inflammatory response, promoting tumor growth. Inflammation promotes a suitable environment for tumor cells to grow, invade, and metastasize through the secretion of multiple inflammatory mediators. This perfect environment for tumor development is often referred to as the "tumor microenvironment" (TME). Inflammation is also associated with the transformation of cells from benign to malignant. Epithelial-mesenchymal transition (EMT) is a process in which normal epithelial cells transform themselves to acquire mesenchymal cell properties, such as invasion, apoptosis resistance, and metastasis (Jaroenlapnopparat, *et al*., 2022).

There is significant evidence supporting the association between chronic inflammation and the onset of cancer. This association is particularly robust for

gastrointestinal cancers in which microbial pathogens by stimulating chronic inflammation can favor malignant transformations. *Helicobacter pylori* is the most prominent well-known example of a pathogen causing chronic gastric inflammation which might progress to atrophy, metaplasia, dysplasia, and eventually, gastric cancer (Figure 1.5).

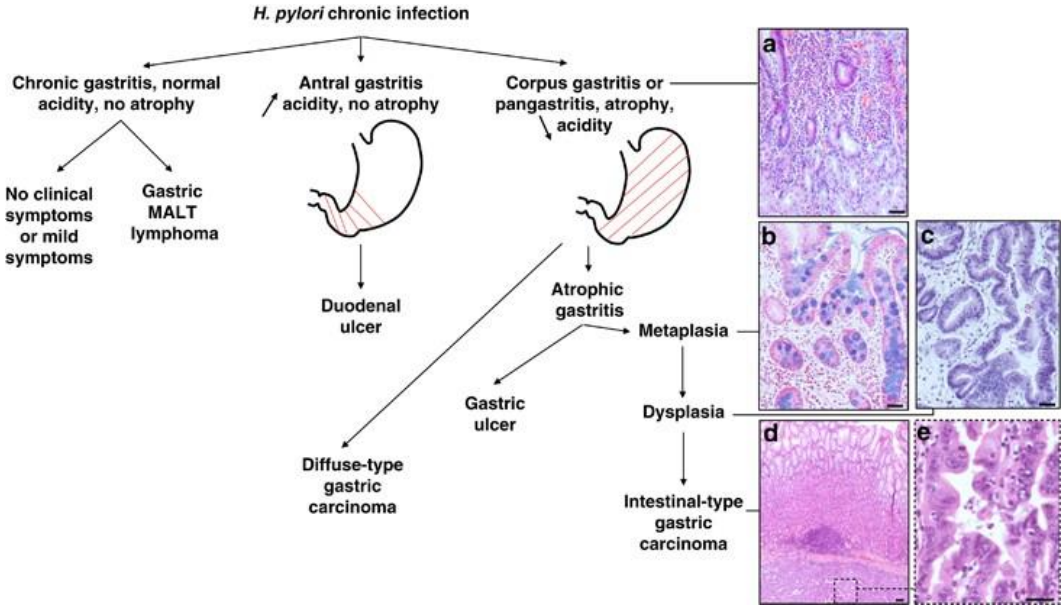


**Figure 1.5. *H. pylori* pathogenesis and the cascade of histopathologic lesions associated with intestinal-type gastric carcinogenesis.** a) Chronic atrophic gastritis: chronic inflammation of the mucosa with local fibrosis and loss of chief and parietal cells. (b) Intestinal metaplasia: specialized parietal and chief gastric epithelial cells are replaced by enterocyte-like cells and intestinal goblet cells producing intestinal mucins (stained in blue). (c) Intraepithelial dysplasia characterized by herniation and glands of irregular and branched shape showing cellular and nuclear atypia. (d) Invasive adenocarcinoma penetrating the submucosa and deeper layers. (e) Enlargement of image D revealing an intestinal-type gastric adenocarcinoma. (a and c–e) Hematoxylin and eosin safran (HES) staining. (b) Alcian blue staining with nuclear fast red counterstaining. (Bessède *et al.*, 2015)

## 1.5. *Helicobacter pylori*

### *1.5.1. History*

The first description of *Helicobacter pylori* was in 1892 when Giulio Bizzozero, a pathologist at the University of Turin, recognized curved shape bacteria within the stomach of animal models. Then, in 1982 Marshall and Warren, two Australian researchers, correlated it with gastric disease by isolating the curved bacilli in the gastric epithelium of patients with chronic gastritis and peptic ulcer. Without a suitable animal model to show that the inflammation was caused by *H. pylori* infection, Marshall drank the live *H. pylori* culture to demonstrate the correlation between the bacterium and gastric disease (Marshall, 2006). In 2005, the two Australian researchers were awarded the Nobel Prize in Medicine for the seminal discovery of this bacterium and its role in peptic ulcer disease. Because of the strong correlation between infection and gastric cancer, in 1994, *H. pylori* was recognized as a type I carcinogen, and now it is considered the most common etiologic agent of infection-related cancers, representing 5.5% of the global cancer burden (Alipour *et al*., 2021).

### *1.5.2. Microbiological characteristics*

*H. pylori* is a Gram-negative bacterial pathogen that selectively colonizes the gastric epithelium. The bacterium is urease, catalase, and oxidase positive, it is spiral-shaped, and possesses 3 to 5 polar flagella that are used for motility. In addition, most *H. pylori* strains express virulence factors that have evolved to affect host cell signaling pathways. Among many unique features of *H. pylori*, one of the most remarkable is its capacity to persist for decades in the harsh gastric environment due to the inability of the host to eliminate the infection. Unlike other viruses and bacteria, *H. pylori* has evolved the ability to colonize the highly acidic environment found within the stomach

by metabolizing urea to ammonia via urease, therefore generating a neutral environment that envelops the bacterium and allows it to reach the epithelium. In addition to this, *H. pylori* employs additional strategies to adapt to the gastric environment's extreme acidic conditions, establish persistent infection, and deregulate host functions, leading to gastric pathogenesis and cancer (Kusters *et al*., 2006; Amieva *et al*., 2016).

### *1.5.3. Helicobacter pylori infection*

Approximately half of the world's population is infected with *H. pylori*, and most colonized individuals develop coexisting chronic inflammation. In most persons, *H. pylori* colonization does not cause any symptoms. However, long-term carriage of *H. pylori* significantly increases the risk of developing site-specific diseases. Among infected individuals, approximately 10% develop peptic ulcer disease, 1 to 3% develop gastric adenocarcinoma, and <0.1% develop mucosa-associated lymphoid tissue (MALT) lymphoma. At early stages, gastric MALT lymphoma can be cured by eradication of *H. pylori*, therefore it is considered the first clonal lesion which can be eliminated by treatment with antibiotics

### *1.5.4. H. pylori pathogenesis: the important role of its virulence factors*

To begin with infection, *H. pylori* enter the mucus layer using flagellar motility. *flaA* and *flaB*, encode the components of the filament of the flagellar system, and *flgE* encodes a hook component that links the body to the flagellar filament (Eaton *et al*., 1996; Clyne *et al*., 2000; Gu *et al*, 2017). *H. pylori* motility is influenced by chemotactic activity in response to various molecules, such as mucin, sodium bicarbonate, urea, sodium chloride, and certain amino acids (Mizote, *et al*., 1997; Worku *et al*., 2004). Chemoreceptors, including T1pA, B, C, and D, a CheA kinase, a

CheY responsive regulator, and various coupling proteins are essential for its chemotaxis (Huang *et al.*, 2017).

*H. pylori*-induced tissue injury begins with bacterial attachment to gastric cells using adhesins and outer membrane proteins, which detect and attach specifically to host receptors expressed on the cell surface (Logan *et al*, 1996). This attachment process can potentially change the epithelial cell's morphology and function or activate specific bacterial proteins. *H. pylori´*s adhesins are BabA (HopS), which facilitates host cell binding to fucosylated Lewis b (Le(b)) blood group antigens (Ilver *et al.*, 1998), OipA (HopH), which promotes inflammation by boosting IL-8 expression (Yamaoka *et al*, 2000), and SabA (HopP), involved in the binding to sialic acid-containing glycoconjugates (Mahdavi *et al*., 2002). Subsequently, *H. pylori* releases enzymes and other microbial products that cause cellular damage. One of the secreted enzymes is urease, which catalyzes the hydrolysis of urea to carbon dioxide and ammonia, a molecule that attenuates the acidity of the stomach environment aiding *H. pylori* in colonization (Eaton *et al*., 1991). The bacterium also secretes Hydrogenase, which is part of a signaling cascade that triggers the formation of an alternate pathway, allowing *H. pylori* to utilize molecular hydrogen as a source of energy for its metabolism (Olson *et al*., 2002). Nickel is essential for *H. pylori* because it is a cofactor for two key enzymes, urease, and hydrogenase, it binds the N-terminal NHE motif of the Ni-metallochaperone HypA, which is essential in the maturation of the two enzymes, critical for the bacteria's acid survival (Hu *et al.*, 2017). *H. pylori* uses important virulence factors to infect the host (Figure 1.6). One of them is the Type IV secretion system (T4SS) encoded by the cag pathogenicity island (CagPAI), which generates a syringe-like pilus structure for injection and translocation of virulent components into host target cells, such as the cytotoxin-associated gene A (CagA) and

peptidoglycans. After assembly of the T4SS and pilus formation, CagA is translocated into host cells where it can undergo phosphorylation of its EPIYA sites (Hayashi *et al*., 2013) by oncogenic tyrosine kinases and mimics host cell factors to activate or inactivate intracellular signaling pathways. Phosphorylated and non-phosphorylated CagA can interact with several host proteins and thus alter host cell signaling, playing a crucial role in *H. pylori*-induced inflammation. Several studies indicate that CagA can directly activate NFκ-B and induce the release of IL-8 (Kang *et al.*, 2013; Papadakos *et al.*, 2013). VacA is a pore-forming protein also secreted via T4SS, which acts as a passive urea transporter that has the potential to increase the permeability of the gastric epithelium to urea, hence facilitating *H. pylori* infection (Blaser *et al*., 1996). Once internalized, VacA accumulates inside different cellular compartments and induces apoptosis (Rassow *et al*., 2012). In addition, VacA disrupts epithelial cell tight connections and is distributed in the lamina propria, where it encounters T cells recruited to the sites of infection. As a result, T cell proliferation and effector functions are inhibited, allowing the persistence of the bacterium (Müller *et al*., 2011). VacA and CagA-producing strains generate more intense tissue inflammation and cytokine production. CagA (+) strains are found in 85–100% of individuals with duodenal ulcers, compared to 30 to 60% of infected patients who do not develop ulcers. Moreover, CagA strains have been linked to an increased risk of precancerous lesions and gastric cancer (Jaroenlapnopparat *et al*., 2022).

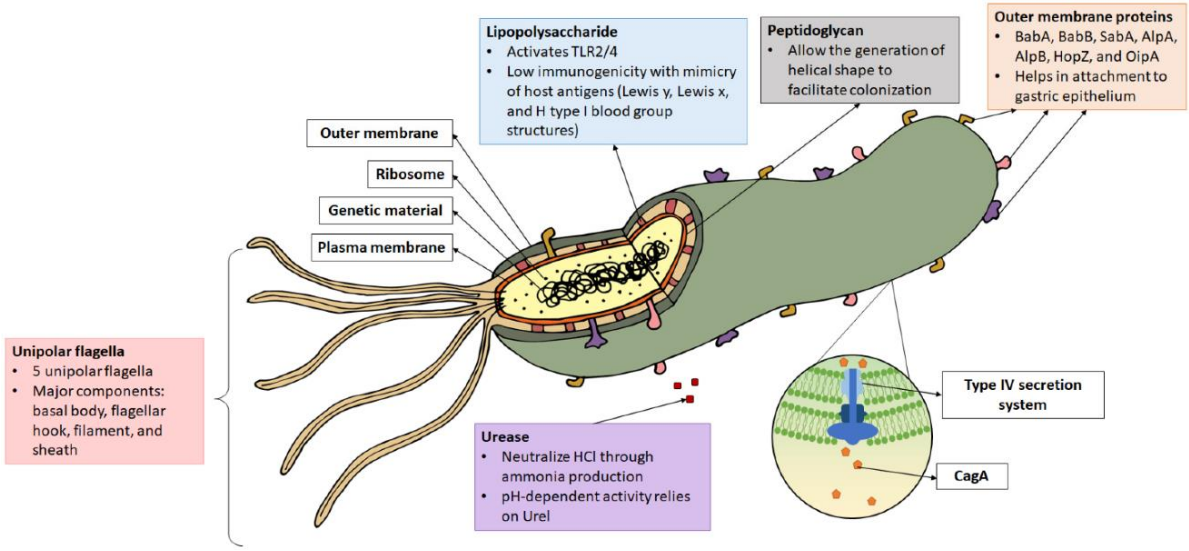


**Figure 1.6. Virulence factors of *Helicobacter pylori*.** Virulence factors such as lipopolysaccharide (LPS), flagella, urease, peptidoglycan, outer membrane protein, CagA, and the type IV secretion system (T4SS) are as indicated. CagA is injected into the host cell via T4SS (Cheok *et al.*, 2021).

*H. pylori* infection triggers various innate and adaptive immunological responses in the host (Crabtree *et al*., 1991a; Yoshikawa *et al*, 2000). Various *H. pylori* antigens, such as lipoteichoic acid, lipoproteins, and lipopolysaccharide, bind the Toll-like receptors (TLRs) and Nod-like receptors (NLRs) of the innate immune cells such as macrophages, dendritic, epithelial cells and intracellular vesicles (Vorobjova *et al*., 2008). This promotes NF-kB and c-jun N-terminal kinase activation, among other signaling pathways, leading to proinflammatory cytokine release (Smith *et al.*, 2003). In addition, CagA injection through T4SS causes cytokine generation, another NF-kB-dependent process (Alandiyjany *et al.*, 2017). Consequently, neutrophils and mononuclear cells infiltrate the gastric mucosa and produce nitric oxide and ROS (Wilson *et al.*, 1996). Adaptive immunity also has a role, especially in CD4+ and CD8+ T-cells involved with the

preferential activation of CD4+ cells (Lundgren *et al.*, 2003). Several studies have revealed a Th1-polarized response in *H. pylori*-positive patients, characterized by low levels of IL-4 (a Th2 cytokine) and increased IFNγ, TNFα, and interleukins, including IL-1β, IL-6, IL-7, IL-8, IL-10, and IL-18. Upregulated cytokines may boost proinflammatory effects during *H. pylori* infection, except for IL-10, which appears to play a role in limiting the inflammatory response (Crabtree, *et al*., 1991b; Lindholm *et al*., 1998). This inflammation is asymptomatic in most infected patients, but it raises the risk of duodenal and gastric ulcer disease and developing gastric cancer in the long term (Lindholm *et al.*, 1998). (Figure 1.7)

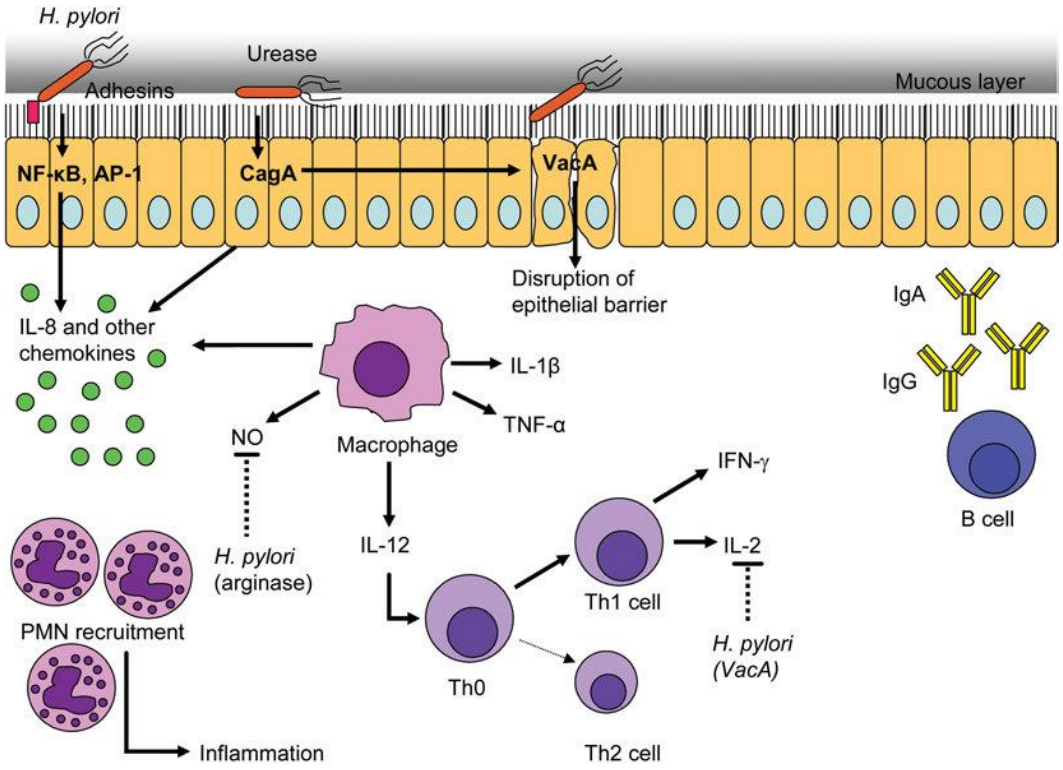


**Figure 1.7. *H. pylori* pathogenesis and the inflammatory response.** *H. pylori* resides in the gastric lumen and colonizes the gastric epithelium using urease. Binding of *H. pylori* to epithelial cells and injection of CagA results in the production of IL-8 and other chemokines, and activation of the innate and adaptive immune systems. (Wilson *et al*, 2007)

One of the gastric tumor suppressor genes responsible for carcinogenesis is TFF1. This protective factor secreted by mucosa as a defense strategy binds *Helicobacter* (Clyne *et al.*, 2004) and reduces inflammation caused by the infection (Soutto *et al.*, 2015).

