



**HAL**  
open science

# Study of the Hippo/YAP1 signaling pathway in gastric carcinogenesis induced by *Helicobacter pylori*

Silvia Molina-Castro

► **To cite this version:**

Silvia Molina-Castro. Study of the Hippo/YAP1 signaling pathway in gastric carcinogenesis induced by *Helicobacter pylori*. Cancer. Université de Bordeaux, 2017. English. NNT : 2017BORD0623 . tel-01956587

**HAL Id: tel-01956587**

**<https://theses.hal.science/tel-01956587>**

Submitted on 16 Dec 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THÈSE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE

**DOCTEUR DE  
L'UNIVERSITÉ DE BORDEAUX**

École Doctorale Sciences de la Vie et de la Santé  
Spécialité Microbiologie Immunologie

Par Silvia MOLINA-CASTRO

**Study of the Hippo/YAP1 signaling pathway in gastric carcinogenesis  
induced by *Helicobacter pylori***

**Etude de la voie de signalisation Hippo/YAP1 dans la carcinogénèse  
gastrique induite par l'infection à *Helicobacter pylori***

Sous la direction de : Dr. Christine VARON

Soutenue le 30 juin 2017

Membres du jury :

M. MOENNER, Michel	PU	Université de Bordeaux	Président
Mme. TOUATI, Eliette	CR	Institut Pasteur	Rapporteur
M. KUPCINSKAS, Juozas	PU-PH	Lithuanian University of Health Sciences	Rapporteur
M. MÉGRAUD, Francis	PU-PH	Université de Bordeaux	Invité
Mme. RAMIREZ, Vanessa	PU	Universidad de Costa Rica	Invité



# **Titre : Etude de la voie de signalisation HIPPO/YAP dans la carcinogénèse gastrique induite par l'infection à *Helicobacter pylori***

## **Résumé :**

Le cancer gastrique (CG) est la 5<sup>ème</sup> cause de cancer et la 3<sup>ème</sup> cause de mortalité par cancer dans le monde. Le diagnostic est tardif et les options thérapeutiques sont limitées. Le CG est une maladie multifactorielle, fréquemment associée à l'infection chronique par des souches CagA<sup>+</sup> d'*Helicobacter pylori*. La transition épithélio-mésenchymateuse (EMT) est un processus réversible dans lequel une cellule épithéliale polarisée acquiert un phénotype mésenchymateux. L'EMT est à l'émergence de cellules souches cancéreuses (CSC), une minorité de cellules capables de division asymétrique et d'autorenouveaulement, à l'origine des cellules différenciées dans la masse tumorale et capables de former des nouvelles tumeurs. Dans le CG, ces CSC expriment CD44 et présentent une activité ALDH élevée. L'infection des cellules épithéliales gastriques humaines (CEGs) par CagA<sup>+</sup> *H. pylori* induit des cellules CD44<sup>high</sup> avec des propriétés des CSCs via une EMT. La voie Hippo est composée par les kinases suppresseur de tumeurs MST et LATS, et leurs cibles de phosphorylation, les oncoprotéines YAP1 et TAZ. Suite à la phosphorylation inhibitrice, YAP1 et TAZ sont retenus aux jonctions cellulaires ou sont dégradés au protéasome. Au noyau, YAP1 et TAZ activés lient les facteurs TEAD pour promouvoir la croissance cellulaire et l'inhibition de l'apoptose.

Notre premier objectif était de rechercher si *H. pylori* change l'état d'activation de la voie Hippo et comment cette régulation modifie l'EMT et la population de cellules avec des propriétés de CSC *in vitro* et *in vivo*. Le deuxième but est la caractérisation du rôle de YAP1/TEAD dans les propriétés de CSCs gastriques *in vitro* et les conséquences de son inhibition pharmacologique dans la croissance des tumeurs *in vivo* pour le développement des thérapies co-adjuvantes pour le CG.

Pour étudier la régulation de la voie Hippo pendant l'infection par *H. pylori*, LATS2, YAP1 et CD44 ont été évalués dans la muqueuse gastrique de sujets non-infectés et infectés par *H. pylori*, qui ont été augmentés avec l'infection et leur surexpression a été associée avec la gastrite et la métaplasie intestinale. Dans les CEGs l'expression de gènes de la voie Hippo a été altérée par l'infection. La régulation de la voie Hippo par *H. pylori* a une cinétique diphasique et dépendante de CagA. Dans l'infection précoce, *H. pylori* déclenche l'activité transcriptionnelle de YAP1. Cette période d'inactivité de la voie Hippo est suivi de son activation progressive, soutenue par l'accumulation de LATS2 et la phosphorylation inhibitrice de YAP1. La répression de LATS2 avec siRNAs a accéléré l'acquisition du phénotype mésenchymateux après l'infection, l'augmentation de marqueurs de l'EMT (Zeb1 et Snail1), et la diminution des miR-200 épithéliaux. Le CD44 et les capacités d'invasion et formation de sphères induites par *H. pylori* ont été potentialisées par l'inhibition de LATS2, ce qui suggère que LATS2 limite l'EMT et le phénotype de

CSC acquis pendant l'infection. L'inhibition de LATS2 ou YAP1 diminue l'expression de ces deux protéines, révélant ainsi une boucle de régulation positive. Dans des coupes de tissu de CG, l'expression de LATS2 et YAP1 est hétérogène et positivement corrélée, fait qui a été confirmé dans 38 CEGs de la CCLE. L'expression LATS2 est fortement corrélée à celle de CTGF et CYR61, ce qui suggère que LATS2 peut aussi être un gène cible de YAP1/TEAD.

La deuxième partie de l'étude correspondait à l'évaluation des propriétés anti-tumorales de la vertéporfine (VP), qui est capable d'interrompre l'interaction YAP1/TEAD, et donc d'inhiber son activité transcriptionnelle. *In vitro*, utilisant CEGs et des cellules de tumeurs de patients amplifiées chez la souris (patient-derived xenograft PDX), le traitement à la VP a diminué la croissance cellulaire, l'expression de gènes cible de YAP1/TAZ/TEAD, l'activité du rapporteur TEAD-luciférase et la capacité de formation de sphères. L'activité de la VP a été testée *in vivo* dans un modèle de greffe sous-cutané des CEGs MKN45 et MKN74 et le PDX GC10 chez la souris NSG. Les tumeurs développées ont été traitées par injection péri-tumorale avec trois doses de VP ou du DMSO comme contrôle. La croissance tumorale a été diminuée à partir du jour 5-12 pour la dose intermédiaire. Le poids des tumeurs, l'analyse par IHC (CD44, ALDH, Ki67) et la capacité de formation de sphères des CSCs résiduelles ont été diminuées dans les tumeurs traitées. Ces résultats montrent une activité inhibitrice de la VP sur les CSCs gastriques *in vitro* et *in vivo*.

Ce travail montre pour la première fois que l'axe LATS2/YAP1/TEAD est précocement activé pendant l'infection chronique avec *H. pylori* et que celui-ci contrôle l'EMT et les propriétés de CSC. Le ciblage de la voie Hippo a été montré comme étant efficace dans la prévention de la croissance tumorale, mettant en évidence le potentiel de son inhibition dans le traitement du cancer gastrique.

**Mots clés :** Cellules souches gastriques, transition épithélio-mésenchymateuse, CD44

# Title: Study of the Hippo/YAP1/TEAD signaling pathway in gastric carcinogenesis induced by *Helicobacter pylori*

## Abstract:

Gastric cancer (GC) is the 5<sup>th</sup> most common cancer overall and the 3<sup>rd</sup> cause of cancer death worldwide. Diagnosis is often late and therapeutic options are limited. It is a multifactorial disease, most frequently associated to chronic infection with CagA-positive *Helicobacter pylori* strains. Epithelial-to-mesenchymal transition (EMT) is reversible process in which polarized epithelial cells acquire a mesenchymal phenotype. EMT is at the origin of cancer stem cells (CSC), a few stem-like cells within the tumors with asymmetrical division and self-renewal capacities. They give rise to the differentiated tumor cells and originate new tumors. In GC, CSCs express the hyaluronate receptor CD44 and high aldehyde-dehydrogenase (ALDH) activity. Infection with *H. pylori* of human gastric cancer cell lines (hGECs) *in vitro* induces the emergence of a population of CD44<sup>high</sup> cells with CSC-properties through an EMT process in a CagA-dependent manner. The Hippo pathway is composed by a tumor suppressor kinases MST and LATS, and their phosphorylation targets, the oncogenes YAP1 and TAZ. Upon phosphorylation by LATS, YAP1 and TAZ are retained in cell-cell junctions or undergo proteasomal degradation. Active nuclear YAP1 and TAZ bind to TEAD transcription factors to promote the expression of genes that positively regulate cell growth and inhibit apoptosis.

The first aim of this work was to investigate whether *H. pylori* affects the activation state of the Hippo pathway, and its effect on the EMT process and the CSC-like cell population *in vitro* and *in vivo*. Second, we intended to characterize the role of YAP1/TEAD in gastric CSC properties *in vitro* and the consequences of its pharmacological inhibition on tumor growth *in vivo*, aiming to the development of adjuvant therapies to GC.

To study the Hippo pathway regulation during infection, LATS2, YAP1 and CD44 were evaluated in gastric mucosae of either non-infected or *H. pylori*-infected patients. They were upregulated in infected mucosae and were associated to gastritis and intestinal metaplasia. In hGECs, expression of Hippo pathway genes was significantly altered by infection. Hippo pathway regulation by *H. pylori* infection has biphasic kinetics and is CagA-dependent. Early in infection, *H. pylori* transiently triggered YAP1 expression and co-transcriptional activity, along with LATS2. This period of Hippo pathway inactivity is followed by a progressive activation, sustained by LATS2 accumulation and inhibitory YAP1<sup>Ser127</sup>-phosphorylation. LATS2 siRNA-mediated repression accelerated the acquisition of the EMT-phenotype upon infection, the up-regulation of EMT-markers ZEB1 and Snail1, and the decrease of the epithelial miR-200. *H. pylori*-induced CD44 upregulation, invasion and sphere-forming capacity were further enhanced upon LATS2 knockdown, suggesting that LATS2 restricts the EMT and CSC-like phenotype in hGECs upon *H. pylori* infection. Inhibition of either LATS2 or YAP1 reduced the expression of both proteins, revealing a positive feedback loop. In tissue sections of GC, LATS2 and YAP1 were heterogeneous and co-expressed. The positive correlation between LATS2 and YAP1 was confirmed in the 38

hGECs of the CCLE. The expression of CTGF and CYR61 was also strongly correlated to LATS2, suggesting that LATS2 could also be a YAP1/TEAD target gene.