## 1.6. Trefoil Factor 1

### *1.6.1. Protein structure*

Trefoil Factor 1 (TFF1), also called pS2, is a small and highly conserved human peptide belonging to the Trefoil Factor Family along with SP/TFF2 and ITF/TFF3, all clustered together on chromosome 21q22.3. Such Trefoil Factors are characterized by a common structural motif, the TFF domain (P- domain), which contains six conserved cysteine residues that form three intramolecular disulfide bonds (CysI-V, CysII-IV, and CysIII-VI) (May *et al.*, 1997; Thim *et al.*, 2002). Specifically, TFF1 (60 amino acids) has a single trefoil domain of 42 amino acid residues and contains a free 7th cysteine (CysVII) residue located outside the TFF domain, which confers the possibility to form dimers (Chadwick *et al.*, 1997; May *et al.*, 2003; Thim *et al.*, 2005; Hoffmann, 2021) (Figure 1.8).

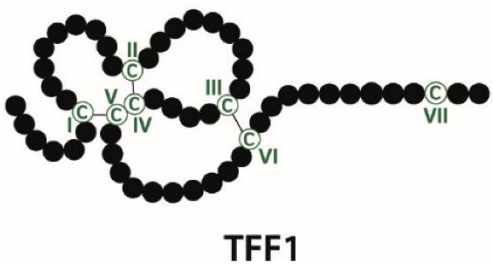


**Figure 1.8. Schematic Human TFF1 structure.** Cysteine residues (C; numbering in Roman numerals) and disulfide bridges are shown in green. (Figure adapted from Hoffmann, 2020).

TFF1 is a site-specific expressed peptide, synthesized in the mucus-secreting foveolar cells and secreted principally in the gastrointestinal mucosa (Xiao *et al*., 2015; Aihara, *et al*., 2017). This factor is co-packaged in the Golgi apparatus into mucus granules and secreted as a mucin-associated peptide, specifically with MUC5AC (Longman *et al*., 2000). This association between TFF1 and MUC5AC is maintained even after secretion (Clyne *et al*., 2019).

TFF1 has been described to be present in normal human gastric mucosa and adherent mucus in different molecular forms: TFF1 monomer (6.67 kDa), TFF1 homodimer (13.33 kDa), and TFF1 heterodimer (25-kDa) (TFF1-GKN2, TFF1-X/60k, TFF1-FCGBP) (Newton *et al*., 2000; Westley *et al*., 2005; Menheniott *et al*., 2010). The monomeric form is predominant, but the homodimeric form contains higher biological activity (Jian-Lin Ren *et al*., 2006; Hoffmann, 2021).

### *1.6.2. Functions*

Trefoil Factors are known to have a protective and reparative action on mucosa and to help maintain mucus integrity by forming stable mucus gel layers (May *et al*., 1997; Thim *et al*., 2002; Thim *et al*., 2005). TFF1 is involved in the reconstruction of the gastric epithelium by enhancing cell migration ("restitution"). In addition, TFF1 could also fulfill an intracellular function as a chaperone to ensure the correct folding and assembly of cysteine-rich glycoproteins (Hoffmann, 2021).

Finally, dimeric TFF1 has lectin activity interacting with a core oligosaccharide portion of the *H. pylori* lipopolysaccharide in a pH-dependent manner. Dimeric TFF1 binding studies with truncated lipopolysaccharides from *H. pylori* mutants demonstrated that N-acetylglucosamine (GlcNAc) is part of the carbohydrate structure

recognized by TFF1. Dimeric TFF1 also binds weakly to the gastric mucin MUC6, probably to a carbohydrate moiety conserved in frogs and humans (Hoffmann, 2022).

This protective factor also has an important role in tumor suppression, signal transduction, and regulation of apoptosis (Hoffmann, 2021).

### *1.6.3. TFF1 in pathological conditions*

Ectopic expression of TFF peptides was detected in pathological conditions, particularly during chronic inflammation, such as gastroesophageal reflux disease, Barrett esophagus, gastric and duodenal ulcers, diverticulitis, inflammatory bowel disease, pancreatitis, hepatolithiasis, cholecystitis, salpingitis, and inflammatory nasal polypi (Hoffmann, 2021). Further pathological expression of TFF1 occurs in metaplasia, as well as in different kinds of tumors. Of note, somatic mutations in the TFF1 gene seem to be associated with gastric cancer and there is a strikingly reduced TFF1 expression in the majority of gastric carcinomas in humans (Park *et al.*, 2000a). TFF1 is considered a gastric tumor suppressor gene; indeed, TFF1 KO mice mainly show a gastric phenotype because all develop antropyloric adenomas with ~30% progressing to carcinomas. TFF1 influences inflammatory processes and in turn, its expression is regulated by inflammatory signals. The TFF1-knockout mouse model provided the first evidence supporting the tumor suppressor role of TFF1 in gastric tumorigenesis, demonstrating that it is essential for normal differentiation of the antral and pyloric gastric mucosa (Feng *et al.*, 2014).

### *1.6.4. TFF1 expression in gastric cells*

The TFF1 gene has been localized to chromosome 21q and consists of three exons, and two introns (Babyatsky *et al.*, 1996). Under normal conditions, the TFF1 gene is

primarily expressed in mucosal epithelial cells in the gastric body and antrum, while under pathological conditions, its expression specificity disappears (Ribieras, *et al*., 2001). Molecular studies have shown frequent loss of TFF1 expression in more than 2/3 of gastric carcinomas (GCs) resulting from a mutation-independent mechanism (Carvalho *et al.*, 2002; Katoh, 2003; McChesney *et al.*, 2006). Multiple evidence has indicated that the occurrence of gastric cancer results from multiple genetic alterations, including activated oncogenes, inactivation of tumor suppressor genes, loss of heterozygosity (LOH), and defects in DNA damage response and repair mechanisms (Tischoff and Tannapfel, 2008). Epigenetic mechanisms, including DNA methylation and histone modifications, also plays a key role in the regulation of TFF1 gene expression (Feng *et al.*, 2014).

The silencing of the TFF1 gene in GCs is due to loss of heterozygosity and methylation of the TFF1 promoter region (Park *et al*., 2000b; Fujimoto *et al*., 2000; Carvalho *et al*., 2002; Tomita *et al*., 2011); however, mutations are observed in ~5% of GCs (Park *et al*., 2000a; Carvalho *et al*., 2002). Silencing of TFF1 may also be triggered by chromatin remodeling associated with histone modifications, including H3K9 methylation and H3 deacetylation at the TFF1 promoter, as observed in N-methyl-N-nitrosourea-induced gastric carcinogenesis mouse model (Tomita *et al*., 2011). In addition, transcriptional repression of TFF1 in gastric epithelial cells by CCAAT/enhancer binding protein-β (Sankpal *et al*., 2005) and cofactor of BRCA1 has been demonstrated (McChesney *et al.*, 2006).

C/EBPβ (CCAAT Enhancer Binding Protein Beta) is a transcription factor belonging to the C/EBP family, comprising a heterogeneous group of transcriptional regulators active in tissue development and regeneration, inflammation, and intermediate metabolism. C/EBPβ exists in three isoforms: NF-IL6, full length; LAP,

which differs for the lack of a few amino acids amino-terminals; LIP, a truncated N-terminal form.

All three isoforms are able to bind the promoter of TFF1 and reduce its gene expression (Sankpal *et al.*, 2005). Several studies have shown that this transcriptional factor is overexpressed in gastrointestinal cancer (Regalo *et al.*, 2016) at the same time as a reduction in TFF1 expression (Sankpal *et al.*, 2005).

### *1.6.5. TFF1 expression in Helicobacter acute and chronic infection*

Previous experiments from our research group demonstrated the differential expression of TFF1 in acute and chronic *H. pylori* infection using gastric antrum of mice (C57BL/6) infected with *H. felis*. *H felis* is another member of the *Helicobacteraceae* family, responsible for the infection in mice, which mimics the damage caused by *H. pylori* in humans better than *H. pylori* SS1 strain modified to infect mice (Hayakawa *et al.*, 2013). The results revealed that TFF1 is up-regulated during the acute phase (3, 5 and 8 days post-infection up to 2 times compared to naïve mice), while it is suppressed when the infection becomes chronic (after 14 days) and remains low up to 6 weeks post-infection (42 days) (Figure 1.9). In addition, it was observed that the acute phase of infection is characterized by an increment of IL-1β and IL-6 and few infiltrates, while after 6 weeks of infection, signs of the chronic phase were observed by immunofluorescence of the gastric mucosa of mice (Esposito *et al.*, 2017).

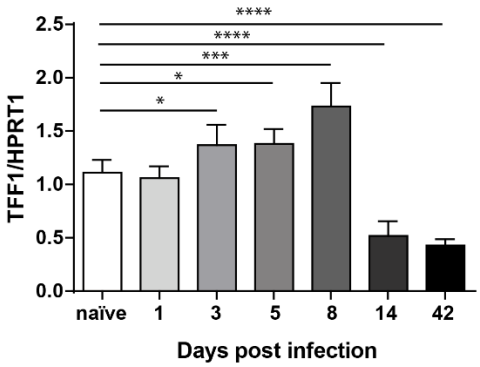


**Figure 1.9. TFF1 transcriptional regulation upon *Helicobacter felis* infection in C57BL/6 mice.** Real Time PCR analysis of TFF1 mRNA in gastric antrum of C57BL/6 mice infected with *H. felis* and sacrificed at the indicated times (d = days; w = weeks) post-infection compared to naïve mice (n: 6–10 for each group). Data are mean ± SEM. (t-test, *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001, ****p ≤ 0.0001) (Esposito *et al.*, 2017).

### *1.6.6. hTFF1 specifically binds H. pylori*

It has been shown that *H. pylori* colocalizes and interacts with the gastric factor TFF1 in the mucus layer. In addition, the bacterium was also found to interact with the mucin MUC5AC (Van Den Brink *et al.*, 2000; Van de Bovenkamp *et al.*, 2003). TFF1 act as a bridge between the MUC5AC and *H. pylori*. Indeed, TFF1 retains its ability to bind the mucin when it interacts with the bacterium probably because the peptide binds *H. pylori* to a different site from the mucin binding site (Dunne *et al* 2014). (Figure 1.10).

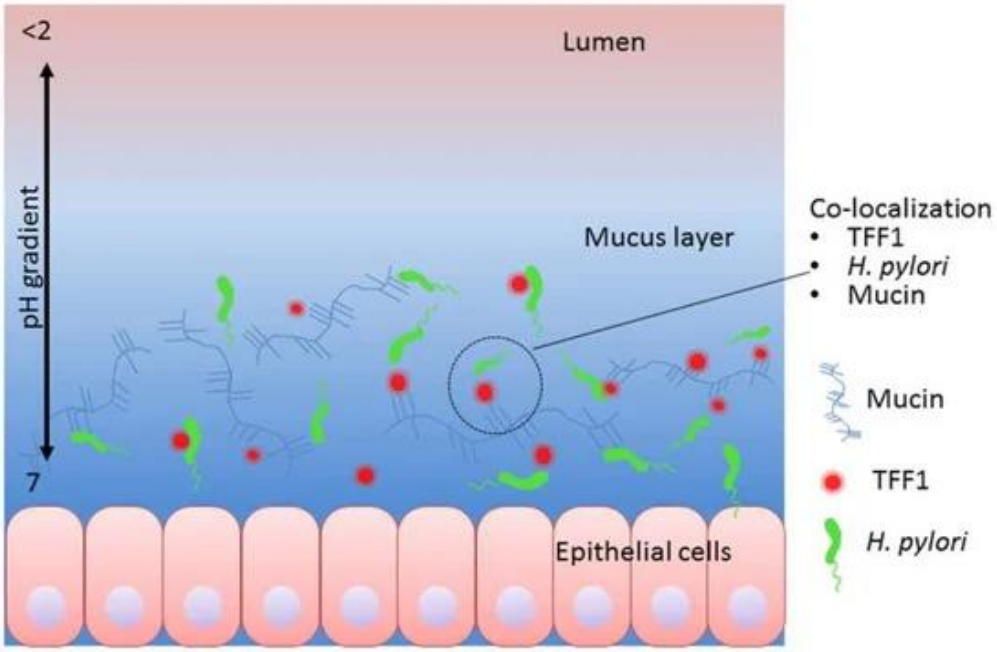


**Figure 1.10. Suggested mechanism whereby TFF1 may help locate *H. pylori* in gastric mucus.** Both TFF1 and *H. pylori* can interact with gastric mucin and with each other.

Deeper investigations showed that the core oligosaccharide portion of *H. pylori* lipopolysaccharides specifically binds the dimeric form of TFF1 (Reeves *et al.*, 2008) at the level of the carboxy-terminal region (Montefusco *et al.*, 2013).

Preliminary data from our group showed that the interaction between TFF1 and *H. pylori* is specific. Indeed pull-down experiments showed a preferential binding of TFF1 to *H. pylori* instead of *H. felis* (Figure 1.11.A). The results also showed that the interaction occurs when the gastric peptide is present in the dimeric form (Figure 1.11.B).

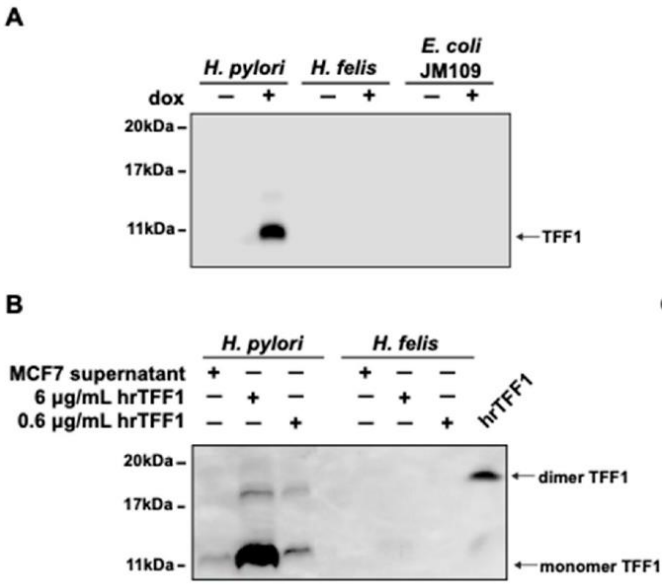


**Figure 1.11. hTFF1 specifically binds *H. pylori*.** Western blot analysis of pull-down experiments using (A) TFF1 protein secreted by AGS-AC1 clone induced (+dox) and not induced (-dox) with doxycycline, and incubated with different Gram-negative bacteria; (B) TFF1 protein secreted by MCF7 cells or hrTFF1, incubated with *H. pylori* or *H. felis*.

## 1.7 Aim of the work

*Helicobacter pylori* is one of the most important pathogenic bacteria associated with various gastric diseases, including cancer. This pathogen induces an acute mucosal inflammatory response; if not eradicated, it might lead to chronic infection and eventually, in a subset of individuals, to gastric cancer. In response to the infection, the gastric mucosa releases several protective factors, including TFF1, which binds *Helicobacter* in the mucus layer and reduces inflammation caused by the infection. TFF1 is a gastro-specific tumor suppressor gene upregulated in the acute phase of the *H. pylori* infection but silenced during the chronic one. Since TFF1 silencing leads to loss of mucosal protection and could be a contributing factor in the development of cancer, my thesis proposal aimed to explore the effects of the interaction between TFF1 and *H. pylori* and the molecular pathways leading to TFF1 silencing.