Second, we evaluated the anti-tumor properties of verteporfin (VP), which disrupts the YAP1/TEAD interaction inhibiting its transcriptional activity. *In vitro*, using hGECs and cells from patient derived primary tumor xenografts (PDXs), we showed that treatment with VP decreased cell growth, expression of YAP1/TAZ/TEAD target genes, TEAD-luciferase reporter activity and sphere-forming capacity. The activity of VP was tested *in vivo*, in a model of subcutaneous graft of hGECs (MKN45 and MKN74) and PDX (GC10) in NGS mice. Resulting tumors were treated daily by peritumoral injection of three doses of VP or DMSO as control. Tumor growth was followed and a decrease starting from day 5-12 was observed at the intermediate dose. Tumor weight measurement, IHC analysis (CD44, ALDH and Ki67), and evaluation of sphere-formation capacity of residual CSCs were decreased in treated tumors. Altogether, these results show the CSC-inhibitory activity of VP both *in vitro* and *in vivo*.

We showed for the first time that the LATS2/YAP1/TEAD axis is early activated during the carcinogenesis process induced by chronic *H. pylori* infection and controls the subsequent EMT and CSC-like features. Targeting the Hippo pathway efficiently prevented tumor growth in a PDX model, highlighting the potential of its inhibition to be implemented in gastric cancer therapy.

**Keywords:** Cancer stem cells, epithelial to mesenchymal transition, CD44

---

## Unité de recherche

**Bordeaux Research in Translational Oncology, INSERM U1053**

146 Rue Léo Saignat, Campus Carreire Zone Nord, 2B RdC

33076 Bordeaux

## Substantial abstract:

Stomach cancer is the fifth most common cancer overall, and despite of a declining incidence, it remains the third leading cause of cancer death worldwide. The diagnosis of the disease is often at advanced stages, the therapeutic options are limited and the survival rates are poor. Gastric cancer is a multifactorial disease, most frequently associated to the chronic infection with *Helicobacter pylori*. *H. pylori* is a curved gram-negative bacillus present in human gastric mucosa linked to dyspeptic conditions and gastric cancer, and it is a WHO-Type I carcinogen since 1994. *H. pylori* infects approximately half of the world's population, especially underdeveloped countries. Upon acquisition of *H. pylori*, a persistent infection is established, ending only by the eradication with antibiotic treatment. Only a small percentage of cases is associated with severe pathologies (10-15 %), such as chronic atrophic gastritis, peptic ulcers and gastric malignancies (1-2 %), notably gastric adenocarcinoma and MALT lymphoma. The main virulence factor of *H. pylori* is the Cytotoxin associated gene A (CagA) whose expression is strongly associated with a higher virulence of the bacterium.

Epithelial-to-mesenchymal transition (EMT) is an evolutionary conserved, reversible process in which a polarized epithelial cell acquires a mesenchymal phenotype through phenotypical and molecular changes, leading to increased capacities of migration, invasion, apoptosis resistance and extracellular matrix production and remodeling. It can occur in association to cancer progression and metastasis of tumors of epithelial nature, tumor-related immunosuppression, and drug resistance. Another interesting aspect of EMT in carcinogenesis is its role in the origin of cancer stem cells (CSC). Tumors are heterogeneous entities in which CSCs represent a minority of stem-like cells with asymmetrical division and self-renewal capacities, as well as the potential to give rise to the more or less differentiated cells within the tumor. These properties combine in the ability to originate new tumors. In gastric cancer, these CSCs express, amongst other markers, the hyaluronate receptor CD44 and a high aldehyde-dehydrogenase (ALDH) activity. Infection with *Helicobacter pylori* of human gastric adenocarcinoma cell lines *in vitro* induces the emergence of a population of CD44<sup>high</sup> cells with properties of CSC through an EMT process. The induction of the EMT and the CD44<sup>high</sup> cell population was demonstrated to be dependent on the bacterial oncoprotein CagA.

The Hippo pathway was first described as a size-controlling pathway in the epithelial precursors in *Drosophila melanogaster*. It is a highly evolutionarily conserved pathway, composed by a tumor suppressor core consisting of a module of two kinases, MST and LATS, and their regulatory proteins SAV and MOB. The second component includes the output elements, which are the targets of phosphorylation by the kinase module, the YAP1 and TAZ proteins. Upon phosphorylation by LATS, YAP1 and TAZ are retained in cell-cell junctions or undergo proteasomal degradation. This sequestration limits their activity

by preventing the nuclear accumulation. Active nuclear YAP1 and TAZ function together with several partners, such as AP-1, p73, and especially TEAD family transcription factors, to promote tissue growth by increasing the expression of genes that positively regulate cell growth and inhibit apoptosis. Their capacity to regulate transcription of genes controlling cell survival and proliferation make YAP1 and TAZ, oncogenes.

Using *in vitro* and *in vivo* models for the study of gastric carcinogenesis induced by *H. pylori* infection, the first aim of this work was to investigate whether *H. pylori* affects the activation state of the Hippo pathway, focusing on the roles of LATS2 and YAP1 and how this regulation influences the EMT process and the CSC-like cell population. As a second goal, the present work intended to characterize the role of YAP1/TEAD in gastric CSC properties *in vitro* and the consequences of its pharmacological inhibition on tumor growth *in vivo* in a model of human tumor xenografts, aiming to the development of adjuvant therapies to gastric cancer.

In the first part, we studied the Hippo pathway regulation during infection. LATS2, YAP1 and CD44 were evaluated in gastric mucosae of either non-infected or *H. pylori*-infected patients, and were found upregulated in the infected mucosa, precisely within the isthmus region of the gastric glands, where gastric epithelial stem cells reside. The over expression was associated to gastritis and intestinal metaplasia. In human gastric epithelial cell lines, expression of genes involved in the Hippo pathway was significantly altered by infection, including the Hippo kinase core genes, the transcriptional co-factors YAP1 or TAZ, the TEAD transcription-enhancer factors, and several target genes involved in cell proliferation and survival, including *cyr61* and *ctgf*. We showed here that Hippo pathway regulation by *H. pylori* infection has biphasic kinetics. In the early period of infection, *H. pylori* transiently triggered YAP1 expression co-transcriptional activity, along with LATS2. This early period of infection corresponds to an inactive Hippo pathway and is followed by a progressive activation, likely sustained by LATS2 accumulation and the subsequent YAP1 Ser127-phosphorylation, preventing its nuclear translocation and TEAD-mediated transcriptional co-activation. Noteworthy, the activation of the Hippo pathway was CagA-dependent and thereby related to the virulence and oncogenic potential of the bacteria.

To study the role of LATS2 in the EMT and CSC-like properties induced by *H. pylori* in human gastric epithelial cell lines *in vitro*, a knock-down of LATS2 using siRNAs was performed. LATS2 repression accelerated the acquisition of the EMT-phenotype upon infection, the up-regulation of the EMT-markers ZEB1 and Snail1, as well as the decrease of the epithelial miR-200 microRNA family members. CD44 upregulation by *H. pylori* infection was further enhanced upon LATS2 knockdown. Invasive properties and tumorsphere-forming capacity of the cells were increased by infection and inhibition of LATS2 as well. These results suggest that LATS2 restricts the EMT and CSC-like phenotype that human gastric epithelial cells acquire upon *H. pylori* infection. Moreover, LATS2 is essential for optimal growth either in monolayer

or in spheroids. The siRNA experiments showed that inhibiting either LATS2 or YAP1 led to a reduced expression of both proteins, revealing a positive feedback loop between them.

In tumor tissue sections of gastric adenocarcinoma patients, LATS2 and YAP1 were found to be heterogeneous and co-expressed, regarding both subcellular localization and expression. The evaluation of the expression of LATS2, YAP1, and CD44 in the 38 gastric cancer cell lines of the Cancer Cell Line Encyclopedia confirmed the positive correlation between LATS2 and YAP1 expression. In addition, the expression of the YAP1/TEAD target genes CTGF and CYR61 is strongly correlated to LATS2 expression, suggesting that LATS2 could also be considered as a YAP1/TEAD target gene.

A second part of the study consisted in the evaluation of anti-tumor properties of verteporfin, a protoporphyrin that is able to disrupt the YAP1/TEAD interaction, and therefore inhibits its transcriptional activity. *In vitro*, using the gastric cancer epithelial cell lines MKN45 and MKN74, and cells from patient derived primary tumor xenografts (PDXs), we showed that treatment with verteporfin decreased cell growth with an accumulation of cells in G0/G1 phase of the cell cycle. The expression of YAP1/TAZ/TEAD target genes and the activity of TEAD-luciferase reporter was also decreased upon treatment, as well as the capacity to form tumorspheres when cells were cultured in non-adherent conditions, a key feature of CSCs. The activity of verteporfin was then tested *in vivo*, in a model of subcutaneous PDXs in immunodeficient mice. Cells from two gastric cancer cell lines (MKN45 and MKN74) and one patient derived gastric carcinoma (GC10) were used. After a period of growth, the developed tumors were treated daily by peritumoral injection of three increasing doses of verteporfin or DMSO as control vehicle, for 15 days. The tumor growth was followed during this period at the end of which, mice were sacrificed and tumors were extracted for weight measurement, histological analysis, and seeding in non-adherent conditions to evaluate tumorsphere-formation capacity of residual CSCs. The growth rate was found to be decreased starting from day 5-12 at the intermediate dose. Tumor weight at sacrifice was also significantly lower in the treated group in comparison with the controls. Tumorsphere formation and self-renewal properties were decreased in treated cells in all cases. Finally, the immunohistochemistry evaluation of cells from treated tumors showed a heterogeneous but decreased expression of CD44 and ALDH in the tumors for the treated group, as well as a downregulation of the Ki67 proliferation marker. Altogether, these results show the CSC-inhibitory activity of verteporfin both *in vitro* and *in vivo*.