# CHAPTER 2

# RESULTS AND DISCUSSION

## 2.1 TFF1 reduces *H. pylori* motility by inducing its aggregation and morphological transformation

Our group recently reported that TFF1 is up-regulated during early *H. pylori* infection (Esposito *et al.*, 2017). Since TFF1 can specifically bind *H. pylori*, we hypothesized that the interaction might serve to protect the gastric epithelium.

### *2.1.1 hrTFF1 induces H. pylori aggregation in a concentration-dependent manner*

To better understand the role of TFF1-*H. pylori* interaction, *H. pylori* was incubated with or without 0.6 μg/mL and 6 μg/mL of the human recombinant protein (hrTFF1) for 6 h. After the incubation, the bacterial morphology was monitored by light microscopy, and *H. pylori* aggregates/mm$^2$ were counted. Our results show that bacteria form large clumps only if incubated with the protein, whereas at the same time of observation without hrTFF1, bacteria were still predominantly in the planktonic form. Furthermore, data suggest that *H. pylori* forms aggregates in a TFF1 concentration-dependent manner (Figure 2.1).

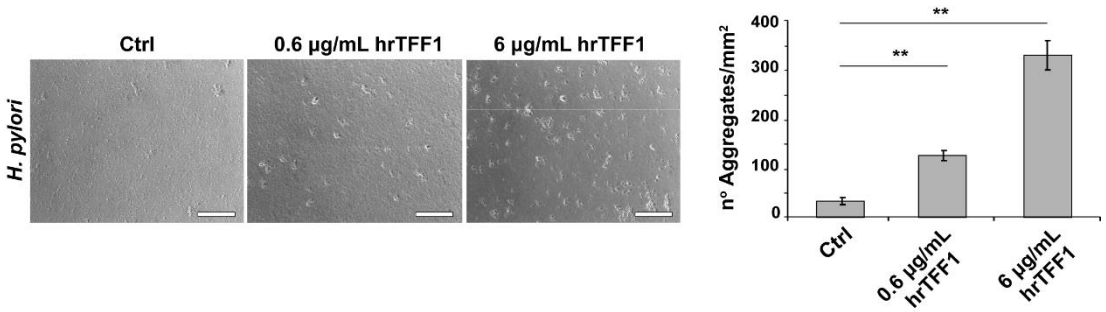


**Figure 2.1. *H. pylori* forms aggregates in a TFF1-concentration dependent manner.** Optical images at 20X magnification of *H. pylori* incubated with or without 0.6 and 6 µg/mL of hrTFF1. Scale bar 150 µm. The panel on the right side reports the histogram quantification of bacterial aggregates. Data are expressed as mean ± SD. (t-test; $^{**}p \leq 0.01$).

To corroborate this result, the formation of aggregates was monitored over time (0, 30, 60, 120 minutes) and with multiple concentrations (0, 0.75, 1.5, 3, 6 µg/mL) of hrTFF1. Consistent with the previous results, hrTFF1 induces *H. pylori* aggregation in a concentration- and time-dependent manner (Figure 2.2).

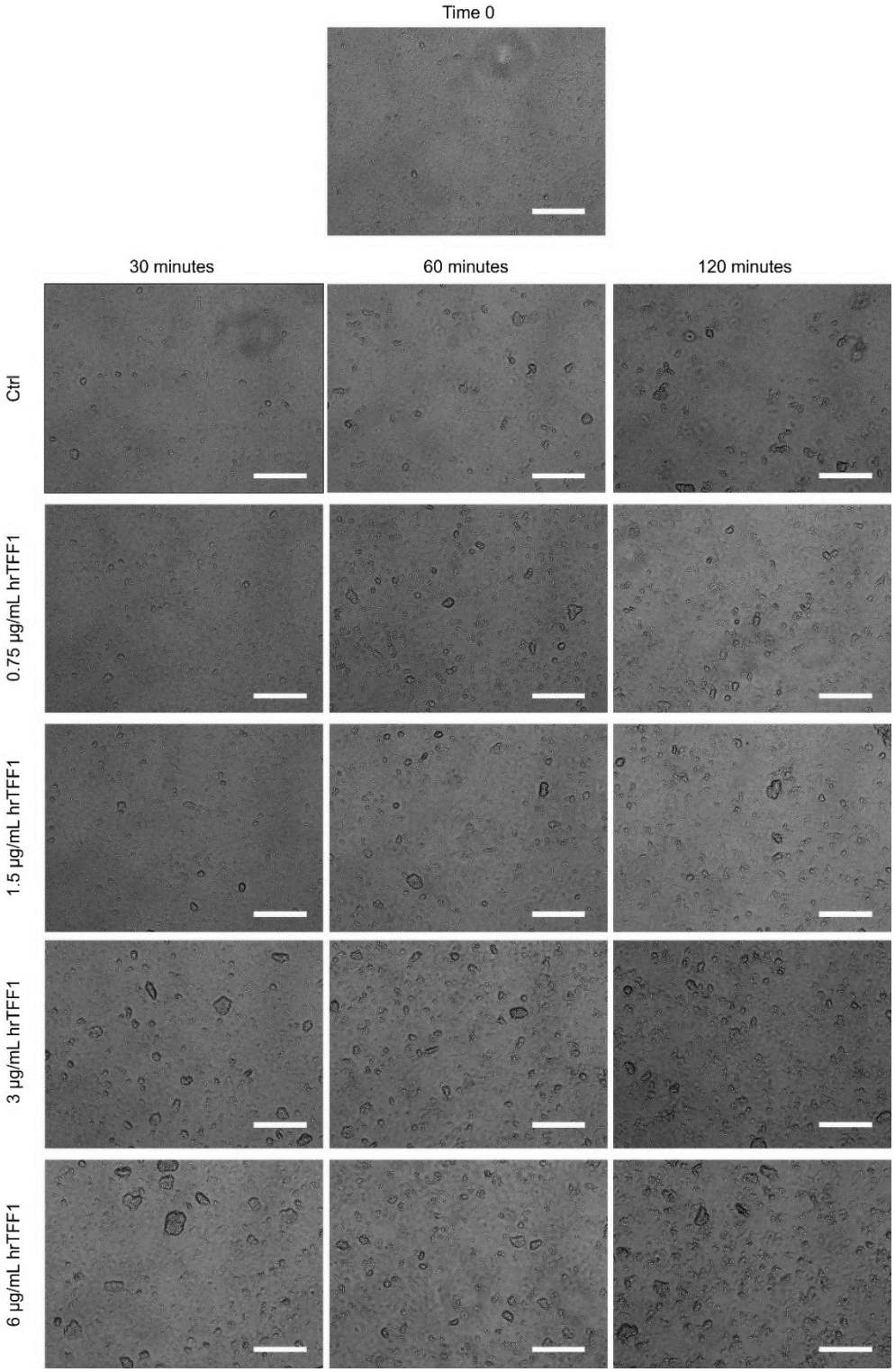


**Figure 2.2**. **hrTFF1 induces *H. pylori* aggregation in a concentration and time-dependent manner.** Optical images at 20X magnification of *H. pylori* incubated with multiple concentrations of hrTFF1 over time.

To evaluate whether the aggregation affects *H. pylori* morphology, the bacterial aggregates were observed by scanning electron microscopy (SEM). hrTFF1-induced aggregation causes the *H. pylori* morphological transformation from spiral-shaped to filamentous (Figure 2.3).

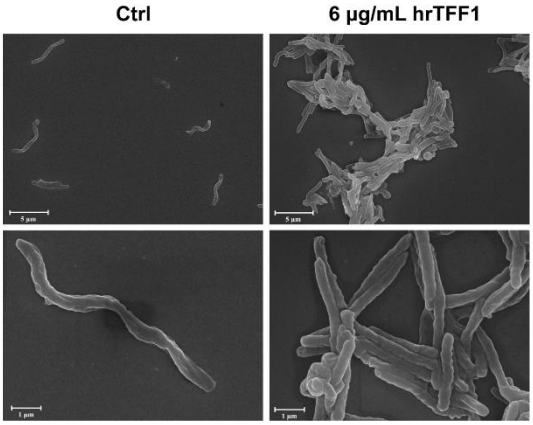


**Figure 2.3. TFF1 induces an *H. pylori* aggregation and morphological transformation.** Scanning electron microscopy (SEM) images of *H. pylori* incubated with or without 6 µg/mL of hrTFF1. Scale bar 5 µm (upper panels), 1 µm (lower panels).

We then asked whether *H. pylori* aggregation induced by TFF1 had affected bacterial viability. To this aim, the growth of *H. pylori* with/without hrTFF1 (0.6 µg/mL and 6 µg/mL) was monitored by measuring optical density at 600 nm ($OD_{600}$) of cell suspension at 6, 24, 30, 48, 54, 70, and 76 h. As shown in Figure 2.4, the viability of *H. pylori* was not affected, as the bacteria showed the same growth rate in any of the tested conditions.

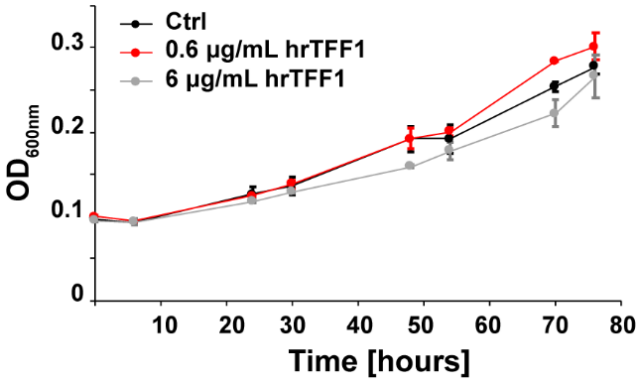


**Figure 2.4**. **TFF1 does not influence *H. pylori* growth.** Growth curves of *H. pylori* incubated w/wo 0.6 and 6 µg/mL of hrTFF1 at 6, 24, 30, 48, 54, 70 and 76 h. The results are expressed as means ± SD.

### *2.1.2 TFF1 does not influence H. pylori chemotactic behavior but affects bacterium motility.*

Once assessed the formation of TFF1-induced aggregates, we hypothesized that this phenomenon could affect the bacterium chemotaxis and/or motility.

To determine whether TFF1 has chemoattractant or chemorepellent activity towards *H. pylori*, a chemotaxis assay as described by Mazumder (Mazumder *et al*, 1999) and revised by Cerda *et al*. (Cerda *et al.*, 2011), was set up. Briefly, a tip-needle-syringe system was used in which bacteria were loaded into a disposable 200 µL pipette tip (yellow), and the solutions tested (10 mM HCl, hrTFF1 6 or 0.6 µg/mL) were drawn up through a stainless-steel needle into a 1-mL tuberculin syringe. The needle-syringe system was positioned to the pipette tip so that it was well embedded. After 45 minutes of incubation, all the bacteria that moved from the disposable tip toward the syringe were recovered and plated on a selective medium for bacterial colony counts. Our results show that TFF1 has no chemotactic effect on *H. pylori* compared to the buffer, while the positive control HCl works as a chemorepellent as expected (Figure 2.5A).

Then, to determine whether TFF1-induced aggregation could influence *H. pylori* motility, we modified the previous procedure and tested whether pre-formed aggregates moved faster or slower toward the syringe compared to planktonic bacteria. In detail, bacteria were pre-incubated with/without 6 µg/mL hrTFF1 for 1.5 h (the minimum time necessary to induce aggregation), placed in the disposable tip, and attached to the syringe needle loaded only with chemotaxis buffer. After 45 minutes of incubation, the bacteria that moved to the syringe were recovered and plated. As shown in Figure 2.5B, when bacteria were pre-incubated with the protein, the number of bacteria recovered from the syringe was significantly reduced compared to the control condition (bacteria without hrTFF1), suggesting that TFF1 reduces bacterial motility towards the chemotaxis buffer by inducing aggregation (Figure 2.5B).

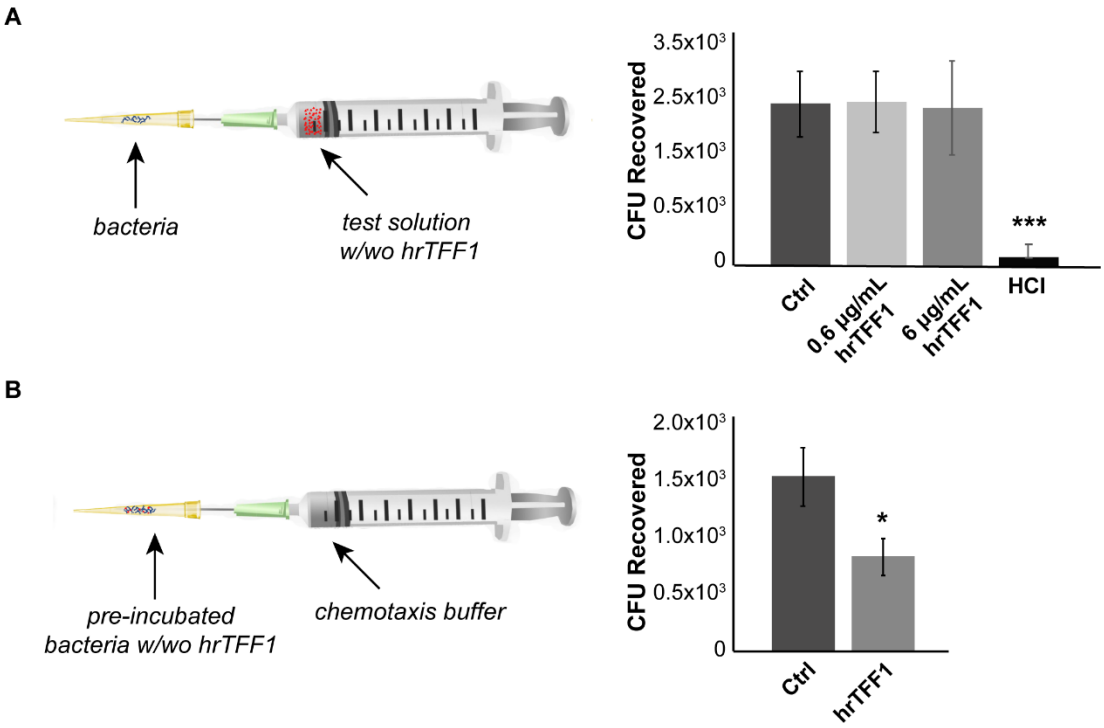


**Figure 2.5. TFF1 has no chemotactic effect on *H. pylori* but reduces bacterial motility by inducing aggregation. (A)** Left side, a cartoon of the experimental procedure: bacterial suspension was put in the tip, while the syringe was filled with chemotaxis solution with or without (w/wo) 0.6 or 6 µg/mL hrTFF1. Right side, bacteria that had moved from the tip toward solutions were recovered and counted as

CFU. **(B)** Left side, a cartoon of the experimental procedure: pre-incubated bacteria, w/wo 6 µg/ml hrTFF1, were put in the tip, while the syringe was filled only with the chemotaxis buffer. Right side, bacteria that had moved toward the syringe were counted as CFU. Data are mean ± SD. (t-test, *$p \leq 0.05$; ***$p \leq 0.001$).

It has been shown that *H. pylori* colocalizes and interacts with TFF1 in the mucus layer. To further investigate the influence of TFF1 protein on *H. pylori* motility, a motility assay was set up using the mucus collected from HT29-E12, a mucus-secreting clone. The mucus was harvested after 21 days of culturing since it has been reported to contain detectable levels of TFFs and mucins (Dolan *et al.*, 2012). Subsequently, the mucus was placed on the upper chamber of a transwell system. Bacteria were preincubated for 1.5 h with/without hrTFF1 (6 µg/mL) to favor the formation of aggregates and were set down on the mucus (Figure 2.6A). After 24 h, the lower chamber medium was plated for CFU counting. Mucus *per se* slowed the migration since most bacteria took 30 minutes to reach the lower chamber when bacteria were directly deposited on the transwell filter (data not shown). Figure 6B shows that the pre-incubation with hrTFF1 protein significantly reduced the number of bacteria, suggesting that TFF1, in combination with the mucus, affects the motility of *H. pylori*.

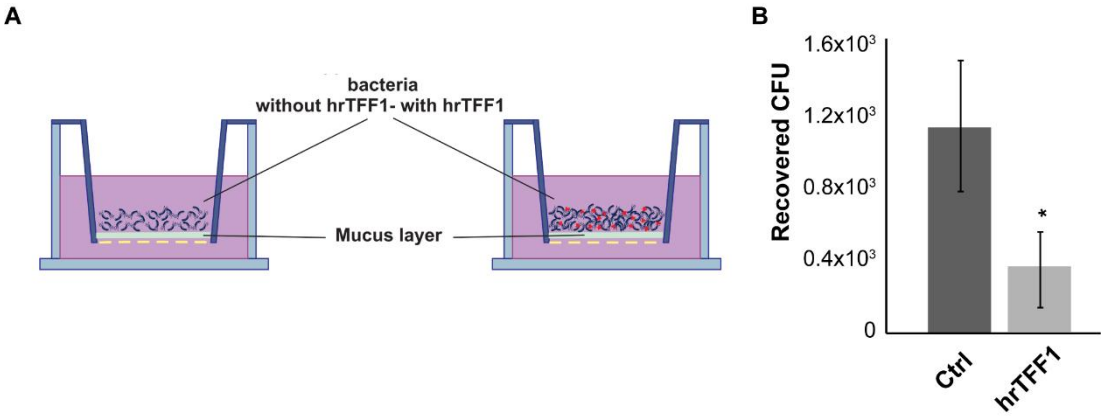


**Figure 2.6. TFF1 in combination with mucus affects the motility of *H. pylori*. (A)** Graphical representation of the mucus motility assay. **(B)** Bacteria recovered from the lower chamber w/wo pre-incubation with 6 µg/mL. Data are mean ± SD. (t-test, *p ≤ 0.05).

### *2.1.3 TFF1 influences the transcriptional regulation of H. pylori motility genes.*

We next hypothesized that TFF1 interaction and the subsequent aggregation might affect *H. pylori* gene expression. To explore this, HT29-E12 cells were infected with *H. pylori*, pre-incubated without or with 6 µg/mL hrTFF1 for about 2 h and then RNA was extracted and analyzed by Real-Time PCR. First, we analyzed the expression of genes involved in bacterial motility, among them *flaA* and *flaB*, components of the flagellar filament, and *flgE*, component of the hook that links the body to the flagellar filament. We observed a significant reduction of *flaB* and *flgE* (0.5-fold), while no variation was observed in the gene expression of *flaA*, compared to the control (Figure 2.7). Furthermore, *virB11*, encoding a component of the secretory apparatus complex virB/D, indispensable to translocate bacterial toxin to eukaryotic cells, was downregulated (0.6-fold) (Figure 2.7). The reduced expression of the flagellum components is consistent with the previous results showing that the motility of *H. pylori* is reduced due to the TFF1-dependent aggregation.

The flagellar filament consists of a copolymer of *FlaA* and *FlaB* flagellins (Leying, *et al*, 1992; Haas *et al*, 1993; Suerbaum *et al*, 1993; Spohn *et al*., 2001), where *FlaA* is the predominant subtype. *FlaB* is a minor constituent (Kostrzynska, *et al*, 1991), but both *FlaB* and *FlaA* are necessary for full motility (Josenhans *et al*, 1995). It has been suggested that *H. pylori* has the ability to alter the relative level of *flaA* and *flaB* in response to different environmental stimuli (Suerbaum *et al*, 1995; Spohn *et al* 2001). This could explain why only *flaB* expression is reduced, resulting in reduced bacterial motility.

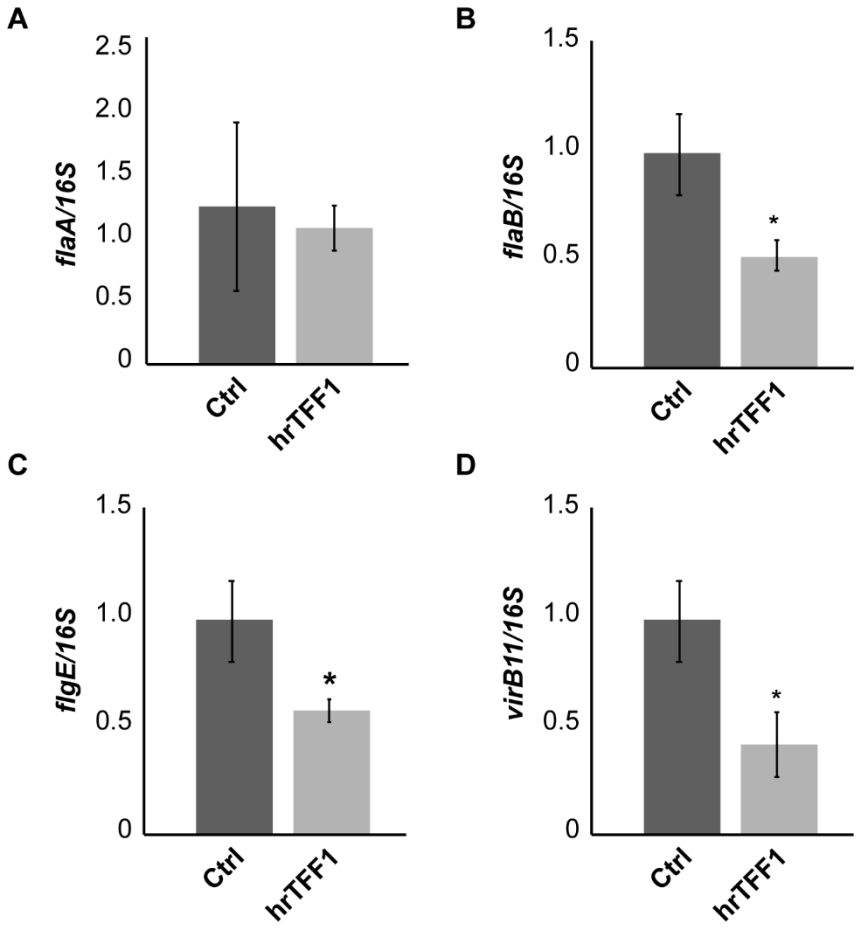


**Figure 2.7. TFF1 produces a significant reduction of *H. pylori* virulence factors involved in its motility.** RT-qPCR analysis of *Helicobacter pylori* genes expression *(flaA, flab, flgE and virB11)* after incubation with recombinant protein TFF1 at a concentration of 6 µg / ml. The results were normalized using *16S* as a reference gene and expressed relative to the control set as 1 SD ( t-test, * $p < 0.05$).

The expression of the other virulence genes did not change compared to the control (data not shown). Among those genes, the expression of *alpA* and *alpB* (contributing to the ability of *H. pylori* to bind host laminin), *hopZ* (outer membrane proteins), *vacA* (vacuolating cytotoxin), and *cheY* (chemotaxis effector) was measured. In addition, genes including the pathogenicity island cagPAI of *H. pylori* were evaluated, such as *cag1* (necessary for the function of T4SS:type IV secretion apparatus), *cagA* (Cag pathogenicity island protein) and *cag25* (membrane protein involved in the interaction of the system with epithelial cell receptors).

## 2.2 The immune system is involved in TFF1 silencing during *H. pylori* chronic infection

Previous results demonstrated that *H. pylori* infection induces TFF1 expression in human gastric tumor cell lines, while in the gastric antrum of C57BL/6 mice infected with *H. felis*, TFF1 was found up-regulated during the acute phase but reduced during chronic inflammation following prolonged *H. pylori* infection (Esposito *et al.*, 2017). In addition, this protective factor is found down-regulated in *H. pylori* chronically infected patients and more than 50% of gastric tumor tissues (van de Bovenkamp *et al.*, 2005; Tomita *et al.*, 2011). The reduced expression of TFF1 during *H. pylori* chronic infection leads to loss of gastric mucosa protection and might become decisive for the development of carcinogenesis, being TFF1 a gastro-specific tumor suppressor.

Based on the data mentioned above, we hypothesized that the host immune response to *H. pylori* infection might trigger molecular pathways that ultimately would lead to TFF1 silencing in the chronic phase of infection.

### *2.2.1 TFF1 expression is reduced mainly by IFNγ proinflammatory cytokine.*

The inflammatory response against *H. pylori* initially consists of the recruitment of neutrophils, followed by T and B lymphocytes, plasma cells and macrophages, along with epithelial cell damage (Velin *et al*., 2006). Antigens derived from *H. pylori* are recognized by the Toll-like receptors (TLRs) and Nod-like receptors (NLRs) of the innate immune cells such as macrophages, dendritic and epithelial cells (Vorobjova *et al.*, 2008). One of the main cytokines released during *H. pylori* infection is IFNγ, along with IL-1β and TNFα (Tanahashi *et al.*, 2000). To explore if the immune system could be involved in TFF1 silencing, we analyzed the effect of the main pro-inflammatory cytokines released during *H. pylori* infection on TFF1 expression. To this aim, KATO III was selected as a cell model of infection since, although a tumor cell line, it exhibits elevated basal levels of TFF1 expression. KATO III cells were incubated with IFNγ (10 ng/mL), TNFα (40 ng/mL), and IL-1β (40 ng/mL), alone or in combination (triple treatment) for 24 h. TFF1 expression was analyzed at transcriptional and protein levels (as intra- and extra-cellular product). TFF1 expression was reduced in cells stimulated with cytokines. In particular, RT-qPCR results showed that both TNFα and IFNγ individually are able to reduce TFF1 levels at different extents (0.2-fold and 0.6-fold, respectively) (Figure 2.8. A). Western blot data also confirmed that TFF1 was decreased at the intracellular level (0.6-fold) (Figure 2.8. B-C) as well at the extracellular one (0.6-fold) (Figure 2.8. D-E) in cells treated with IFNγ or with cytokines' combination.

Taken together, these results revealed that the reduction of TFF1 expression was mainly due to IFNγ treatment and, to a lesser extent, to TNFα.

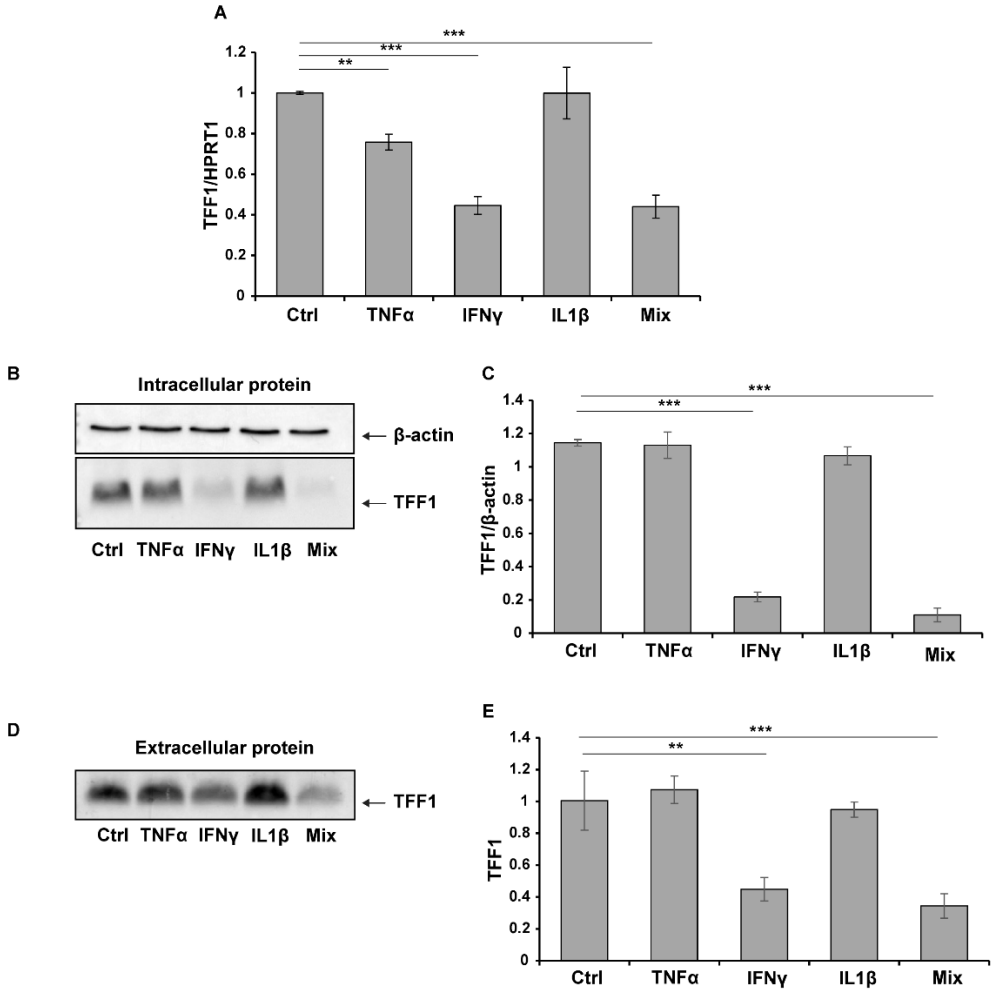


**Figure 2.8**. **Proinflammatory cytokines reduce TFF1 expression. (A)** RT-PCR analysis of TFF1 expression in KATO III cells before and after treatment with the indicated cytokines (40 ng/mL TNFα, 10 ng/mL IFNγ, 40 ng/mL IL-1β), individually or in combination. HPRT1 was used as the reference gene. Experiments were performed at least in triplicate and data are expressed as mean ± SD (t-test, **p ≤ 0.01; *** p ≤ 0.001). **(B)** Western blot analysis of intracellular TFF1 protein after treatment of KATO III cells with the indicated cytokines individually or in combination. **(C)** Densitometric analysis of intracellular TFF1 normalized to β-actin. (t-test, *** p ≤ 0.001) **(D)** Western blot analysis of TFF1 secreted by KATO III cells after treatment with the indicated cytokines individually or in combination. **(E)** Densitometric analysis of extracellular protein signals (t-test, **p ≤ 0.01; *** p ≤ 0.001).

To verify whether the IFNγ effect is maintained over time, KATO III cells were incubated with IFNγ (10 ng/mL) for 24h, 72h, and 7 days. The reduction of TFF1 expression by IFNγ has been observed up to 7 days (Figure 2.9).

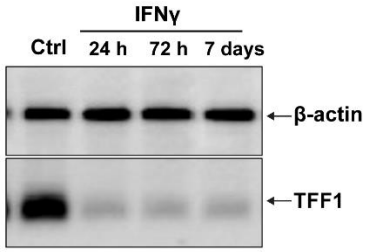


**Figure 2.9**. **IFNγ reduces TFF1 expression up to 7 days.** Western blot analysis of TFF1 protein after treatment of KATO III cells with IFNγ (10 ng/mL) for 24h, 72h and 7 days. β-actin was used as a loading control.

### *2.2.2 IFNγ acts on the TFF1 gene promoter inhibiting its expression*

Once determined that cytokines inhibit TFF1 expression, the next step was to explore whether this phenomenon was due to a direct effect on TFF1 promoter using a luciferase assay in the gastric KATO III cell line. A pGL3 commercial plasmid (Promega) containing a 1 kb long TFF1 promoter fragment (-931 bp) upstream of a Luciferase reporter gene (Figure 2.10.A) was co-transfected in the abovementioned cells along with a plasmid containing the β-galactosidase gene, whose expression is used as a normalization parameter of transfection efficiency. Subsequently, cells were treated with TNFα, IFNγ, and IL-1β cytokines, alone or in combination, as described above. In agreement with our previous results, a significant reduction in the relative luciferase activity following triple treatment or with IFNγ alone was observed (0.6-fold) (Figure 2.10.B). Therefore, IFNγ affects TFF1 expression by directly inhibiting its promoter.

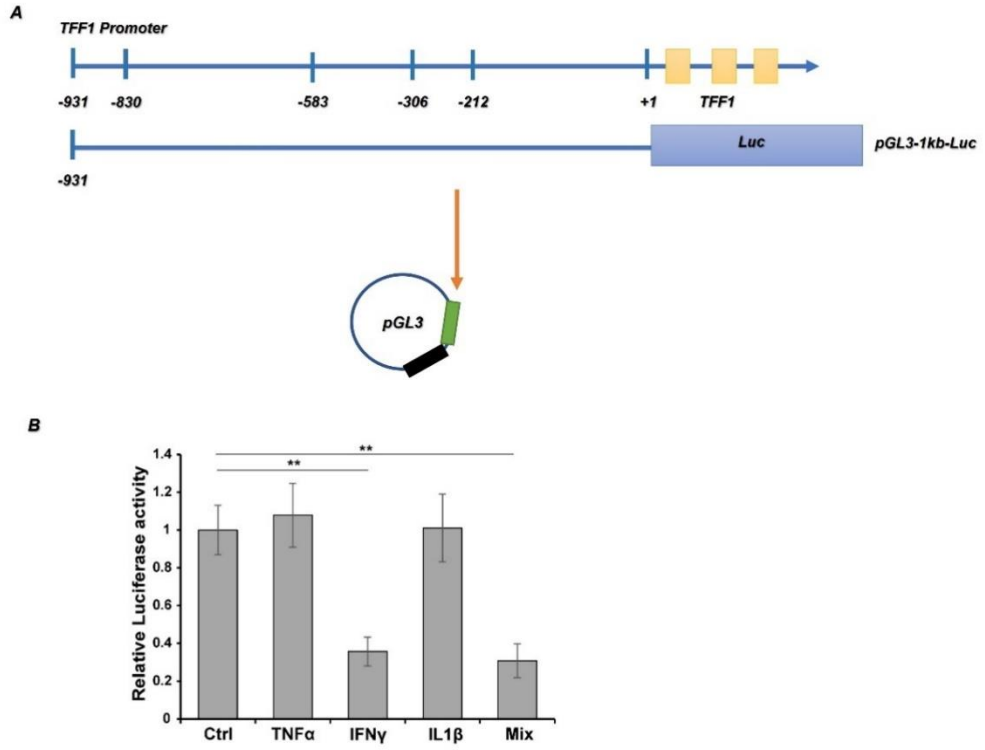


**Figure 2.10**. **IFNγ acts directly on the TFF1 promoter. (A)** Schematic representation of pGL3- (1kb TFF1 promoter) -Luc reporter construct. **(B)** Luciferase assay in KATO III cells co-transfected with pGL3- (1kb) -Luc and β-galactosidase and successively treated with the cytokines indicated (40 ng/mL TNFα, 10 ng/mL IFNγ, 40 ng/mL IL-1β), individually or in combination. All data are representative of experiments performed in quadruplicate and are reported as mean ± SD. (t-test, ** $p \leq 0.01$ compared to untreated cells having pGL3- (1kb) -Luc).