This work shows for the first time that the Hippo pathway and particularly the LATS2/YAP1/TEAD axis is early activated during the carcinogenesis process induced by chronic *H. pylori* infection and controls the subsequent EMT and CSC-like features. Targeting this pathway was shown to be efficient in preventing tumor growth in a PDX model, therefore highlighting the potential of its inhibition to be implemented in gastric cancer therapy.



## **Remerciements - Acknowledgements - Agradecimientos**

To the jury members, for agreeing their precious time to analyze, criticize, and help me to improve the scientific quality of my work.

A ma directrice de thèse Mme le Dr. Christine Varon pour son guide et son soutien pendant ces quatre ans, et pour me conseiller et me permettre d'apprendre à son côté, mais aussi pour me donner la liberté de poursuivre la curiosité scientifique et être toujours ouverte au débat des idées et opinions, ce qui m'a permis de croître comme professionnel et comme personne.

A Mme le Dr. Cathy Staedel pour ses conseils, son expertise et sa gentillesse, sans lesquels ce travail n'aurait pu être possible. Merci pour être un exemple de ce qu'on aime dans la science. A Mme le Dr. Hélène Bœuf pour ses conseils, sa gentillesse et les riches discussions pour améliorer mon travail.

A M le Pr. Francis Mégraud pour son soutien, sans lequel je ne serais pas venue en France, pour son accueil chaleureux depuis mon arrivée au laboratoire, et toutes les opportunités scientifiques et personnels données dans ces années.

A M le Pr. Philippe Lehours, Mme le Dr. Emilie Bessède, Mme le Dr. Armelle Menard et Mme le Dr. Claire Roubaud pour leurs conseils, leurs critiques, et leur soutien tout au long de ma thèse.

Aux assistants ingénieurs de notre laboratoire : Elodie Sifré, Lucie Chambonnier, Julien Izotte, Alban Giese, Alice Buissonière, Astrid Ducournau, Lucie Bénéjat et Salha Ben Amor, c'était vraiment un plaisir de travailler avec vous, toujours disponibles pour partager vos compétences à la paillasse et ailleurs.

A mes chers collègues Sarah, Pauline, Christelle et Julie pour leur amitié, leur solidarité, leurs conseils et les expériences partagées, essentiels pour tenir jusqu'au bout. A Luis et Amandine, les « M2 originaux », cette expérience n'aurait été la même chose sans vous. A Camille et Solène, pour son travail et pour être les « sujets expérimentales » de mon apprentissage pédagogique, j'espère de ne pas avoir été trop décevante.

A Ann et Isabelle pour leur aide professionnel et personnel, leur gentillesse et leur amitié.

En fin, un grand merci à tous pour faire une belle ambiance de travail au laboratoire.

A Mme Lindsay Mégraud pour son aide et sa gentillesse qui m'ont fait me sentir bienvenue en France.

A mis jefes y compañeros del Programa de Epidemiología del Cáncer del INISA, Dra. Vanessa Ramirez, Dr. Clas Une, M.Sc. Wendy Malespín y Dr. Warner Alpízar, por todo el apoyo brindado, fundamental en el crecimiento profesional y personal que me permitió comenzar con la etapa que hoy termina. Gracias a la Universidad de Costa Rica y el Ministerio de Ciencia, Tecnología y Comunicaciones, así como al Instituto Francés para América Central por el apoyo financiero.

A mis papás y mis hermanitos de dos y cuatro patas, a toda mi familia y mis queridos amigos, los de siempre y los que me he ido encontrando en el camino, en Costa Rica y de este lado del charco. Les debo todo.



## Table of contents

I.	Introduction.....	19
a.	Gastric cancer.....	21
1.	Macro-anatomic classification .....	22
2.	Histological classification.....	23
3.	Molecular classification.....	24
4.	Pathologic staging .....	25
1.	Dietary factors .....	28
2.	Tobacco and alcohol intake.....	29
3.	Autoimmune gastritis.....	29
4.	Gastro-esophageal reflux disease .....	30
1.	Signaling pathways in cancer .....	30
a.	Wnt/ $\beta$ -catenin signaling pathway .....	30
b.	Signaling pathways associated with growth factors .....	31
c.	Notch signaling pathway .....	32
d.	Epithelial to mesenchymal transition (EMT) .....	33
2.	Genomic alterations and driver mutations .....	39
3.	Somatic copy number aberrations .....	39
4.	Epigenetic alterations.....	40
1.	In vivo models.....	44
a.	Chemical carcinogenesis .....	46
b.	Genetically engineered mice.....	46
c.	Xenotransplantation in immunodeficient mice .....	47
2.	In vitro .....	49
b.	Stem cells and Cancer stem cells.....	51
1.	Mechanisms of cell renewal .....	51
2.	Gastric stem cells.....	52
1.	Concept of cancer stem cell and the CSC model for tumor heterogeneity .....	54
2.	Evidence .....	58
3.	Origin of cancer stem cells .....	58
a.	Gastric cancer stem cells .....	59
i.	Bone marrow derived cells (BMDCs).....	59
ii.	Gastric stem cells (GSC).....	59
iii.	Dedifferentiated epithelial cells .....	60

4.	Markers of CSCs.....	60
a.	General .....	60
b.	CD44 (Cluster of differentiation 44) .....	61
i.	Structure and function .....	61
ii.	Role in cancer .....	63
5.	Methods of identification and isolation of CSCs .....	63
a.	Colony and sphere formation assays .....	63
b.	Side-population assay.....	64
c.	Aldehyde dehydrogenase (ALDH) activity assay .....	64
d.	Isolation based on marker expression .....	64
e.	In vivo xenotransplantation.....	65
c.	<i>Helicobacter pylori</i> .....	66
1.	Colonization.....	68
a.	Adaptation to acid environment survival: Urease .....	68
b.	Bacterial motility and chemotaxis.....	69
c.	Adhesion: Outer membrane proteins .....	70
2.	CagA and cagPAI .....	70
3.	VacA.....	73
4.	LPS .....	74
1.	In vitro models.....	76
a.	Cell lines.....	76
b.	Organoids .....	76
2.	In vivo models.....	77
a.	Murine models .....	77
b.	The Mongolian gerbil model .....	77
d.	Hippo pathway .....	79
1.	FERM- and WW-containing proteins upstream MST .....	81
2.	Merlin and angiostatins .....	81
3.	Willin/FRMD6 .....	82
4.	KIBRA .....	82
5.	Cell polarity and adhesion .....	82
a.	Apical cell polarity .....	82
b.	Basolateral cells polarity .....	83
c.	Cell adhesion proteins .....	84
6.	Cytoskeleton, extracellular matrix and mechanical forces .....	85

1.	MST 1/2 .....	85
2.	LATS1/2.....	86
1.	Structure.....	89
2.	Phosphorylation and proteasomal degradation .....	90
3.	Physical regulation .....	91
4.	Nuclear functions and TEAD-transcription factor co-transcriptional activity .....	91
5.	YAP1/TAZ/TEAD target genes.....	92
1.	Crosstalk with other oncogenic signaling pathways .....	96
a.	YAP1/TAZ - Wnt/ $\beta$ -catenin crosstalk.....	96
b.	YAP1/TAZ - TGF- $\beta$ /Smad crosstalk.....	97
2.	Hippo pathway targeting in cancer therapy.....	97
a.	Verteporfin .....	97
b.	VGLL4-mimicking “Super-TDU” inhibitor peptide .....	99
II.	Results/Articles.....	101
a.	Article 1: LATS2 and YAP1 are co-upregulated in response to <i>Helicobacter pylori</i> infection and control cancer stem cell properties in gastric epithelial cells .....	103
b.	Article 2: Deciphering and targeting YAP1/TAZ/TEAD activity in human gastric adenocarcinomas 151	
III.	Discussion .....	173
a.	The LATS2/YAP1/TEAD axis controls EMT and CSC-properties.....	175
1.	Is LATS2 linked with pro-inflammatory signaling pathways?.....	176
2.	Is the Hippo pathway activation status correlated with the histological subtype of gastric carcinomas?.....	180
b.	Verteporfin targeting of the YAP1/TAZ/TEAD transcriptional program inhibits CSC-properties and tumor growth .....	180
1.	TAZ role in gastric cancer .....	182
2.	Hippo pathway status in response to ATRA treatment.....	182
IV.	References.....	185
V.	Annexes .....	211
a.	Deletion of IQGAP1 promotes <i>Helicobacter pylori</i> -induced gastric dysplasia in mice and acquisition of cancer stem cell properties <i>in vitro</i> .....	213



## Table Index

Table 1. Macroanatomical classification of gastric cancer .....	23
Table 2. TNM-staging of gastric cancer .....	26
Table 3. Hereditary conditions associated with gastric cancer .....	28
Table 4. Markers of EMT .....	34
Table 5. Comparative anatomy and histology of the murine and human stomach.....	45
Table 6. Genetically altered murine model of gastric cancer.....	47
Table 7. Commercially available gastric epithelial cell lines.....	50
Table 8. Markers of gastric stem cells identified by lineage tracing .....	54
Table 9. CSC markers in hematological malignancies and solid tumors .....	61
Table 10. Hippo pathway components in Drosophila and human homologs .....	80
Table 11. Overview of the regulation of mammalian LATS kinases by phosphorylation.....	88

## Figure Index

Figure 1. Key features of gastric cancer subtypes according to molecular classification.....	25
Figure 2. Hereditary gastric tumors.....	27
Figure 3. Xenograft murine models.....	49
Figure 4. Architecture of the gastric glands and localization of stem/progenitor cells of the stomach.....	53
Figure 5. Models explaining the heterogeneity of tumor cells.....	55
Figure 6. Unified model of clonal evolution and cancer stem cells.....	56
Figure 7. Unified model for gastric cancer stem cells.....	57
Figure 8 CD44 gene and protein structure .....	62
Figure 9. cagPAI T4SS structure and CagA effects on the host cells.....	72
Figure 10. Hippo pathway.....	79
Figure 11. Cell polarity systems involved with Hippo regulation.....	83
Figure 12. Structure of LATS1 and LATS2.....	87
Figure 13. Structure of YAP and TAZ.....	90
Figure 14 Features of cancer cells controlled by the Hippo pathway .....	94
Figure 15. YAP1/TAZ in human tumors.....	95
Figure 16. Chemical structure of verteporfin.....	98
Figure 17. Hippo pathway regulation by <i>H. pylori</i> infection.....	176
Figure 18. Regulation of anti-microbial peptide response in <i>Drosophila</i> .....	177
Figure 19. Functional categories enriched upon LATS2 inhibition in MKN74 cells.....	178
Figure 20 NLR signaling pathway associated genes differentially expressed upon LATS2 inhibition.....	179

# I. Introduction



## a. Gastric cancer

### i. Epidemiology

According to the latest report of the International Agency for Research on Cancer (IARC), stomach cancer is the fifth most common cancer overall, with an estimate of 952 000 new cases in 2012<sup>1</sup>. Despite of a declining incidence, it remains the third leading cause of cancer death in both sexes worldwide, with approximately 723 000 stomach cancer deaths in 2012 which represent 8.8% of the death burden due to all types of cancer (except non-melanoma skin cancers). This cancer is more frequent in men than in women and more common in developing countries<sup>1,2</sup>. In general, gastric cancer rates are twice as high in men as in women, and the risk of gastric adenocarcinoma is lower at a young age, and gradually increases, reaching a plateau between 55 and 80 years<sup>3</sup>. The highest incidences are found in Eastern and Western Asian countries (Korea, Japan, and Iran) and Eastern Europe (Russia and other former URSS countries), as well as in some Latin American countries (Chile, Costa Rica, Ecuador, and Colombia)<sup>4,5</sup>. In countries with very heterogeneous populations, like the United States, there are differences in the cancer incidence rates associated with the ethnical background of the population. The incidence of gastric cancer is two times higher in Hispanics, Korean Americans, and African Americans than in non-Hispanic whites and other Asian Americans<sup>6,7</sup>. This may be because these groups tend to have lower socioeconomic conditions, limited access to quality healthcare and screening and preventions campaigns, as well as different genetic backgrounds<sup>6,8</sup>.

Unfortunately, the diagnosis of the disease is often at advanced stages, generally in elderly people, making the therapeutic options limited at best. Consequently, the 5-year survival is poor, usually less than 30%, and as low as 4.3-9.2% when untreated<sup>3,9</sup>. As expected, there is a difference between survival rates in developed and developing countries. A recent meta-analysis reported a 5-year survival in Iran, whilst values between 26 and 32% were reported for Western European countries in the SUDCAN study<sup>10,11</sup>.

Gastric cancer is a multifactorial disease, it can be hereditary or associated with a specific mutational profile, but most frequently, it is associated to the chronic infection with *H. pylori*<sup>3</sup>. In a study of the Eurogast-EPIC project, more than 93% of the non-cardia gastric cancer were positive for *H. pylori*<sup>12</sup>. Moreover, of the 2.2 million new cases of cancer attributable to infectious agents in 2012, *H. pylori* infection occupied the first place with 770 000 cases, representing 81% of the total new cases of gastric cancer for that year and being the only bacterial pathogen included in the list<sup>13</sup>.

Epidemiological features of the different types of gastric adenocarcinomas vary regarding their localization and histological classification. The most frequent type of gastric adenocarcinoma is the non-cardia carcinoma of intestinal type, which has the stronger association with *H. pylori* infection, but a tendency towards decrease has been observed<sup>1,14</sup>. The diagnosis for this entity is normally carried at older

male patients (55-80 years old, male to female ratio 2:1), after a long history of precancerous lesions<sup>14-16</sup>. Still in the non-cardia zone, the diffuse type incidence is, on the contrary, increasing<sup>16,17</sup>. The non-cardia diffuse type can be associated with the *H. pylori* infection but the main factor is the loss of E-cadherin, due to mutations in the *CDH1* gene<sup>17</sup>. It is diagnosed at a younger age than the intestinal type (40-60 years old) and frequently there is an autosomal dominant pattern of inheritance, and it is equally distributed between men and women<sup>16</sup>. Incidence of adenocarcinomas of the cardia region is increasing as well<sup>18</sup>. This type of cancer is not associated with *H. pylori*, but mostly with gastroesophageal reflux disease (GERD) for which *H. pylori* has been described as a protective factor, and it is strongly predominant in white males (male to female ratio of 6:1)<sup>18,19</sup>.

## **ii. Classification of gastric cancer**

### **1. Macro-anatomic classification**

The anatomical localization of gastric adenocarcinomas often reflects the pathological mechanisms laying behind the disease. Intestinal-type adenocarcinomas usually differ in etiology regarding whether they are proximal, which are associated with reflux, or distal, associated with *H. pylori* infection instead, while diffuse type adenocarcinomas are usually located in the middle third and the body of the stomach<sup>20</sup>. The macroscopic appearance of the tumors is also a feature used for classification. Non-invasive adenocarcinomas confined to the mucosa, regardless of the presence of lymph node metastasis and size, are considered as early<sup>21</sup>. The Japanese system classifies these tumors into Types I to IV, and the more recent Paris classification recognizes three gross patterns for superficial neoplastic lesions in the gastrointestinal tract<sup>21</sup>. The prognosis of early gastric carcinoma is excellent, with a 5-year survival rate as high as 90%, contrasting with the 60% or less observed for the advanced cases, that is, when there is an invasion of the muscularis propria or beyond<sup>21</sup>. All three classification systems are detailed in table 1.

**Table 1. Macroanatomical classification of gastric cancer<sup>21</sup>**

<b>Early</b>	
<b>Japanese classification</b>	
<b>I</b>	Protruding growth
<b>II</b>	Superficial growth. Further classified in IIa (elevated), IIb (flat), and IIc (depressed)
<b>III</b>	Excavating growth
<b>IV</b>	Infiltrating growth with lateral spreading
<b>Paris classification</b>	
<b>0-I</b>	Polypoid growth further divided in 0-Ip (pedunculated) and 0-Is (sessile)
<b>0-II</b>	Non-polypoid growth further divided in 0-IIa (slightly elevated), 0-IIb (flat), and 0-IIc (slightly depressed)
<b>0-III</b>	Excavated growth
<b>Advanced</b>	
<b>Borrmann classification</b>	
<b>I</b>	Polypoid, 25% more common in the greater curvature of the corpus
<b>II</b>	Fungating, 36% of all cases, usually located in the lesser curvature of the antrum
<b>III</b>	Ulcerating, 25% more common in the greater curvature of the corpus
<b>IV</b>	Diffusely infiltrating, also referred as linitis plastica when it involves a very significant portion of the stomach and is associated with the diffuse histological type

## 2. ***Histological classification***

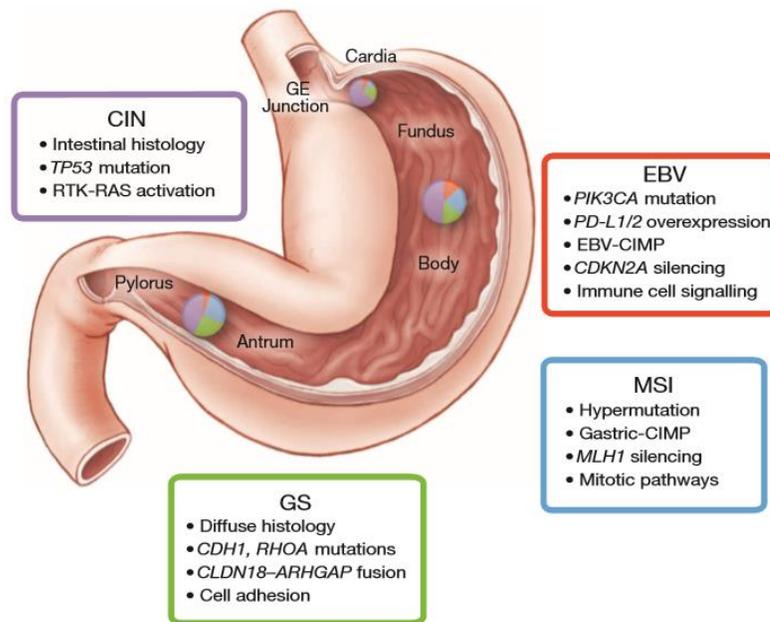
There are two main systems used to classify the gastric adenocarcinomas regarding their microscopic features, the Laurén classification and the WHO system. The Laurén classification is the most widely used and was described in the 1965, based on the examination of 1344 cases of gastric cancer<sup>22</sup>. It distinguishes two subtypes of gastric adenocarcinomas: the intestinal type, characterized by the formation of glands with or without the production of extracellular mucin, and the diffuse, which has poorly cohesive cells that often contain intracytoplasmic mucin and are therefore known as signet ring cells<sup>20</sup>. Tumors with a combination of the two subtypes, known as mixed-type adenocarcinomas are included in this system as well. The WHO system, updated in 2010, recognizes four main categories for gastric adenocarcinomas: tubular, papillary, mucinous and poorly cohesive, and it includes also the possibility of mixed carcinomas when the tumors present components of the four main groups<sup>23</sup>.