To determine the specific region of the TFF1 promoter responsive to IFNγ treatment, KATO III cells were transfected with pGL3 plasmids containing fragments of different lengths of TFF1 promoter (from -931 bp, -830 bp, -586 bp, -306 bp and -212 bp to +17, named pGL3-1kb-Luc, pGL3-0.8kb-Luc, pGL3-0.5kb-Luc, pGL3-0.3kb-Luc, and pGL3-0.2kb-Luc respectively) (Figure 2.11.A) and treated with IFNγ. Luciferase activity was significantly reduced by IFNγ treatment in cells transfected with all constructs, but one, the pGL3-0.2kb-Luc plasmid (Figure 2.11.B). Based on this evidence, we identified a region (including -931 /-212) containing specific

elements responsive to IFNγ treatment. Specific potential transcriptional inhibitors are currently under investigation.

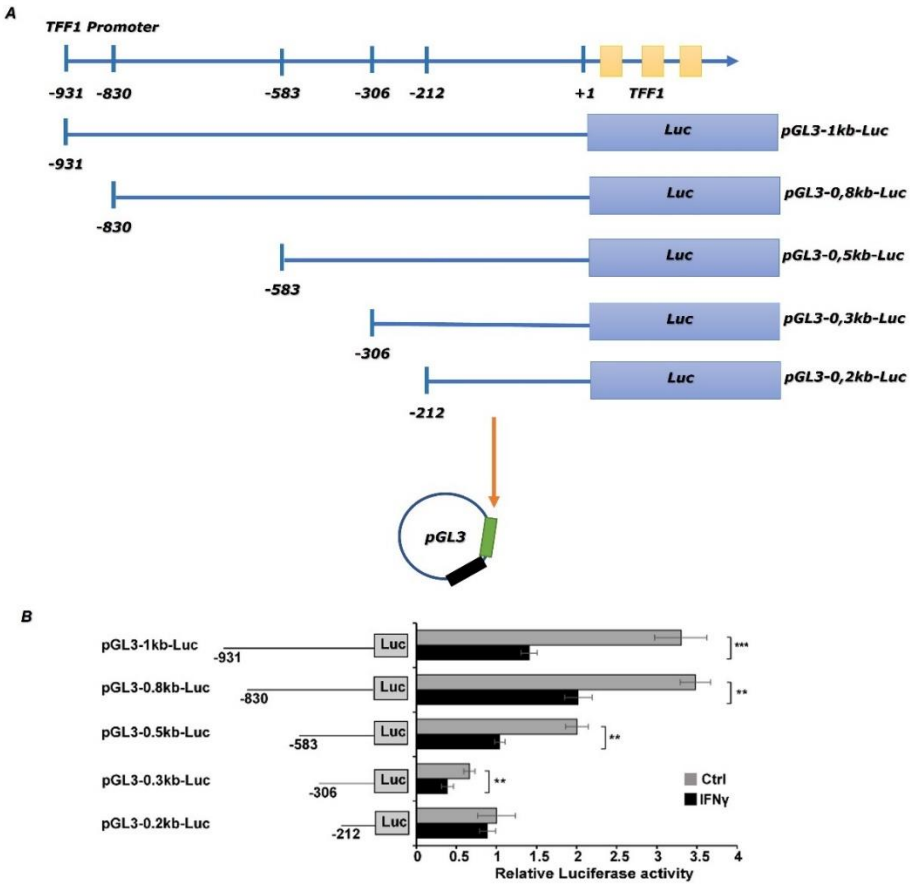


**Figure 2.11**. **IFNγ acts in the -931 /-212 region of TFF1 promoter. (A)** Schematic representation of luciferase reporter constructs containing fragments of different lengths from −931 to −212 of the TFF1 promoter upstream of a Luciferase reporter gene. **(B)** Luciferase assay in KATO III cells transfected with the indicated plasmids and treated with IFNγ (10 ng/mL) for 24 hours. All data are representative of experiments performed in quadruplicate and are reported as mean ± SD. (t-test, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to untreated cells).

### *2.2.3 Analysis of TFF1 promoter*

TFF1 expression is controlled at different levels by genetic and epigenetic mechanisms. It depends on the methylation status of its promoter (Métivier *et al.*,

2008) and on the regulatory activity of several transcription factors, including EGF, GATA6, AP-1, HNF3, C/EBPβ and the copper-sensing transcription factor SP1 (Wright *et al.*, 1990; Al-Azzeh *et al.*, 2000; Lacroix *et al*, 2004; Sankpal *et al*, 2005; Esposito *et al.*, 2015).

To identify the IFNγ-induced TFF1 silencing mechanisms, we looked for the consensus binding sites of transcriptional modulators in the TFF1 promoter. One of these is C/EBPβ, a transcription factor with three binding sites on the TFF1 promoter, one previously reported (Sankpal *et al*, 2005) and two putative binding sites retrieved from the TRANSFAC software (Dossinger *et al.*, 2002) (Figure 2.12). Since C/EBPβ is overexpressed in gastric cancer, reported to be stimulated by IFNγ (Xiao *et al.*, 2001; Stark *et al*, 2012) and reported to cause TFF1downregulation (Sankpal, *et al*, 2005; Regalo *et al.*, 2016), we started analyzing its involvement in our system.


**Figure 2.12**. **TFF1 promoter sequence Analysis**. TFF1 promoter sequence (-1000bp) with binding sites of different transcription factors. C/EBPβ binding sites are highlighted in red.

*2.2.3.1 IFNγ positively regulates C/EBPβ transcription factor expression.*

Several studies have shown that inflammatory stimuli, including cytokines and bacterial LPS, produce the C/EBP*β* transcriptional activation by phosphorylation of its transactivation domain (Trautwein C *et al.*, 1993; Cloutier *et al.*, 2009) followed by nuclear accumulation. One of the cytokines reported to induce C/EBPβ expression is IFNγ (Xiao *et al.*, 2001). IFNγ triggers a signal through the membrane into the cell via the JAK-STAT pathway, leading to transcriptional activation of the IFN-stimulated gene (ISGs) (Stark *et al*, 2012). Since C/EBPβ is reported as an ISG, we investigated whether C/EBPβ expression is induced by IFNγ also in our cellular system. KATO III cells were incubated with IFNγ (10 ng/ml) for 2, 6, and 24 h. Real-time PCR and Western blotting analyses showed basal high C/EBPβ levels in KATO III cells, further raised (2.5-fold in TFF1 transcriptional level and 0.8-fold in TFF1 protein level) following 24h IFNγ treatment (Figure 2.13.B-D). At the same time point, we observed a reduced expression of TFF1 (0.4-fold; Figure 2.13.A). These data indicate that C/EBPβ is an IFNγ-stimulated gene also in the KATO III cellular system.

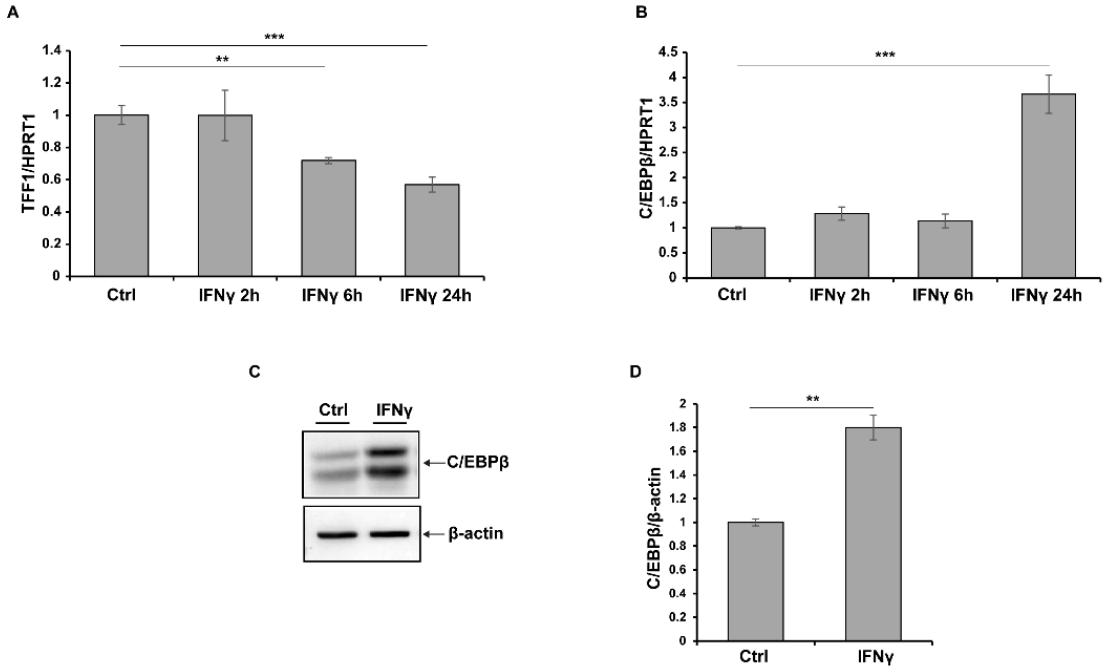


**Figure 2.13**. **IFNγ increases C/EBPβ expression.** RT-PCR analysis of TFF1 **(A)** and C/EBPβ **(B)** gene expression in KATO III cells treated with IFNγ (10 ng/mL) over time up to 24h. HPRT1 was used as the reference gene. Experiments were performed at least in triplicate, and data are expressed as mean ± SD (t-test, **p ≤ 0.01, *** p ≤ 0.001). **(C)** Western blot analysis of C/EBPβ protein after treatment of KATO III cells with 10 ng/mL IFNγ for 24 h. β-actin was used as a loading control. **(D)** Densitometric analysis of C/EBPβ protein signals normalized to β-actin. Experiments were performed at least in triplicate. Data are expressed as mean ± SD (t-test; **p ≤ 0.01).

### *2.2.3.2 IFNγ induces C/EBPβ nuclear accumulation.*

The human genome encodes 1,639 annotated transcription factors (TFs), of which 54 contain a basic-leucine zipper (bZIP) domain (Lambert *et al.*, 2018), a basic region mediating sequence-specific DNA-binding followed by a leucine zipper region required for dimerization (Hurst, 1995). The activity of several TFs is regulated by the modulation of their intracellular location (Vandromme *et al.*, 1996; Whitmarsh *et al.*, 2000). Indeed, nuclear translocation is one of the steps at which transcription factor activity may be controlled. C/EBP*β* is a bZIP domain transcription factor reported to

undergo translocation from the cytoplasm to the nucleus (Metz *et al*, 1991; Katz *et al.*, 1993; Yin *et al.*, 1996) by increased phosphorylation (Metz *et al*, 1991) leading to increased transcriptional activity (Williams *et al.*, 1997). To answer whether IFNγ stimulation promotes C/EBPβ translocation, nuclear and cytosolic fractions were purified from cell lysates of KATO III upon IFNγ exposure and C/EBPβ expression analyzed by Western Blot. As shown in Figure 2.14, C/EBPβ is localized mostly in the nucleus even in unstimulated conditions. Following 24 h of IFNγ stimulation, the nuclear levels of C/EBPβ are higher than untreated ones, as expected (Figure 2.14). These data confirm the stimulatory effect of IFNγ and show a preferential localization of C/EBPβ in the nucleus.

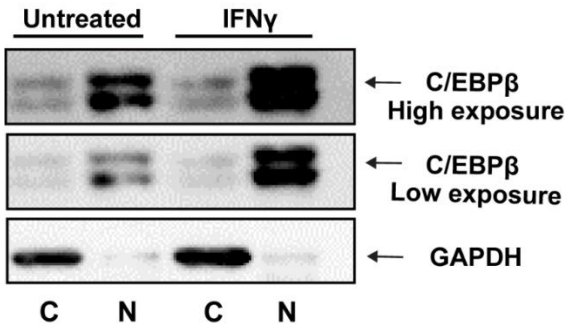


**Figure 2.14**. **IFNγ induces the C/EBPβ nuclear accumulation.** Western blot analysis of C/EBPβ protein levels in the nuclear (N) and cytosolic (C) extracts from KATO III cells stimulated or not with IFNγ (10 ng/mL) for 24h. GAPDH was used as a marker for the purity of the cytosolic (C) fraction.

*2.2.3.3 IFNγ induces C/EBPβ-mediated TFF1 down-regulation.*

Subsequently, to confirm the role of C/EBPβ in IFNγ-induced TFF1 down-regulation, a gene-silencing experiment was performed using siRNA gene silencers that selectively inhibit the expression of the target protein. To verify whether reduced C/EBPβ levels would impair the IFNγ-depending silencing of TFF1, KATO III cells were transfected with C/EBPβ siRNA or a control siRNA and stimulated with IFNγ

(10 ng/ml) for 24 h. The control siRNA is a non-targeting RNA designed as a negative control with no matches to any known mRNA sequences. Successful siRNA-mediated C/EBPβ knockdown was confirmed by Western blot. The results revealed that IFNγ-induced TFF1 repression was prevented when C/EBPβ was knocked down by siRNA, while no change in the TFF1 expression was observed with control siRNA (Figura 2.15), confirming the involvement of C/EBPβ in the IFNγ-induced TFF1 suppression.

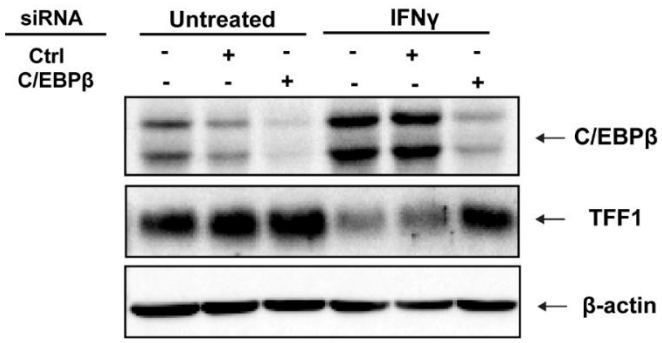


**Figure 2.15**. **C/EBPβ siRNA reverts the IFNγ-induced TFF1 reduction.** The Western blot analysis of C/EBPβ and TFF1 protein in KATO III transfected with siRNA control (Ctrl) or C/EBPβ and with/without stimulation with IFNγ (10 ng/mL) for 24h. β-actin served as a loading control.

*2.2.3.4 IFNγ stimulation promotes the binding of C/EBPβ to the TFF1 promoter.*

C/EBPs affect gene expression by binding to a DNA binding site (consensus sequence "CCAAT"), which is present in many promoters and enhancer regions. Previous studies have demonstrated the existence of a cis-acting repressor region between −241 and −84 bp of the TFF1 promoter containing a consensus binding site of the C/EBPβ transcriptional modulator at −216 (referred to as site 1 in Figure 2.16.A) (Sankpal *et al.*, 2005). Moreover, C/EBPβ has two additional putative binding sites retrieved from the TRANSFAC software (referred to as site 2 and site 3 in Figure 2.16.A) (Dossinger *et al.*, 2002). To verify whether the role of C/EBPβ under IFNγ

stimulation occurs via a direct interaction with the *cis*-acting TFF1 promoter elements, a ChIP-qPCR assay in KATO III cells unstimulated or stimulated with IFNγ for 24 h or left untreated was performed using a ChIP-grade anti-C/EBPβ antibody. The qPCR analysis using TFF1 promoter primers showed higher binding of C/EBPβ to all three sites in response to IFNγ compared to untreated cells (Figure 2.16.B-D). These data suggest that TFF1 might be a C/EBPβ direct downstream target in the presence of IFNγ, promoting its silencing during *H. pylori* chronic infection.