There is a significant correlation between the two classification schemes. The Laurén's diffuse type corresponds to the poorly cohesive, and it is characterized by a proliferation of isolated cancer cells which do not form glandular structures and usually affects younger subjects (mean age of 48 years in comparison with 55 for intestinal type)<sup>24,25</sup>. These cancers are more associated with genetic factors of the host than with environmental triggers and are present in populations of low risk. They may be inherited

in an autosomal dominant pattern due to mutations in CDH1<sup>26</sup>. The more prevalent intestinal subtype includes the tubular, papillary and mucinous variants. This form of adenocarcinoma is preceded by a long-term process where different types of precancerous lesions succeed each other in a model proposed by Correa in 1975<sup>27</sup>. After some modifications the model recognizes the following steps from a healthy gastric mucosa to invasive adenocarcinoma: non-atrophic gastritis, multifocal atrophic gastritis without intestinal metaplasia, complete or small bowel-type intestinal metaplasia, incomplete or colonic-type intestinal metaplasia, low grade dysplasia, high grade dysplasia, and invasive adenocarcinoma<sup>28</sup>.

### **3. *Molecular classification***

The study of genetic alterations in gastric cancer is not new, and different methods have been used to this end, from detection of single nucleotide polymorphisms (SNP) to whole genome sequencing. However, up until 2014 a systematic global assessment of the molecular features of gastric cancer was not available. In order to develop a robust molecular classification of gastric cancer The Cancer Genome Atlas (TCGA) carried on a large scale, multicenter study that included material from 295 patients<sup>29</sup>. Sample assessment by different techniques such as whole-exome, whole-genome, mRNA, and miRNA sequencings, SNP arrays, somatic copy number analysis, DNA-methylation profiling allowed not only the establishment of a classification system of gastric carcinomas according to molecular criteria, but also the identification of dysregulated pathways and candidate drivers for the distinct classes of gastric cancer. The resulting classification system includes four categories: Epstein-Barr virus (EBV) infected tumors, tumors with microsatellite instability (MSI), genomically stable tumors (GS), and chromosomally unstable tumors (CIN)<sup>29</sup>. Further details on this classification are shown in figure 1.



**Figure 1. Key features of gastric cancer subtypes according to molecular classification.**  
Anatomical distribution of each type is shown in pie-charts<sup>29</sup>.

#### 4. **Pathologic staging**

The process of staging is the determination of how much cancer is present in the body and where it is located, and it describes the severity of cancer based on criteria such as the characteristics of the primary tumor, and the spreading of cancer through the body<sup>30</sup>. Pathologic staging, that is, after tumor resection or exploratory surgery, follows the guidelines established by the American Joint Committee on Cancer Staging (AJCC) and the Union for International Cancer Control (UICC) known as the TNM system. The TNM system is based on the size of the primary tumor (T), lymph node involvement (N) and the presence or absence of distant metastasis (M)<sup>20</sup>. The 2010 update (7<sup>th</sup> edition) is detailed in table 2.

**Table 2. TNM-staging of gastric cancer<sup>31</sup>**

<b>Stage</b>	<b>T</b>	<b>N</b>	<b>M</b>
<b>Stage 0</b>	Tis	N0	M0
<b>Stage IA</b>	T1	N0	M0
<b>Stage IB</b>	T2	N0	M0
	T1	N1	M0
<b>Stage IIA</b>	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
<b>Stage IIB</b>	T4a	N0	M0
	T3	N1	M0
	T2	N2	M0
	T1	N3	M0
<b>Stage IIIA</b>	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
<b>Stage IIIB</b>	T4b	N0 or N1	M0
	T4a	N2	M0
	T3	N3	M0
<b>Stage IIIC</b>	T4b	N2 or N3	M0
	T4a	N3	M0
<b>Stage IV</b>	Any T	Any N	M1

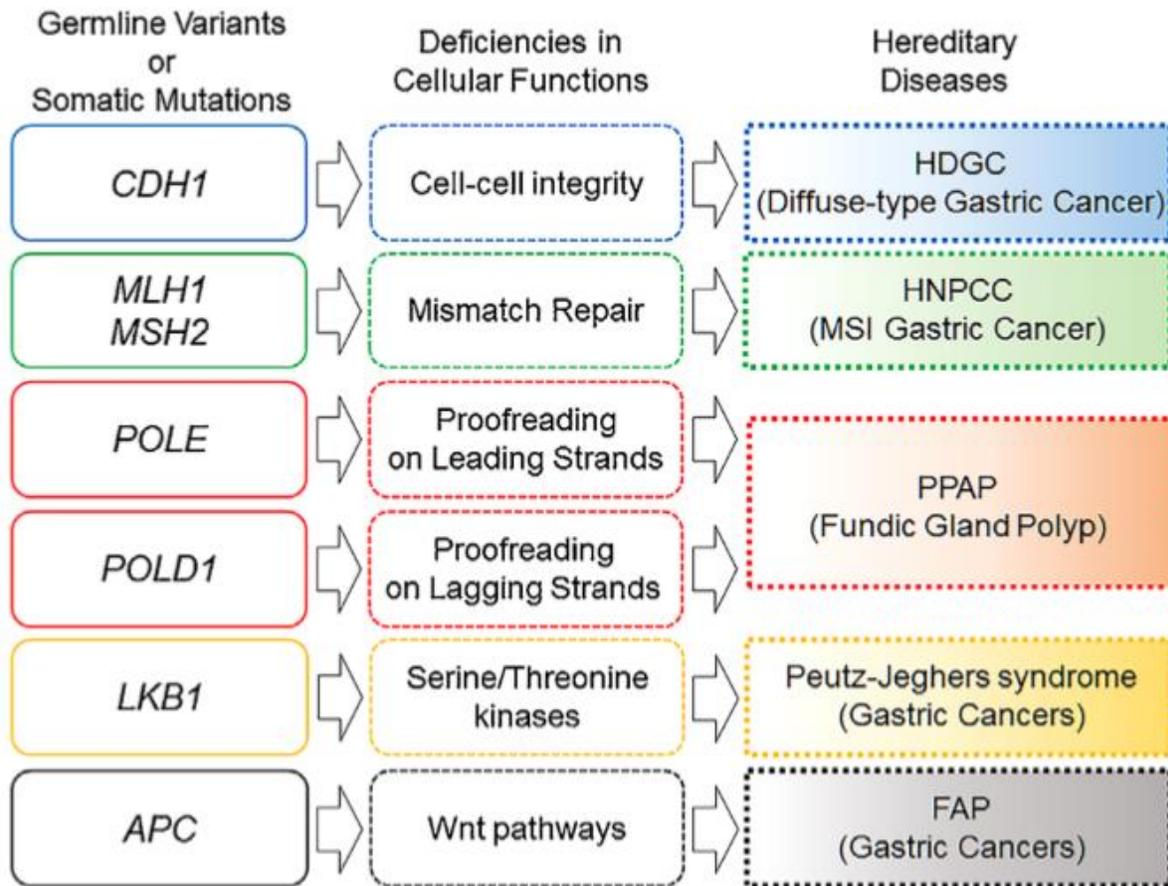
**T category definitions.** **Tis:** Carcinoma in situ, **T1:** Tumor invades lamina propria, muscularis mucosae, or submucosa, **T1a:** Tumor invades lamina propria or, muscularis mucosae, **T1b:** Tumor invades submucosa, **T2:** Tumor invades muscularis propria, **T3:** Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures, **T4:** Tumor invades serosa (visceral peritoneum) or adjacent structures, **T4a:** Tumor invades serosa (visceral peritoneum), **T4b:** Tumor invades adjacent structures (spleen, colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine, and retroperitoneum).

**N category definitions.** **N0:** No regional lymph node metastasis, **N1:** Metastasis in 1 to 2 regional lymph nodes, **N2:** Metastasis in 3 to 6 regional lymph nodes, **N3:** Metastasis in 7 or more regional lymph nodes.

**M1:** Positive peritoneal cytology.

### iii. Pathogenesis

Multiple environmental, behavioral and genetic factors can contribute to the development of gastric cancer. Most gastric cancers are sporadic, however there are also approximately 10% of cases that are familial, associated to heritable mutations listed in table 3 and figure 2. As mentioned before, *H. pylori* infection is a major risk factor for the development of cancer and the bacterial virulence factors and pathological mechanisms involved are described in detail in chapter 3.



**Figure 2. Hereditary gastric tumors.** Germline mutations can cause hereditary gastric cancer. HDGC, hereditary diffuse gastric cancer; HNPCC, hereditary non-polyposis colorectal cancer; PPAP, polymerase proofreading-associated polyposis; FAP, familial adenomatous polyposis.

**Table 3. Hereditary conditions associated with gastric cancer<sup>32</sup>.**

<b>Syndrome</b>	<b>Genes (% case association)</b>	<b>Inheritance</b>	<b>Gastric cancer risk (%)</b>	<b>Gastric cancer histology</b>	<b>Features of gastric polyps</b>	<b>Onset (years)</b>
<b>Hereditary diffuse gastric cancer</b>	CDH1 (45)	Autosomal dominant	56-70	Diffuse	-	14
<b>Hereditary nonpolyposis colon cancer</b>	MLH1 (30), MSH2 (60), MSH6 (<10), PMS2 (<10)	Autosomal dominant	2-30	Intestinal	-	-
<b>Peutz-Jeghers</b>	STK11 (70)	Autosomal dominant	29	Intestinal	Hamartomatous polyps and melanocytic macules (antrum, pylorus)	16
<b>Juvenile polyposis</b>	SMAD4 (4-20), BMPR1A (20-25)	Autosomal dominant	21	Intestinal and diffuse	Hamartomatous and heterogenous	41
<b>Familial breast cancer</b>	BRCA1, BCRA2	Autosomal dominant	2.6-5.5	-	-	<50
<b>Li-Fraumeni</b>	TP53 (70)	Autosomal dominant	3.1-4.9	Intestinal (70%) and diffuse (30%)	-	12
<b>Familial adenomatous polyposis</b>	APC (≤90)	Autosomal dominant	2.1-4.2	Intestinal	FGPs (body/fundus) and GAs (body/antrum junction)	8

### **1. Dietary factors**

Several factors associated with nutritional behavior have been associated to gastric cancer. While an adequate intake of fruit, vegetables, and other foods rich in antioxidant compounds and dietary fiber is considered to decrease the risk of gastric cancer, the intake of high quantities of salt and nitrous compounds may increase it. There is strong epidemiological evidence for a link between increased risk of gastric cancer and the high intake of salt and salt-preserved foods, some studies have even found progressive increased risk across consumption levels<sup>33</sup>. Mechanistically, it has been described that intake of salted foods enhance the risk of *H. pylori* infection and CagA expression, therefore promoting gastric cancer, and high concentrations of sodium chloride have been shown to induce inflammatory and erosive damage in the gastric epithelium, cell death and regenerative hyperplasia in animal models<sup>34</sup>. Nitrous

compounds are recognized mutagens and carcinogens associated with high risk of gastric cancer, and they can originate from diet or be produced endogenously, especially from red meats as nitric oxide reacts with hemoglobin and myoglobin in the acid environment of the stomach<sup>34</sup>. The nitrous compounds scavenging capacity of dietary fiber have been demonstrated experimentally and may be one of the reasons why an intake of dietary fiber has been described as a protective factor against gastric cancer in a dose dependent fashion<sup>33</sup>.