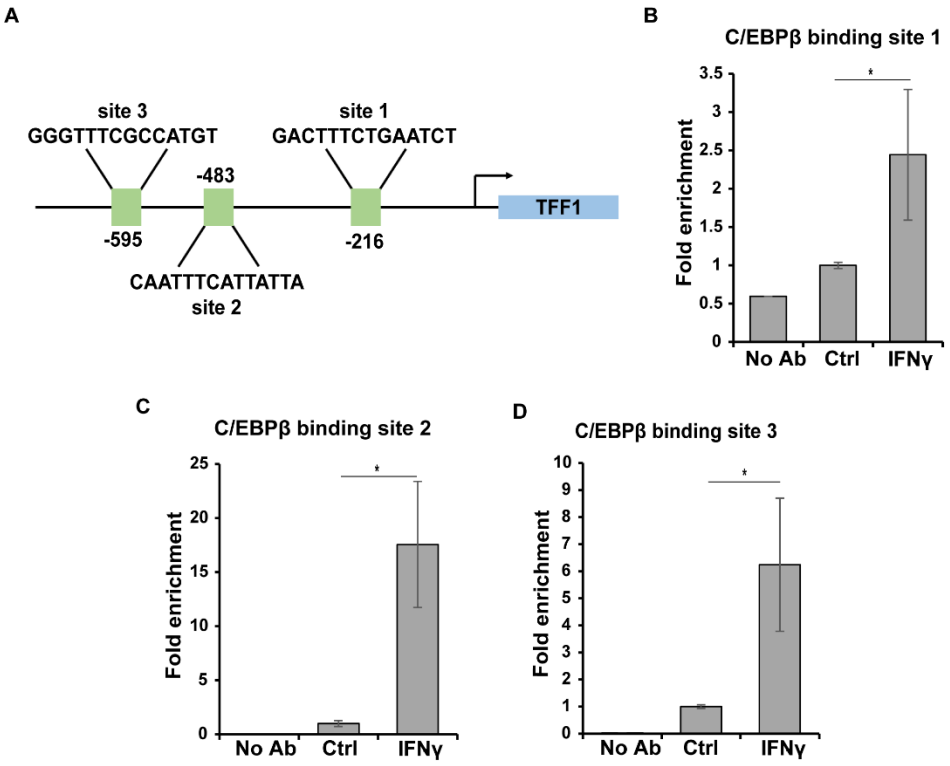


**Figure 2.16. C/EBPβ binds to all three binding sites on TFF1 promoter.** ChIP assay on KATO III cells with/without IFNγ (10 ng/mL) for 24 hours. The binding capacity of C/EBPβ protein to the indicated TFF1 promoter binding sites was analyzed by RT-q-PCR (*p≤ 0.05).

To ensure a successful ChIP experiment, we used different controls. Since Histone H3 is a core component of chromatin and is bound to most DNA sequences throughout the genome, including the ribosomal protein L30 (RPL30) locus, an anti-Histone H3

antibody and human RPL30 primers set were used as a positive control. In addition, to measure the non-specific binding, a normal Rabbit IgG antibody which does not recognize specific epitopes, was used as a negative control (Figure 1.17.A). Moreover, the COX-2 promoter was used as a positive control locus (Figure 2.17.B) because several studies have shown that C/EBPβ binds the COX-2 promoter once induced by proinflammatory mediators in murine and human cells (Wadleigh *et al.*, 2000; Saunders *et al.*, 2001; Zhu *et al.*, 2002) and the TFF1 coding region as a negative control locus (Figure 2.17.C).

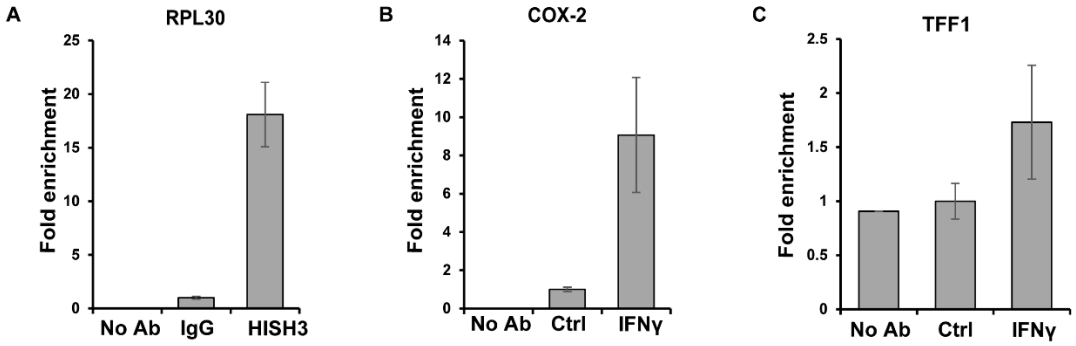


**Figure 2.17. Different controls of ChIP assay.** ChIP assay on KATO III cells with/without IFNγ (10 ng/mL) for 24 hours. The binding capacity of Histone H3 and IgG to RPL30 locus, and of C/EBPβ to COX-2 or TFF1 coding region was analyzed by RT-q-PCR.

### *2.2.3.5 Methylation is involved in TFF1 reduction upon IFNγ-stimulation*

The effect of *H. pylori* on the oncogenesis process has been ascribed to two main mechanisms: an indirect inflammatory reaction to *H. pylori* infection on the gastric mucosa and a direct epigenetic outcome of *H. pylori* on gastric epithelial cells (Machlowska *et al.*, 2020). *H. pylori* is able to induce hypermethylation of DNA, especially on CpG islands, thereby silencing genes associated with tumor suppression (Correa, 2013). On the other hand, TFF1 expression is strongly influenced by the methylation status of its promoter (Carvalho *et al.*, 2002; Tanka *et al.*, 2013; Feng *et*

*al.*, 2014). It is reported that deletions, mutations, or methylation of the TFF1 promoter produce a down-regulation of its expression (Fujimoto *et al.*, 2000; Park *et al.*, 2000a; Carvalho *et al.*, 2002). To evaluate if methylation is involved in TFF1 down-regulation promoted by the chronic infection, we looked whether IFNγ-depending silencing of TFF1 is affected by the inhibition of DNA methylation. To this aim, KATO III cells were exposed to a combined treatment with IFNγ and 5-Aza-2′-deoxycytidine (5-AZA) and TFF1 expression measured by RT-qPCR and Western blot. 5-AZA, also known as Decitabine, is a deoxycytidine analog typically used to inhibit DNA methyltransferases (Seelan *et al.*, 2018). KATO III cells were treated with 5-AZA for 72 h and in the last 24 h co-treated with IFNγ. Our results showed an increase of TFF1 levels in 5-AZA-treated cells (1.3-fold) and a level comparable to control cells in the combined treatment (MIX) (Figure 2.18). These data suggest that DNA methylation might also play a role in the IFNγ-induced silencing of TFF1.

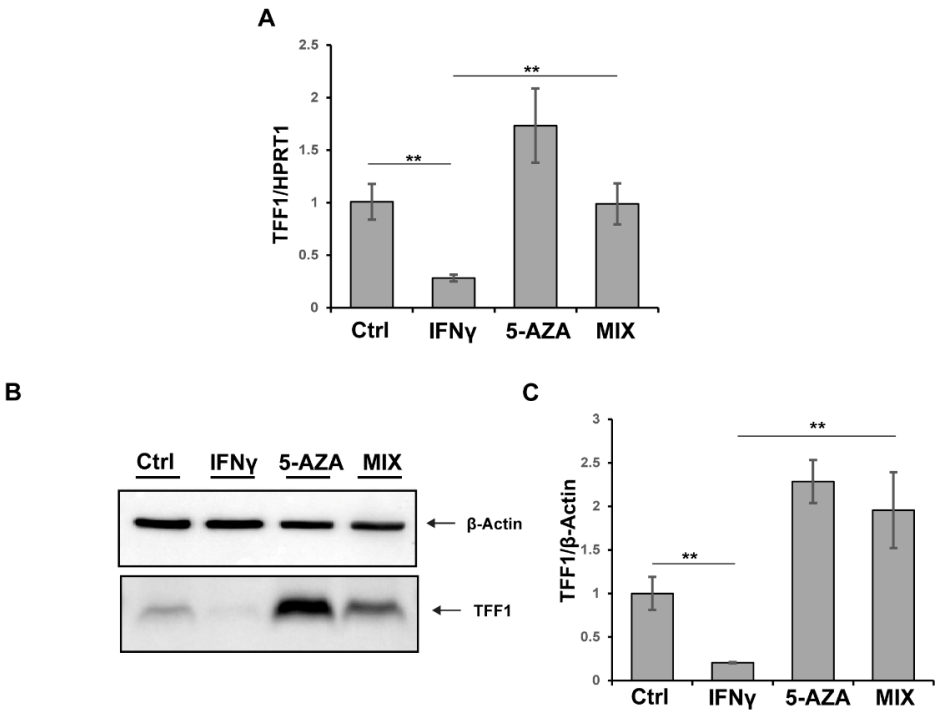

**Figure 2.18. 5-AZA reverts the IFNγ-induced TFF1 reduction. (A)** RT-PCR analysis of TFF1 expression in KATO III cells pre-treated with 5-aza-2′-deoxycytidine (5-AZA, inhibitor of DNA methylation) (10 μM) for 72 hours and then stimulated with IFN-γ (10 ng/mL) for the last 24 hours. HPRT1 was used as the reference gene. Experiments were performed at least in triplicate, and data are expressed as mean ± SD (t-test, **p ≤ 0.01;). **(B)** Western blot analysis of TFF1 protein in KATO III treated as in A. **(C)** Densitometric analysis of TFF1 protein signals normalized versus β-actin signals (t-test, **p ≤ 0.01;).

To assess whether 5-AZA had an effect also on the TFF1 promoter, KATO III cells were transfected with 1.0 kb TFF1 promoter plasmid containing luciferase reporter and treated with IFN-γ and/or 5-AZA for 24 hours. Luciferase reporter activity was normalized versus beta-galactosidase. IFNγ treatment caused a significant reduction of luciferase activity as expected, a robust increase with 5-AZA treatment and a level comparable to the control condition with the IFNγ/5-AZA combination (Figure 2.19).

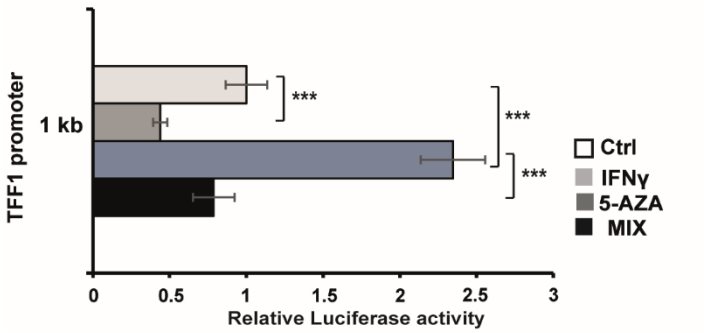


**Figure 2.19. 5-AZA prevents the IFNγ-induced TFF1 reduction acting on its promoter.** Luciferase assay in KATO III cells transfected with 1.0 kb TFF1 promoter plasmid containing luciferase reporter and treated with IFNγ (10 ng/mL) and/or 5-AZA (10 μM). Luciferase reporter activity was normalized versus beta-galactosidase. All data are representative of experiments performed in quadruplicate and are reported as mean ± SD. (t-test, *** p ≤ 0.001).

*2.2.3.6 Chemical inhibition of DNA methylation reduces IFNγ-induced C/EBPβ expression and reverts C/EBPβ binding to TFF1 promoter*

To verify whether the combined treatment 5-AZA+ IFNγ affected the expression and as consequence the repressor activity of C/EBPβ on TFF1, we first analyzed the expression of C/EBPβ in cells treated with 5-AZA and/or IFNγ by RT-q-PCR and Western blot. As shown in Figure 2.20, IFNγ-induced C/EBPβ expression is reduced in cells treated with 5-AZA (1.2-fold), suggesting a hypothetical reduction also of its repressor activity (Figure 2.20).

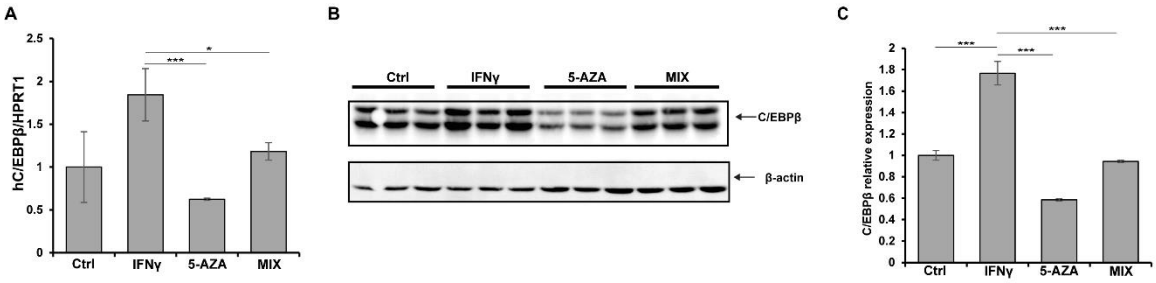


**Figure 2.20**. **5-AZA reverts the IFNγ-induced C/EBPβ increase. (A)** RT-PCR analysis of C/EBPβ expression in KATO III cells pre-treated with 5-AZA (10 μM) for 72 h and the last 24h stimulated with IFNγ (10 ng/mL) for 24 h. HPRT1 was used as the reference gene. Experiments were performed in triplicate and data are expressed as mean ± SD (t-test; * $p \leq 0.05$, *** $p \leq 0.001$). **(B)** Western blot analysis of C/EBPβ protein in KATO III treated as in A. β-actin was used as a loading control. **(C)** Densitometric analysis of C/EBPβ protein signals normalized with respect to β-actin signals. (t-test; *** $p \leq 0.001$).

To evaluate the influence of DNA methylation on IFNγ -induced C/EBPβ binding on TFF1 promoter, we performed a ChIP-qPCR assay in KATO III cells treated with the combination of 5-AZA and IFNγ. The results are different according to the binding sites on the TFF1 promoter in response to DNA methylation inhibition. A significant

increase of C/EBPβ binding to Site 1 was observed with 5-AZA treatment and the combination with IFNγ (Figure 2.21.B), indicating that DNA methylation reduces the ability of C/EBPβ to bind the site 1 on the promoter under investigation. Since the binding site 1 on the TFF1 promoter (in KATO III cells) is known to be unmethylated at steady state conditions, the ChIP-qPCR results can be explained only if we consider another factor, sensitive to methylation, that would promote the recruitment of C/EBPβ on TFF1 promoter. In addition, the increased C/EBPβ enrichment observed with 5-AZA and IFN-γ in combination, compared to 5-AZA alone treatment, may be justified by the increased C/EBPβ expression induced by IFN-γ and, therefore, the greater number of transcription factor molecules that bind to TFF1 promoter binding site 1.

Conversely, an opposite trend was observed in the ability of C/EBPβ to bind sites 2 and 3 on the TFF1 promoter. A significant reduction of C/EBPβ binding to sites 2 and 3 was observed with 5-AZA and IFN-γ in combination compared to IFN-γ stimulation (Figure 2.21.C-D). Since 5-AZA inhibits DNA methylation, the result seems to suggest that C/EBPβ preferentially binds those consensus sites when methylated. Two hypotheses can be made to explain this:

1. IFN-γ induces an increased state of DNA methylation by recruiting DNMT, favoring C/EBPβ binding to TFF1 promoter sites 2 and 3.

2. A C/EBPβ binding protein, probably belonging to the RUNX family, could be down-regulated under IFNγ treatment, releasing C/EBPβ which in turn would bind TFF1 promoter. When methylation is inhibited with 5-AZA, this C/EBPβ repressor protein could enhance its expression and prevent C/EBPβ from binding to the TFF1 promoter.

The results of these experiments confirm the functionality of putative C/EBPβ binding sites in the region of the analyzed TFF1 promoter and suggest a crucial role

of DNA methylation in the expression and binding of C/EBPβ to the promoter of TFF1.

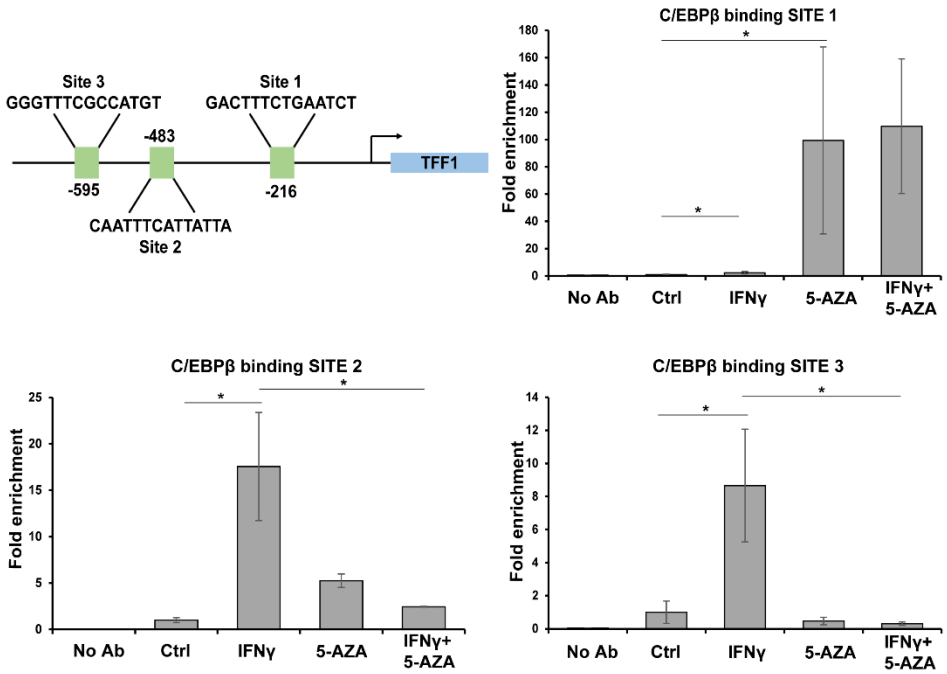


**Figure 2.21. 5-AZA increases C/EBPβ binding on TFF1 promoter binding site 1 and reduces it on site 2 and 3.** ChIP assay of KATO III cells incubated with 5-AZA (10 μM) for 72 hours and then stimulated with IFN-γ (10 ng/mL) for 24 h. The binding capacity of C/EBPβ protein to the indicated TFF1 promoter binding sites was analyzed by RT-q-PCR ( t-test; *p≤ 0.05).

*2.2.3.7 Helicobacter infection increases TFF1 promoter methylation in gastric antrum tissues of mice.*

To further investigate the implication of promoter methylation in the silencing of TFF1 during chronic *H. pylori* infection, we measured the methylation status of the TFF1 promoter in the gastric antrum of mice by bisulfite sequencing to characterize the critical CpGs. C57BL/6 mice were infected with *H. felis* (10^7 total bacteria per mouse in 100 μL every other day for three days) for 6 weeks. This model represents a more suitable system compared to *H. pylori* mouse-adapted strain as it develops gastric metaplasia, dysplasia, and invasive cancer recapitulating *H. pylori* human infection

(Hayakawa *et al.*, 2013). Infected mice were sacrificed 6 weeks post-infection, which can be considered a time-point of chronic infection. *Naïve* mice received only the vehicle. The stomach was isolated, and the antrum was used for DNA analysis. Treated mice showed a gradual reduction of TFF1 expression during the infection (Esposito *et al.*, 2017). Genomic DNA was purified from tissues, bisulfite-converted, and amplified by PCR using sequence-specific primers to CpG-rich region of the TFF1 promoter (from -215 to +21). Finally, PCR products were cloned into a pGEM-T easy vector and sequenced by Sanger analysis.

Our results revealed that infected mice present a higher % of methylated CpGs in the TFF1 promoter; in particular, the ones at - 56 and -14 sites were fully methylated compared to control mice (*naïve*) (Figure 2.22). It has been reported that the methylation of only a few CpGs is related to TFF1 gene expression (Martin *et al.*, 1995). Indeed, it has been observed that both in cell lines and gastric biopsies, the TFF1 expression is closely correlated with the methylation of CpGs present in a minimal region responsible for the regulation (between -85 and +20). The CpGs found methylated in gastric antrum cells of mice infected with *H. felis* are included in this region, suggesting therefore that *Helicobacter* chronic infection promotes TFF1 silencing by methylating its promoter.

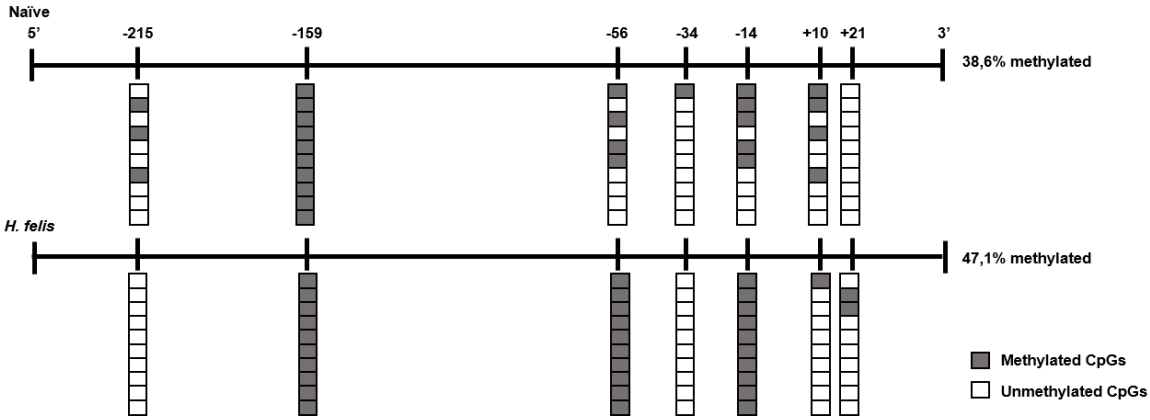


**Figure 2.22**. ***H. felis*** **infection increases the CpGs methylation in the TFF1 promoter.** Comparison of TFF1 promoter bisulfite-sequenced from murine gastric antrum tissues infected or not with *H. felis* for 6 weeks. Experiments were performed in replicates (n=10).

# CHAPTER 3

# CONCLUSIONS

*Helicobacter pylori* colonizes the gastric mucosa in over 50% of humans worldwide, causing acute gastritis. Despite triggering vigorous host immune response, *H. pylori* clearance might be ineffective leaving the pathogen in the stomach throughout the individual's lifetime and favoring the development of chronic inflammation. A long-standing infection can further lead to gastric lesions that can silently progress to gastric cancer in a subgroup of patients. As a defense strategy, the gastric mucosa releases protective factors, among which TFF1, along with mucin 5AC, plays a crucial role by interacting and retaining *H. pylori* in the mucus layer, thus preventing its migration towards the gastric epithelium. Previous *in vivo* studies from our laboratory revealed that TFF1 expression is stimulated during the acute phase but gradually silenced during chronic *Helicobacter* infection. Moreover, TFF1 expression has been observed to get lost in about 40–60% of gastric cancer by extensive promoter methylation. Initially, my Ph.D. project was focused on determining the effects of *H. pylori*-TFF1 interaction at the molecular and morphological levels. By means of light microscopy and scanning electron microscopy (SEM), we observed that TFF1 induces *H. pylori* aggregation in a concentration-dependent manner and bacterial morphological change without affecting cell viability.

Furthermore, following the hypothesis that TFF1 could affect bacterial movements, we investigated the potential chemotactic effect of the protein on the bacteria. To

answer this question, we set up a motility assay through a mucus layer and analyzed the amount of bacteria passing through the mucus and bacterial gene expression by Real-Time PCR. Our results showed that TFF1 reduces bacterial motility in the mucus, slowing its migration toward the epithelial layer and influencing the expression of *H. pylori* motility genes. These data confirm the protective role of TFF1 during *H. pylori* infection, which help gastric cells to counteract bacteria colonization and prevent the development of chronic inflammation.

As a second aim, we explored the molecular pathways leading to TFF1 silencing during chronic *Helicobacter* infection. Since the immune system plays a crucial role in *H. pylori* infection, we investigated the effect of the main proinflammatory cytokines released by activated immune cells during *H. pylori* chronic infection, on human TFF1 expression in tumor gastric cell lines. RTq-PCR, Western blot, and luciferase assays identified IFN-γ as the cytokine mainly responsible for TFF1 downregulation. To deeply investigate the silencing mechanisms, I explored the potential role of C/EBPβ, a transcription factor reported as negative regulator of TFF1. RT-qPCR, Western blot, nuclear and cytosolic fractionation, and siRNA assays indicated that IFNγ induces the expression of C/EBPβ, which is accumulated in the nucleus. ChIP-qPCR assays revealed that under IFNγ stimulation C/EBPβ binds to three different sites on the TFF1 promoter, inhibiting its transcription. Since TFF1 expression is strongly influenced by the methylation status of its promoter, the possible involvement of methylation as an epigenetic mechanism has been investigated as an additional mechanism for IFN-γ-induced TFF1 silencing. By multiple means, the use of methylation inhibitor (5-AZA) in KATO III cells and the bisulfite sequencing performed on gastric antrum DNA from *H. felis*-infected mice, methylation resulted to be one of the mechanisms involved in regulating TFF1 transcription. However, these

data require to be explored in a more suitable and physiological system, such as primary human gastric cells.

In conclusion, this work lays the foundations for understanding the intracellular mechanisms activated by IFN-γ released during *H. pylori* chronic infection, that leads to reduction of TFF1 expression. Given the key role of this cytokine in gastric inflammation, supported by previous evidence reporting that infected IFN-γ knockout mice (IFN-$\gamma^{-/-}$) develop gastritis with less extent compared to naïve mice (Sawai *et al*., 1999; Smythies *et al*., 2000; Akhiani *et al*., 2002) and given the significant reduction of TFF1 in gastric cells exposed to IFN-γ, it is clear that the immune response to bacterial infection is crucial for determining the fate of the cell.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 Cell cultures and culture condition

HT29-E12, a mucus-secreting subclone of the human colorectal adenocarcinoma cell line HT29-MTX (generous gift from Professor Per Artursson, Uppsala University, Sweden) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Euroclone), supplemented with 10% (v/v) fetal bovine serum (FBS, Euroclone), 100 U/mL penicillin and 100 μg/mL streptomycin (Euroclone). KATO III cells (gastric carcinoma, derived from a metastatic site, poorly differentiated) were maintained in RPMI 1640 (Euroclone, #ECM2001L, Italy), supplemented with 20% (v/v) fetal bovine serum (FBS, Euroclone, #ECS0180D, South America, origin EU approved) and Penicillin- Streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin) (Euroclone, #ECB3001D, Italy). All cell lines were tested free of *Mycoplasma* and grown at 37°C with 5% $CO_2$ in a humidified incubator.

## 4.2 Bacterial strains and culture condition

*H. pylori* P12 strain from a German patient with a duodenal ulcer was kindly provided by Dr. Marguerite Clyne (University College Dublin) and cultured on selective Columbia agar (Oxoid, Basingstoke, Hampshire, UK) containing 7% (v/v) defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) supplemented with

antibiotic mix (DENT, Oxoid, Basingstoke, Hampshire, UK). Bacteria plates were incubated for 3-4 days in a capnophilic atmosphere with 10% $CO_2$. Once confluent on the plate, bacteria were scraped using brain heart infusion (BHI, Oxoid, Basingstoke, Hampshire, UK) with 10% FBS and measured at an optical density at 600 nm ($OD_{600}$) considering 1 $OD_{600}$ = 1 x $10^8$ bacteria/mL.

### 4.3 Human recombinant TFF1 production and purification

E. coli BLR (DE3) pLysS containing the hrTFF1-pIVEX vector, encoding the human recombinant TFF1, were inoculated in LB liquid medium supplemented with 100 μg/mL of Ampicillin (Amp) and incubated for 16 h at 37 °C under shaking. After 24 h, 20 mL of bacterial culture was diluted in 1 L of LB liquid medium supplemented with 100 μg/mL of Amp and incubated at 37 °C under shaking. When the culture reached an OD600 of ≈0.8, protein expression was induced by adding 1 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG), and growth was allowed for 2 more hours. After IPTG-induction, bacteria were harvested by centrifugation at 4000 g for 20 min at 4 °C and washed twice with 30 mL of PBS. The bacteria were resuspended in buffer A (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, and 30 mM imidazole) containing a protease inhibitor and sonicated for 40 min (1 min on/1 min off, at 24% of amplitude) on ice. The lysate was clarified by centrifugation at 10000 g for 30 min at 4°C, and filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). The his-tagged hrTFF1 protein was initially purified by affinity chromatography on 1-mL HisTrap HP column (Amersham Biosciences, Uppsala, Sweden) using an AKTA Purifier chromatographic system (Amersham Biosciences, Uppsala, Sweden). The eluted fractions corresponding to the chromatographic peaks were analyzed by SDS-PAGE on 18% acrylamide gel, stained with Coomassie blue, and those containing the his-tagged protein were pooled and subsequently digested with the Factor Xa

(Promega, Madison, WI, USA) for 30 h at 25 °C to remove the his-tag. After digestion, the protein was loaded again on the His-trap HP column to separate it from the cleaved tag.

### 4.4 Bacterial aggregation assay

*H. pylori* (7,5×.$10^6$/mL) was incubated w/wo hrTFF1 (0.6 and 6 μg/mL) for 6 h in a 12-well plate. The recombinant protein is mainly dimeric (≈90%) and was used at 6 μg/mL, roughly the supposed concentration within the gastric mucus, and 0.6 μg/mL (a ten-fold dilution). The experiment was repeated three times in technical duplicates. At 6h post-incubation, six pictures per condition were taken by AME-3206 Digital inverted Microscope (AMG/EVOS, Mill Creek, WA, USA), at 20× magnification. *H. pylori* aggregates were quantified by ImageJ 1.49 software by using the following method—convert to 8-bit image, apply a moments threshold (0 to 100 boundaries), and analyze clumps with a size between 100 to infinity and a circularity between 0.10–1.00.

### 4.5 Scanning Electron Microscopy Analysis

*H. pylori* was incubated in BHI broth supplemented with 10% FBS, at 0.1 $OD_{600}$. After 24 h, $1 \times 10^6$ bacteria were put on coverslips pre-treated with 0.01% (v/v) poly-l-lysine (Sigma–Aldrich, Saint Louis, MO, USA) in 24-well plates and incubated with or without hrTFF1 6 μg/mL. After 16 h, the medium was removed and bacteria were fixed by 25% glutaraldehyde (v/v) for 16 h at room temperature. Samples were then washed and dehydrated in graded alcohol of 25%, 50%, 75%, 95% for 5 min and three times with 100% ethanol for 10 min. Finally, each coverslip was mounted on the SEM stage and analyzed. Samples were sputtered with gold using a LEICA EMSCD005 metallizator producing a deposition of a 100–200 Å thick gold layer. SEM images of each sample were then acquired using a Tescan Solaris UHR microscope equipped

with secondary electron and backscattered electron detectors (TESCAN, Brno, Czech Republic). Analyses were performed at 5 keV.

### 4.6 Growth curve of *Helicobacter pylori*

*H. pylori* suspension of $1.25 \times 10^7$/mL was incubated w/wo hrTFF1 (0.6 and 6 μg/mL) or with an equal volume of vehicle (PBS), in a 24-well plate. At 6, 24, 30, 48, 54, 70, and 76 h bacterial growth was monitored by measuring optical density at 600 nm ($OD_{600}$) (Multiskan spectrum, Thermo, Vantaa, Finland). The experiment was repeated three independent times in technical duplicates. The results are expressed as means ± SD. Statistical differences between the groups were evaluated by Two-way ANOVA. Statistical analyses were done using PRISM4 software (GraphPad Software, La Jolla, CA, USA). A $p<0.05$ was considered statistically significant.

### 4.7 Analysis of TFF1 chemotactic properties

Chemotaxis assays were performed as described by Mazumder (Mazumder *et al.*, 1999) and revised by Cerda *et al*. (Cerda *et al.*, 2011). Briefly, bacteria were inoculated in BHI broth supplemented with 10% FBS, starting from 0.8 OD600. After 24 h, bacteria were diluted in chemotaxis buffer (10 mM potassium phosphate, pH 7.0; 3.0% polyvinylpyrrolidone) up to a concentration of $4 \times 10^7$ bacteria/mL (0.4 $OD_{600}$) and 100 μL of bacterial suspension was drawn into a disposable 200 μL pipette tip(yellow). A 100 μL volume of solution to be tested (10 mM HCl, hrTFF1 6 μg/mL, hrTFF1 0.6 μg/mL diluted in chemotaxis buffer) was drawn up into a 1-mL tuberculin syringe and a 23 G × 1¼ stainless-steel needle (0.6 × 30 mm) was used as the chemotaxis capillary. The chemotaxis buffer alone was included as a control. The needle-syringe system was positioned into the pipette tip and incubated horizontally at 37 °C under microaerophilic conditions for 45 min. At the end of incubation, bacteria recovered from the syringe were appropriately diluted and plated onto Columbia Blood Agar

(Oxoid, Basingstoke, Hampshire, UK) plates supplemented with 10% FBS (Euroclone) and DENT (Oxoid, Basingstoke, Hampshire, UK). Colonies were counted after 4–5 days of incubation.