## **2. Tobacco and alcohol intake**

Both tobacco smoking and high alcohol intake have been described as risk factors for gastric cancer in various prospective studies and meta-analysis. In the case of smoking, a prospective study of more than 18 000 middle-aged men in Shanghai showed a 60% more risk of gastric cancer in smokers in comparison with non-smokers after correction for the confounder variants, and a meta-analysis including 23 studies showed 50% more risk for smokers than for never smokers<sup>33,34</sup>. Mechanistically, tobacco smoke induced an epithelial to mesenchymal transition in the gastric epithelium in a model of BALB/c mice exposed during twelve weeks, with increased expression of Snail-1, Vimentin, and N-cadherin, and concomitantly reduced the expression of E-cadherin, ZO-1, and CK5<sup>35</sup>. This was mediated by an activity of ERK5 that can be inhibited with a pharmacological inhibitor<sup>35</sup>. In the prospective study cited before, heavy drinkers also showed a significant increase in the risk of gastric cancer, which has also been found in similar studies in European populations, especially with non-cardia intestinal-type cancer<sup>33,34</sup>. Not only high alcohol consumption leads to the production of acetaldehyde that may cause DNA damage incrementing the risk of gastric cancer, but it also interact with host factors such as polymorphisms in genes associated with its metabolism further elevating the risk in this individuals<sup>36</sup>.

## **3. Autoimmune gastritis**

Autoimmune gastritis (AIG) is a chronic inflammation process in the gastric mucosa that can result in atrophy<sup>37</sup>. AIG is restricted to corpus and fundus, because it originates from an autoimmune reaction against parietal cells, which are absent from the antrum, and maybe totally lost during the progression of disease. In this case, the autoimmune response is mediated by CD4<sup>+</sup> T cells against the gastric H<sup>+</sup>/K<sup>+</sup> ATPase, causing the loss of parietal cells that results in increased pH of the stomach and loss of intrinsic factor, impairing the uptake of vitamin B12<sup>37</sup>. There are two proposed mechanisms for the autoimmune response: first, a cross reaction of the immune response elements generated against *H. pylori* infection with antigens within the proton pump, and second, a primary autoimmune response independent of *H. pylori* infection<sup>38</sup>.

#### **4. Gastro-esophageal reflux disease**

Gastro-esophageal reflux disease (GERD) has long been recognized as a major risk factor for esophageal adenocarcinoma as it exposes the distal esophageal mucosa to gastric juices containing acid, pepsin, and bile<sup>39</sup>. It has also been identified as a precursor lesion of cardia adenocarcinoma of intestinal-type resembling esophageal adenocarcinoma in *H. pylori*-negative patients, with OR (95% CI) ranging between 8.02 (2.25-28.58) and 10.08 (2.29-44.36)<sup>40</sup>. The pathophysiological mechanisms involved are suggested to be similar to those in esophageal adenocarcinoma, the reflux of gastric contents leading to a columnar intestinal metaplasia, dysplasia and adenocarcinoma.

#### **iv. Molecular Mechanisms**

##### **1. Signaling pathways in cancer**

##### **a. Wnt/ $\beta$ -catenin signaling pathway**

The Wnt family is widely conserved family of secreted glycoproteins which includes at least 19 members in humans, and the signaling they induce upon binding to receptors of the Frizzled (Fz) family is a key mechanism that direct multiple cellular processes such as cell proliferation, differentiation, cell-fate decision, migration, and apoptosis, playing a crucial role during embryonic development and tissue homeostasis<sup>41-43</sup>. Therefore, mutations in the Wnt pathway are often linked to human birth defects, cancer, and other diseases, and abnormal activation of this pathway is a common feature of many types of cancer, including gastric adenocarcinomas<sup>42,44,45</sup>.

The canonical activation involves the  $\beta$ -catenin protein as a key signaling intermediate, therefore is known as the Wnt/ $\beta$ -catenin signaling pathway. In the absence of Wnt ligands,  $\beta$ -catenin is bound in a destruction complex with Axin, APC, CK1 $\alpha$ , and GSK3 $\beta$ , where it is phosphorylated and targeted for proteosomal degradation<sup>46,47</sup>. On the other hand, if Wnt ligands are bound to the Fz/LRP receptor complexes, the destruction complex is dissociated by the recruitment of the Dvl protein, the phosphorylation of LRP, and the delocalization of cytoplasmic Axin<sup>45</sup>. These events prevent the phosphorylation of  $\beta$ -catenin, stabilizing it and allowing the activation of transcription mediated by the  $\beta$ -catenin/TCF/LEF complex, whose target genes include CCDN1, gastrin and *c-myc*<sup>45</sup>. Non-canonical or  $\beta$ -catenin-independent activation of Wnt signaling has also been described, including the planar cell polarity (PCP) pathway and the Wnt/Ca<sup>2+</sup> pathway. In the later, the intracellular calcium concentration mediates the signaling, and it is known to regulate stem cells in development<sup>48</sup>.

Both canonical and non-canonical Wnt signaling are not mutually exclusive and predominance of one or the other depends on the cell type, the activation signaling and the extracellular context<sup>49</sup>, and

both have been found in association to gastric carcinogenesis<sup>50,51</sup>. However, the  $\beta$ -catenin dependent pathway seems to be the one with the stronger association, hence the more studied.  $\beta$ -catenin is overexpressed in most gastric cancers, especially in those of the intestinal type<sup>52</sup>, and a role for the Wnt/ $\beta$ -catenin signaling pathway in the self-renewal properties of gastric cancer stem cells has been shown<sup>53</sup>. Furthermore, studies have demonstrated mutations in genes coding for pathway elements are frequent in gastric cancers, 26% of gastric cancers with nuclear accumulation of  $\beta$ -catenin had mutations in *CTNN1*<sup>54</sup> whilst 22% of sporadic cases presented mutations in APC<sup>55</sup>. Evidence suggests that *H. pylori* infection, the main environmental factor associated with gastric carcinogenesis, activates the Wnt/ $\beta$ -catenin pathway in a CagA-dependent manner<sup>56</sup> and induces the transcription of  $\beta$ -catenin and its nuclear accumulation via LRP6 phosphorylation<sup>57</sup>. Moreover, this pathway seems to be responsible for the upregulation of the stemness genes *NANOG* and *OCT4* observed during *H. pylori* infection<sup>58</sup>.

#### *b. Signaling pathways associated with growth factors*

When growth factors bind to their cognate receptors, they initiate the transduction of extracellular signals through the activation of intracellular messengers or directly through receptor translocation to the nucleus<sup>59</sup>. Generally, as a result of this activation, there is a stimulation of processes that are tightly regulated in order to maintain the tissue homeostasis, such as cell proliferation and differentiation. Hence, alterations in growth factor-induced signaling lead to imbalances in the tissue equilibrium and could be implicated in carcinogenesis. Two of these pathways frequently dysregulated in gastric cancer are the fibroblast growth factor (FGF) and the epidermal growth factor (EGF) signaling pathways.

The FGF/FGFR pathway play a key role during embryogenesis and its alterations have been related to developmental disorders and carcinogenesis<sup>60,61</sup>. The FGF proteins bind to the FGFR receptor on the cell surface, and have high affinity for heparin and the heparin sulfate glycosaminoglycans of the extracellular matrix, which protect the FGF from degradation and increase their capacity to bind the FGFR<sup>62</sup>. Once activated, FGFRs stimulate the intracellular PI(3)K-ERK-AKT signaling, increasing mitogenic activity and preventing apoptosis<sup>63</sup>. As a consequence, FGF/FGFR are considered to be oncogenes and are overexpressed in a wide variety of cancers<sup>62</sup>. Amplifications in the FGFR have been reported in gastric cancer, resulting in deregulation of FGF signaling<sup>64,65</sup>. Interestingly, *H. pylori* is capable of activating FGF signaling via FGFR2 upregulation and SHP2 activation<sup>66</sup>.

EGFR is a tyrosine-kinase receptor of the ERbB family and, upon activation by its ligands EGF or transforming growth factor alpha (TGF- $\alpha$ ), it can initiate diverse intracellular signaling cascades such as PI(3)K/Akt/mTOR and Ras/Raf/MAPK, and therefore stimulate the transcription of genes associated with cell cycle activation, cell survival, and proliferation<sup>67</sup>. EGFR and other members of the ERbB family are

overexpressed in gastric cancer, especially in the advanced stages of the disease<sup>68</sup>. *H. pylori* is able to induce the transcription of EGF and EGFR, causing an activation of the EGFR signaling and contributing to neoplastic transformation of gastric epithelial cells<sup>69,70</sup>. During the infection, this activation may counterbalance the bacterium-induced apoptosis<sup>71</sup>.

### c. *Notch signaling pathway*

The Notch signaling is an evolutionary conserved pathway, first described in fruit flies presenting notched wings, and controls crucial cellular processes such as self-renewal of stem cells, proliferation, differentiation, and apoptosis<sup>72,73</sup>. The four Notch receptors (Notch 1,2,3, and 4) are EGF like domains containing, single pass transmembrane proteins that interact with Delta-like and Jagged ligands, which are also transmembrane proteins with EGF-like repeats<sup>73</sup>. There are five of these ligands in mammals, Dll1, Dll3, Dll4, Jagged1, and Jagged2. Upon activation by the ligands and as a result of a tightly controlled series of proteolytic events, the intracellular portion of the Notch receptor translocates to the nucleus to form an active transcriptional complex that controls the expression of several genes including Hes family genes, NF- $\kappa$ B, VEGF and *c-myc*<sup>74</sup>. Hence the Notch pathway regulates expression of genes associated with carcinogenesis and, unsurprisingly, deregulations of this pathway are found in various malignant diseases including T-cell leukemia, breast, prostate, colorectal, lung, and central nervous system cancers and associated to esophageal and breast cancer stem cells<sup>75</sup>.

Regarding gastric carcinogenesis, the presence of the four types of Notch receptors and their ligands, Jagged1 and Jagged 2, has been described in gastric tumors<sup>76,77</sup>. Moreover, the overexpression of Jagged1 in gastric tumors is associated with a worse prognosis<sup>78</sup>. Notch1 is of particular importance as it is not present in non-cancerous gastric mucosa, but is upregulated in both intestinal and diffuse type cancers<sup>77</sup>. The increased expression of Notch1 and its target gene *hes1* in gastric cancer cells has been associated to the upregulation of the DLL1 and DLL4 ligands<sup>79,80</sup>. Experimental studies have demonstrated the Notch signaling ability to promote gastric cancer progression, as cells transfected with an activated form of Notch1 presented an enhanced colony formation capacity, migration and invasion abilities and increased growth in xenografted tumors, all mediated by the induction of COX-2 expression<sup>78</sup>. Furthermore, similar results were found for Notch2 and the effect was downregulated by an inhibitor of COX-2<sup>81</sup>. Notch and other signaling pathways of the stem cell signaling signature are thought to be activated during *H. pylori* chronic infection<sup>82</sup>, but one study suggests that, on the contrary, Notch1 and Notch2 activity is repressed during infection in a GES-1 cells *in vitro* model<sup>83</sup>.

#### d. *Epithelial to mesenchymal transition (EMT)*

The transformation of epithelial cells towards mesenchymal cells was first described by Elizabeth Hay using a chick model in the 80's and early 90's<sup>84-86</sup>. Later renamed as a transition to distinguish it from a malignant transformation of the cells, it is an evolutionary conserved, reversible process in which a polarized epithelial cell acquires a mesenchymal phenotype through phenotypical and biochemical changes that leads to increased capacities of migration, invasiveness, apoptosis resistance and extracellular matrix production and remodeling<sup>87</sup>. Several molecular events have been described at the initiation of EMT. Transcription factors such as Snail, Slug, zinc-finger E-box binding (ZEB1/2) and FOXC2 are activated at the beginning of the process, accompanied by the expression of specific microRNAs, for instance the miR-200 family, changes in the expression of cell surface proteins, cytoskeletal reorganization, and activation of Wnt/ $\beta$ -catenin, growth factor-mediated, and Notch signaling<sup>78,87,88</sup>. A critical feature of EMT is the downregulation of E-cadherin<sup>89</sup>, a surface glycoprotein expressed in the epithelial cells as a key component of the adherens junctions in epithelial tissues<sup>90</sup>. Expression of E-cadherin can be repressed directly or indirectly by multiple transcription factor including ZEB1/2, Snail, Slug, NF- $\kappa$ B, E47, and KLF8, and also the proteins SIX1 and FOXC2<sup>91-93</sup>. These and other features of cells undergoing an EMT, are used as markers of the process and are further detailed in table 4. Various signaling pathways and the cross talks between them can also influence the expression of E-cadherin such as the TGF $\beta$  signaling, hypoxia-induced response, Wnt/ $\beta$ -catenin, Notch, and PI3K/Akt, therefore playing a role in EMT and becoming potential targets for therapy<sup>94,95</sup>. Even if the EMT is usually depicted as a binary switch that moves cells from a fully epithelial to a fully mesenchymal state, this is a misrepresentation the normal action of the EMT program, which most frequently moves cells from a fully epithelial state to a partially mesenchymal, retaining some epithelial markers, but nonetheless has conferred a subset of mesenchymal traits with profound effects on the cell biology<sup>96</sup>.

The EMT process is far from exclusive of malignant transformation, it is very important in other contexts, both physiological and pathological. Hence, for the sake of clarity, a classification of EMT in three categories was proposed in 2007.

Type I EMT refers to the processes occurring during implantation of the embryo, embryogenesis, and organ development<sup>87</sup>. In absence of EMT, the incipient organism would not go beyond the blastula, because the epithelial cell in the blastoderm require to go through an EMT to give origin to the primary mesenchyme needed to form the mesoderm during gastrulation<sup>97</sup>. Also, this type of EMT is especially important during the formation of the neural crest from the neuro-ectoderm<sup>98</sup>, which serves as a precursor for the nervous system, melanocytes and the suprarenal medulla, and in the cardiac morphogenesis, where there are three waves of EMT<sup>99</sup>. After the first stages of embryogenesis, the trophoctoderm cells

undergo an EMT which facilitates the invasion in the endometrium that assures the anchorage in the placenta<sup>100</sup>.

**Table 4. Markers of EMT. Modified from** <sup>101</sup>

<b>Proteins with increased expression</b>	<b>Proteins increased in the nucleus</b>
Collagen (Types 1 and 3)	$\beta$ -catenin
Fibronectin	NF- $\kappa$ B
FOXC2	Smad 2/3
Goosecoid	Snail 1/2/3
Integrins $\alpha$ V $\beta$ 6 and $\alpha$ 5 $\beta$ 1	Twist
Laminin 5	ZEB1/2
MMP-2	
MMP-3	
MMP-9	
N-cadherin	<b>Proteins with decreased expression</b>
Slug (Snail 2)	Claudins
Snail 1	Cytokeratins
Snail 3	Desmoplakin
Sox10	E-cadherin
Twist	Occludins
Vimentin	Osteopontin 1
ZEB1	
ZEB2	
<b>Proteins with increased activity</b>	<b>Functional properties <i>in vitro</i></b>
ILK	Increased migration capacity
GSK-3 $\beta$	Increased invasion capacity
Rho	Elongated morphology
	Apoptosis resistance

On the other hand, type II EMT is associated with tissue regeneration and fibrosis<sup>87</sup>. Cutaneous wound-healing exhibits EMT features, especially during the process of re-epithelialization, in which the keratinocytes at the wound edge lose their intercellular adhesions and migrate across the wound, with changes that are strikingly similar to the first steps of EMT<sup>102</sup>. Also the myofibroblasts present in the wound and essential in the remodeling and maturation phases of healing, are derived from resident epithelial cells transformed through EMT to synthesize extracellular matrix components and contract the wound bed<sup>102</sup>. In addition to the process in skin, the involvement of EMT in wound healing has been described in other tissues, such as mammary epithelial cells *in vitro*<sup>103</sup>, a murine model of lacrimal gland injury<sup>104</sup>, and a myocardial injury model in zebrafish<sup>105</sup>. Unsurprisingly, signaling pathways activated during both wound healing and EMT are significantly overlapped, including activation of EGF, FGF, HGF and TGF- $\beta$  signaling, which in turn switch on MAPK pathways to trigger simultaneously wound healing and EMT

promoting factors<sup>102</sup>. Deposition of collagen and other ECM components is part of the normal scar formation during tissue repair. When this deposition is excessive due to persistent injurious stimuli and turns pathological it is referred as fibrosis, and it occurs mainly in parenchymal organs such as the liver, the kidney, and the lung<sup>106</sup>. Myofibroblasts, a proportion of which is derived from organ epithelial cells via an EMT process, are the cells that produce ECM components to create the scar necessary to complete the wound healing, and they usually undergo apoptosis once the process is over<sup>102</sup>. However during pathological activation there is a dysregulation of injury trigger-EMT and TGF- $\beta$ , a key regulator of wound healing, is also implicated in fibrosis.

The last category, type III EMT, is always pathologic and occurs in association to cancer progression and metastasis of tumors of epithelial nature<sup>87</sup>. Most of the malignancies that affect humans are of epithelial origin, and in the early stages, tumors continue to express phenotypical traits of the epithelium, such as polarization, lack of motility, and an ability to form continuous sheets. They also express markers of the untransformed cells of origin, for example cytokeratins and E-cadherin, hallmarks of the epithelial state<sup>96</sup>. As the tumors continue to progress to more advanced stages, the neoplastic cells start showing an “undifferentiated” phenotype where they no longer resemble the cells of origin, morphologically or otherwise, and display mesenchymal features, including motility and invasiveness. Cells with mesenchymal properties are frequently located at the invasive front of primary tumors and express mesenchymal markers such as FSP1,  $\alpha$ -SMA, desmin, and vimentin<sup>107</sup>.

Tumors with these characteristics are deemed to be more aggressive, more likely to disseminate and form distant metastases and carry a worst prognostic for the patient. The acquisition of these malignant traits is proposed to be due to the reactivation within carcinoma cells of previously latent EMT programs during tumor progression<sup>108</sup>. The cells undergo an EMT in the primary tumor, then disseminate throughout the body, where they newly acquire features include resistance to apoptosis and anoikis, which facilitate survival after intravasation<sup>109</sup>. Upon arrival to distant sites, the cells reverse their mesenchymal phenotype in a process of mesenchymal-to-epithelial transition to form epithelial metastases<sup>108</sup>. While the signaling pathways and the transcriptional programs involved in cancer progression-associated EMT remain fundamentally the same as in other types of EMT, the heterogeneity and constant evolution of the tumor microenvironment requires that the EMT program in metastasis must be able to adapt itself to these conditions to allow the tumor to metastasize<sup>110</sup>. The plasticity of the epithelial cells allows a rapid response to EMT-inducing signals, but also implies that there must be molecular and cellular mechanisms operating to ensure long-term residence of the cells in one state or another<sup>96</sup>. Recently, mechanical signals from the tumor microenvironment have been proposed as key regulator of the induction of EMT during metastasis. A non-invasive primary tumor generates a desmoplastic stromal response that increases the stiffness of the ECM, which in turn causes the release of

TGF- $\beta$ , a well-known inductor of EMT, hence increasing tumor invasiveness<sup>111</sup>. Once in the secondary site, the unaffected local microenvironment attenuates the EMT-inducing signaling cascade allowing the re-epithelialization of tumor cells and the formation of epithelial metastasis. This response to the microenvironment is accompanied by biochemical changes in order to adapt the nutrient uptake and cellular metabolism to the needs of the cells undergoing the transformation<sup>112</sup>. The metabolic reprogramming that favors the pathways of aerobic glycolysis, a hallmark of cancer cells, is becoming increasingly recognized as important in maintaining the undifferentiated state during EMT.