### 4.8 Analysis of the effect of TFF1- induced aggregation on *H. pylori* motility behavior

The experiment was performed as the above paragraph, with a different concentration of starting bacteria that here was of $1 \times 10^7$ bacteria/mL ($OD_{600} = 0.1$) and preincubated for 1.5 h w/wo hrTFF1 (6 μg/mL), in order to favor the formation of aggregates. Bacterial suspension diluted in water was used as a control. Proper dilutions of the syringe content were plated for CFU (colony forming unit) counting. The experiment was repeated three independent times in technical duplicates.

### 4.9 Motility Assay: Transwell Migration Assay

HT29-E12, mucus-secreting cells, were used as mucus producers. Once they reached confluence, day 1 started and after 20 days of culturing, replacing the medium every two days, the antibiotic-containing medium was replaced with an antibiotic-free medium and the mucus was collected every 2 days, avoiding mechanical stress. Once we obtained enough mucus, 100 μL of slurry (1:1, mucus: BHI broth) was carefully set down on the filter of a transwell system (Falcon® 353097; pore size 8 μm, Merck, Millipore). The lower chamber of the transwell was filled with 500 μL of BHI supplemented with 10% FBS.

Bacteria were grown in BHI liquid broth supplemented with 10% FBS, starting from 0.1 OD. After 24 h, 100 μL of $1 \times 10^6$ bacteria/mL was preincubated for 1.5 h with or without hrTFF1 (6 μg/mL), in order to favor the formation of aggregates and then layered on the previously prepared mucus. The system was incubated at 37 °C

under microaerophilic conditions and after 24 h 50 µL of the lower chamber medium was plated for CFU counting.

### 4.10 Analysis of TFF1 influence on transcriptional regulation of *H. pylori* motility gene.

HT29-E12 cells were cultured for 20 days before enrolling in this experiment. 24 h prior to infection, the antibiotic-containing medium was replaced with an antibiotic-free medium, and cells were infected with *H. pylori* with MOI 1:30 ($7.5 \times 10^8$ bacteria/well) in a final volume of 500 µL RPMI +10% FBS without antibiotics. Before adding bacteria to the cells, *H. pylori* was pre-incubated without or with 6 µg/mL hTFF1 for about 2 h. Bacterial RNA was extracted and the Real-Time PCR was performed.

### 4.11 Cell treatments

For cytokine stimulation experiments, KATO III cells were seeded into a 6-well plate ($6x10^5$ cells/well) or 12-well plate ($3x10^5$ cells/well) and allowed to grow for 24 h. When 70-80% confluence was reached, cells were stimulated with IFNγ (Invitogen, #BMS303, Austria) (10 ng/mL), TNFα (Invitogen, #BMS301, Austria) (40 ng/mL) and IL-1β (Invitogen, #RIL1BI, USA) (40 ng/mL) for 24 h alone or together as triple treatment.

For 5-AZA and IFNγ treatment, KATO III cells were seeded in 100 mm plates ($3x10^6$ cells/well). After 24 h, the cells were treated with 5-AZA (10 µM), also known as Decitabine, which is an analog of deoxycytidine typically used to inhibit DNMT.

Every 24 h, up to 72 h, the medium supplemented with 5-AZA has been refreshed to maintain the concentration of 5-AZA constant, since a degradation over time is described. After 48 h of 5-AZA treatment, the cells received a medium containing IFN-γ (10 ng/ml) for 24h.

### 4.12 RNA extraction and qRT-PCR.

Bacterial and human RNA was extracted using TRIzol reagent (Invitrogen, #15596018, New Zealand) following the manufacturer's instructions and reverse transcription of 1 µg di RNA was performed to obtain cDNA by using M-MLV Reverse Transcriptase (GeneSpin S.r.l, Milan, Italy).

The Real-time PCR was performed using the Light Cycler 480 II instrument (Roche, Basel, Switzerland). Suitable dilutions of cDNA were used for each gene in a 12 µL reaction using Luna Universal qPCR Master Mix (New Englands BioLabs, USA). Results from 3 independent experiments, in technical duplicates, were analyzed using the Delta-Delta CT method. 16S was used as the bacterial reference gene and HPRT1 as a human reference gene. Primer sequences are reported in Table 4.1 (*H. pylori)* and Table 4.2 (Human)

**Table 4.1** *H. pylori primers used for Real-Time PCR analyses*

| Gene | | Sequence |
|---|---|---|
| *flaA* | Fw | 5'-CGGGCAAGCGTTATTGTCTG-3' |
| | Rv | 5'-GCGATACGAACCTGACCGAT-3' |
| *flaB* | Fw | 5'-ATTCGCAACGCTAATGACGC-3' |
| | Rv | 5'-GGCTTGAACGGCTTTGGTTT-3' |
| *flgE* | Fw | 5'-GCAGAAAACAGCACGCTTGA-3' |
| | Rv | 5'-ATCGCATTTTTCGCCGCTAC-3' |
| *virB11* | Fw | 5'-TTAGGCGAAATTGACACGCA-3' |
| | Rv | 5'-ATCATTCCGCTATGCCCAGT-3' |
| *alpA* | Fw | 5'-ACTACGGCACGAACACCAAT-3' |
| | Rv | 5'-GACCATCTGAACCAGTCGCA-3' |
| *alpB* | Fw | 5'-CAATAACCAAGCGGGTGGGA-3' |
| | Rv | 5'-TAAAGCGGCGTCCAAAAACG-3' |

| | | |
|---|---|---|
| *hopZ* | Fw | 5’-TGGGGCTGTGGAATGTCATC-3’ |
| | Rv | 5’-ATACTCGTGGAATGCGACCC-3’ |
| *ureA* | Fw | 5’-AGACATCACTATCAACGAAGGC-3’ |
| | Rv | 5’-TTTCTTCGCCAGGCTCAAAC-3’ |
| *vacA* | Fw | 5’-TGGATAGTGCGACTGGGTTT3’ |
| | Rv | 5’-GGCGCTCTTTGAATTGCTCT-3’ |
| *cag1* | Fw | 5’-CGGTGCTATGGGGATTGTTG-3’ |
| | Rv | 5’-GCTTCAGTTGGTTCGTTGGTAA-3’ |
| *cagA* | Fw | 5’-AGAGCCTACTGGTGGGGATT-3’ |
| | Rv | 5’-AGCCTTGTATGTCGGTGGTG-3’ |
| *cag25* | Fw | 5’-CAAGAATCACTGACAGCTACAAGAA-3’ |
| | Rv | 5’-TTTGACCCCTAAAGCGCAAC-3’ |
| *16S* | Fw | 5’-ACGCATAGGTCATGTGCCTC-3’ |
| | Rv | 5’-GTGTCCGTTCACCCTCTCAG-3’ |

**Table 4.1** *Human primers used for Real-Time PCR analyses*

| **Gene** | | **Sequence** |
|---|---|---|
| HPRT1 | Fw | 5’-GACCAGTCAACAGGGGACAT-3’ |
| | Rv | 5’-CCTGACCAAGGAAAGCAAAG-3’ |
| TFF1 | Fw | 5’-CCCAGTGTGCAAATAAGGGC-3’ |
| | Rv | 5’-TGGAGGGACGTCGATGGTAT-3’ |
| C/EBPβ | Fw | 5’-CAAGCACAGCGACGAGTACA-3’ |
| | Rv | 5’-AGCTGCTTGAACAAGTTCCG-3’ |
| TFF1 (C/EBPβ binding Site 1) | Fw | 5’-GGATTAAGGTCAGGTTGGAGGA-3’ |
| | Rv | 5’-ACGACATGTGGTGAGGTCAT-3’ |
| TFF1 (C/EBPβ binding Site 2) | Fw | 5’-GTGTTGGGATTACAGGCGTG-3’ |
| | Rv | 5’-AGTGAGAGATGGCCGGAAAA-3’ |
| TFF1 (C/EBPβ binding Site 3) | Fw | 5’-TGATTCTCCTGACTTAACCTCC-3’ |
| | Rv | 5’-TCACGCCTGTAATCCCAAC-3’ |

| | | |
|---|---|---|
| *IFNγ* | Fw | 5'-TCGGTAACTGACTTGAATGTCCA-3' |
| | Rv | 5'-TCGCTTCCCTCTTTTAGCTGC-3' |
| *TNFα* | Fw | 5'-CTGCTGCACTTTGGAGTGAT-3' |
| | Rv | 5'-AGATGATCTGACTGCCTGGG-3' |
| *IL1β* | Fw | 5'-GAAGCTGATGGCCCTAAACA-3' |
| | Rv | 5'-AAGCCCTTGCTGTAGTGGTG-3' |
| *RPL30* | | SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling, #9003, USA). Sequences not shown |
| *COX-2* | Fw | 5'-AGCTTCCTGGGTTTCCGATT-3' |
| | Rv | 5'-GCCCATGTGACGAAATGACTG-3' |

### 4.13 Western blot analysis

To obtain total proteins extracts, cells were resuspended with 1X gel-loading buffer (50 mM Tris-Cl pH 6.8; 2% w/v SDS; 0.1% bromophenol blue; 10% (v/v) glycerol and 100 mM β-Mercaptoethanol), and sonicated (1 minute, 10 sec pulse on, 10 sec pulse off, amplitude 30%, Vibra-Cell Sonics). Protein samples were then incubated at 100 °C for 5 minutes, centrifuged at 10,000g for 30 seconds, loaded onto a polyacrylamide gel for electrophoretic separation and then transferred AmershamTM ProtranTM Premium 0,45 μm NC (GE Healthcare Life Sciences, #GE10600003, Germany) nitrocellulose membrane. Ponceau red staining for 10 min was used to evaluate the transfer efficiency (0.1% solution in 1% v/v acetic acid). After blocking with 5% w/v of non-fat dry milk (BioRad) for 1 h, the membranes were incubated overnight with primary antibodies at 4°C. After washing three times with TBST (Tris-Buffered Saline with 0,05% Tween 20) for 10 min each, the membranes were incubated with secondary antibodies (Donkey anti-Rabbit (Jackson) e Donkey anti-Mouse (Jackson) respectively) for 1 h at room temperature, washed three times, and then visualized by LAS 4000 (GE Healthcare, Life Sciences) digital imaging system.

The band densities were quantified by ImageJ (National Institutes of Health, USA). The antibodies are shown in table 4.3.

**Table 4.3** *Antibodies used for western blot analyses*

| Antibody | |
|---|---|
| β-actin (C4) | Santa Cruz sc-47778 |
| C/EBPβ rabbit Ab | Cell Signaling 90081S |
| C/EBPβ antibody | Genetex GTX100675 |
| GSpAb-HTFF1 | GenScript Corp. |
| LS-BIO ANTI-TFF1 | LS-155659 |
| GAPDH (6C5) | Santa Cruz sc-32233 |
| Anti-Rabbit Antibody HRP-linked IgG (H&L) | Cell Signaling #7074 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling 7076S |

### 4.14 Cell Transfection

For the reporter-gene assays, KATO III cells (1,5 x $10^5$ cells/well) were seeded in a 24-well plate and after 24h were transfected using Lipofectamine 2000 reagent (Invitrogen, #11668-027, USA) following the manufacturer's instruction. Different pGL3 plasmids (Promega, #E1751, USA) (pGL3-1kb-Luc, pGL3-0.8kb-Luc, pGL3-0.5kb-Luc, pGL3-0.3kb-Luc, pGL3-0.2kb-Luc) constructed previously in our lab containing sequential deletions of the TFF1 promoter sequence (from -931 bp, -830 bp, -583 bp, -306 bp and -212 bp) upstream of a Luciferase reporter gene, were used for these experiments. Cells were co-transfected with 1 μg of each plasmid and 0.1 μg of a plasmid containing the β-galactosidase gene, whose expression was used as a normalization parameter of transfection efficiency.

For Small-interfering RNA knockdown experiments, KATO III cells were seeded into a 12-well plate at a density of $1 \times 10^5$ cells/well and transfected with C/EBP-β siRNA (60 nM) (Santa Cruz Biotechnology, #sc-29229, USA) or control siRNA (60 nM) (Santa Cruz Biotechnology, #sc-37007, USA) according to the manufacturer using Lipofectamine™ 2000.

### 4.15 Dual-Luciferase Reporter Assay

The luciferase activity of transfected cells was measured using the Luciferase/β-Galactosidase Luciferase Assay Kit, 96 Dual-Light (Applied Biosystems). The relative transcriptional activity was calculated as Firefly-Luc/β-galactosidase. The analysis was performed in quadruplicate and the results are reported as the ratio of firefly luciferase activity and β-galactosidase (relative transcriptional activity). The light emission was measured with EnSpire Alpha Multimode Plate Reader (PerkinElmer).

### 4.16 Chromatin Immunoprecipitation

ChIP assays were performed using a SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling, #9003, USA), according to the manufacturer's protocol. Briefly, KATO III cells ($2 \times 10^5$ cells/well) were treated with 1 % paraformaldehyde for protein-DNA crosslinking and then with glycine (Cell Signaling, #7005S, USA) for neutralization. Samples were treated for nuclei extraction and DNA shearing with Micrococcal Nuclease (Cell Signaling, #10011, USA) for 20 min at 37°C to obtain DNA fragments of approximately 150-900 bp. Nuclei were sonicated to break the nuclear membrane and chromatin was immunoprecipitated with anti-C/EBPβ antibody (Cell Signaling, #90081S, USA), or control IgG overnight at 4 °C, and then incubated for 2 h with ChIP-Grade Protein G Magnetic Beads (Cell Signaling, #9006, USA). DNA was eluted, de-crosslinked for 2 h at 65 °C and purified.

The binding of C/EBPβ to the TFF1 promoter was assessed by PCR using the primers reported in Supplementary Table 4.1.

### 4.17 Nuclear and Cytoplasmic Fractionation

KATO III cells (6 x $10^5$ cells/well in a 6 well plate) were collected and washed twice in ice-cold PBS. The cell pellet was resuspended in 100 µL of buffer A (10 mmol/L Hepes pH 7.9, 60 mmol/L KCl, 1 mmol/L EDTA pH 8, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, 0,2% NP40, and protease inhibitors), incubated on ice for 30 minutes. The cell suspension was forced through a tuberculine syringe-needle and nuclei were pelleted by centrifugation at 660 × g for 5 minutes at 4°C. The supernatant was collected as the cytoplasmic fraction, resuspended in 50 µL of buffer C (250 mmol/L Tris-HCl, pH 7.8, 60 mmol/L KCl, 1 mmol/L dithiothreitol, 2 mmol/L PMSF, 20% glycerol) and centrifugated at 9500 × g for 15 minutes at 4°C. The supernatant corresponds to the cytoplasmatic fraction.

The nuclei pellet was washed three times in 300 µl of Buffer B (10 mmol/L Hepes pH 7.9, 60 mmol/L KCl, 1 mmol/L EDTA pH 8, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, and protease inhibitors), then resuspended in 50 µL of buffer C (250 mmol/L Tris-HCl, pH 7.8, 60 mmol/L KCl, 1 mmol/L dithiothreitol, 2 mmol/L PMSF, 20% glycerol) and incubated in dry ice. The suspension was centrifuged at 9.500g for 15 min at 4°C. The supernatant was collected as the nuclear fraction. The amount of β-actin and CEBPβ in the fractionated nuclear and cytoplasmic cell extracts were analyzed by western blot.

### 4.18 Analysis of the TFF1 promoter methylation status: bisulfite conversion and bisulfite sequencing.

Genomic DNA was purified from mouse tissues cells using NucleoSpin® Tissue kit (Macherey Nagel) and quantized with NanoDrop 1000 (Thermo Scientific). 0.5 µg

of DNA were converted with EZ DNA Methylation™ kit (Zymo Research, Irvine, CA, USA), a method that allows to discriminate unmethylated cytosines from methylated. Converted DNA was amplified by PCR using sequence-specific primers to CpG-rich region of TFF1 promoter (from -215 to +21). Finally, PCR products were cloned into pGEM-T easy vector and sequenced by Sanger analysis. FASTA data were analyzed by BISMA software. The primers are shown in table 4.4.

Table 4.4 TFF1 promoter primers used for Real-Time PCR analyses

| Gene | | Sequence |
|---|---|---|
| ***mTFF1*** | Fw | 5'-GATTTTTTAGTTAAGATGATTTTATTATATG-3' |
| | Rv | 5'-ATTTTATAAAACAAACTCTATTTACTTAAAA-3' |

### 4.19 Statistical Analysis

The experiments were repeated three independent times in technical duplicates. The results are expressed as means ± SD. Data were statistically analyzed using an unpaired Student t-test. Statistical analyses and graphing were done using PRISM4 software (GraphPad Software, La Jolla, CA, USA). A p-value $< 0.05$ was considered statistically significant.

# CHAPTER 5

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