Epigenetic mechanisms also play a role in epithelial plasticity during metastasis. Hypermethylation of E-cadherin promoter has been described as responsible to the loss of its expression during cancer progression, and the heterogeneous pattern of methylation present in some cancer cell lines is compatible with the variability of the protein expression<sup>113</sup>. Alternatively, hypomethylation of the promoters of transcription factors that favor EMT has also been described, as well as changes occurring in the histone marks in cell undergoing EMT during cancer progression<sup>114</sup>. For example, it has been shown that the EMT-associated transcription factor Snail and Slug can recruit histone deacetylases to the E-cadherin promoter region and thus repress gene transcription<sup>115,116</sup>.

The role of EMT in metastasis have also been disputed as it is been difficult to distinguish *in vivo* between the tumor cells with mesenchymal properties derived from EMT and the mesenchymal cells of the tumor stroma<sup>117</sup>. However, a study showed used a flox-stop-flox LacZ transgenic mouse model combined with a Cre recombinase controlled by an epithelial promoter to track epithelial cancer cell fate and demonstrated that mesenchymal cells arise from an epithelial origin and can be located close to the tumors in c-Myc driven tumors. This study, together with numerous demonstrations *in vitro* and histologic observations of epithelial marker loss in the invasive front of the tumors, provides sufficient evidence to justify the role of EMT in cancer progression.

Besides metastasis, there are other roles of EMT in the context of cancer, for instance immunosuppression. Snail-induced EMT has been shown to impair the activation of dendritic cells and induce regulatory T cells both *in vitro* and *in vivo* through thrombospondin-1<sup>118</sup>, thus causing an immunosuppression that could be beneficial for the tumor development and dissemination. Furthermore, ZEB1 has been found to induce the expression of PD-L1, a ligand for the inhibitory receptor PD-1 in CD8+ tumor infiltrating lymphocytes<sup>119</sup>. PD-L1 is repressed by miR-200, and when ZEB1 relieves this repression, it leads to CD8+ T-cell immunosuppression and metastasis. The importance of this regulation *in vivo* has been confirmed by robust correlations between EMT, miR-200 levels and PD-L1 expression in multiple human cancer datasets.

Another important trait in cancer progression acquired via EMT is the drug resistance. Drug resistance is frequently accompanied by EMT in several types of cancer, including pancreatic, bladder, and

breast<sup>120</sup>, and recently, the causal role of EMT in resistance to chemotherapy was demonstrated experimentally using a transgenic mice model for lung cancer<sup>121</sup>. GFP under the control of a fibroblast specific protein 1 promoter was used to trace EMT, and mice were treated with cyclophosphamide, resulting in a successful reduction of the tumor mass. The remaining cell fraction was enriched in cells expressing GFP, which were resistant to apoptosis induction and showed no significant reduction in cell number compared to epithelial cells. Other observations have contributed to this hypothesis, for example, the restoration of sensibility to alkylating agents when TGF- $\beta$  signaling is blocked, and the increase of its expression induced by the treatment with doxorubicin, as well as the promotion of a trastuzumab-resistant phenotype when Wnt3 is overexpressed in breast cancer cells, among others<sup>120</sup>. Further evidence arises from various studies in breast cancer models where the Snail/Slug transcription factors were able to attenuate cell cycle and confer resistance to cell death induced by pro-apoptotic signals, and overexpression of these transcription factors induced EMT phenotypes with multidrug resistance<sup>122</sup>. Moreover, the promoters of ABC transporters, some of which are in part responsible for the multidrug resistance phenotypes, carry several binding sites for EMT-inducing transcription factors, and overexpression of Twist, Snail, and FOXC2 increases the promoters' activity<sup>123</sup>.

But perhaps the most interesting aspect of EMT involvement in carcinogenesis is the role in the origin of cancer stem cells (CSC). CSC are a minority of self-renewing, stem-like cells within tumors, functionally defined by their ability to originate new tumors. Guided by the fact that during the metastatic process, disseminated cancer cells undergoing EMT would require self-renewal capability to spawn macroscopic metastases, Mani *et al* studied the link between the mesenchymal-appearing cells generated through EMT and stem cells *in vitro* in breast cancer cell lines<sup>124</sup>. EMT was induced in nontumorigenic, immortalized human mammary epithelial cells (HMLEs) by ectopic expression of either Twist or Snail. As a result, the cells not only acquired mesenchymal traits such as fibroblast-like appearance, downregulated epithelial markers, and upregulated mesenchymal markers, but also stem cell features, including CD44<sup>high</sup>/CD24<sup>low</sup> expression profile and the capacity to form mammospheres *in vitro*. These results were corroborated when TGF- $\beta$ 1 was used as EMT-inductor. These cells were also able to originate bipotential progenitors, luminal cells and myoepithelial cells when cultured in Matrigel. The study showed for the first time that cells that have undergone an EMT behave in many respects similarly to stem cells isolated from normal or neoplastic cell populations. Shortly after, Morel *et al* published a study showing that CD44<sup>+</sup>/CD24<sup>-</sup> cells with CSC properties can originate from primary CD44<sup>-</sup>/CD24<sup>+</sup> human mammary epithelial cells after activation of the Ras-MAPK signaling pathway and that this process was accelerated by induction of EMT with TGF- $\beta$ 1<sup>125</sup>.

These findings show that EMT programs provide a ready-to-use source of CSC, as neoplastic cells don't need to invent novel programs for the dedifferentiation of epithelial cells within carcinomas, but

appropriate of strategies already operative in normal antecedent cell populations<sup>126</sup>. Since the first studies in mammary tissues suggesting that EMT programs generate cells with traits of CSCs, this have been recognized as a common mechanism in various malignancies, including pancreatic, colorectal, and hepatocellular carcinoma. The association has been recognized for gastric cancer as well. One of the first studies used *in vitro* isolated CSC from the gastric cancer cell line SGC7901 to show that these cells, aside from the stem cell properties of enhanced invasion and tumorsphere formation capability, also showed features of an EMT such as reduced expression of E-cadherin, increased levels of vimentin and MMP-2<sup>127</sup>. Further evidence of the potential link was demonstrated *in vivo* in primary gastric cancer tissue samples by Ryu et al. Immunohistochemistry staining was performed for Snail, ZEB1, E-cadherin, vimentin and  $\beta$ -catenin and for CD44 as CSC marker, and the combination of Snail-1, vimentin, E-cadherin, and CD44 was found to be an important and independent factor associated with aggressiveness of primary gastric cancer<sup>128</sup>. Furthermore, the Wnt5a-induced EMT was found to maintain CSC properties in MKN7 cells<sup>129</sup>, and as did hypoxia-induced EMT in BGC823 and SGC7901 cell lines<sup>130</sup>.

In agreement with these findings, in a model of infection with *Helicobacter pylori* in AGS, MKN45, and MKN74 human gastric adenocarcinoma cell lines *in vitro*, our group has shown the emergence of a population of CD44<sup>high</sup> cells with properties of CSC through an EMT process<sup>131</sup>. The cells acquired an elongated phenotype, the expression of mesenchymal markers (Snail1, ZEB1, and vimentin) and CD44 was increased, and they showed a higher ability to migrate, invade, and form tumorspheres *in vitro* and tumors when grafted in immunodeficient mice, compared to the CD44<sup>low</sup> cells. Moreover, the induction of the EMT and the CD44<sup>high</sup> cell population was demonstrated to be dependent on the bacterial oncoprotein CagA, as isogenic mutant strains depleted for *cagA* or *cagE* were unable to produce the same result. Recently, the production of MMP-3, a matrix metalloprotease also related to the EMT process, was showed to be not only CagA-dependent, but also to correlated with the level of phosphorylation of CagA on its EPIYA motifs, which are directly linked to the bacterial virulence<sup>132</sup>. IHC staining on human and murine gastric mucosa tissue samples confirmed a high expression of CD44 and EMT markers in *H. pylori* infected cases, in gastric dysplasia and in carcinoma<sup>131</sup>. Regarding the mechanisms involved in the process, this same work showed that ERK and JNK are involved in *H. pylori* *cagA*-positive strains and induced EMT-like changes, CD44 overexpression and the ability to form tumorspheres *in vitro*<sup>131</sup>. Other studies have addressed the matter. It has been shown that the Wnt/ $\beta$ -catenin activation was necessary for the increased expression of Nanog and Oct4 induced after infection with *H. pylori* in gastric epithelial cell lines<sup>58</sup> and *in vivo* observations of a follow-up study on patients with gastric dysplasia and early gastric cancer, before and after eradication of *H. pylori*, showed a relation between the expression of TGF- $\beta$ 1 and EMT markers mRNA and expression of CD44, suggesting that *H. pylori* infection may trigger a TGF- $\beta$ 1-induced EMT and the emergence of CSC<sup>133</sup>.

## **2. Genomic alterations and driver mutations**

A recent study by Wang *et al*, outlined the genetic changes associated with a cohort of 100 gastric cancer cases and control using whole genome sequencing<sup>134</sup>. The authors found 3.5 million somatic mutations, of which almost 18 000 were predicted to be protein altering, and MSI tumors were the group with more SNPs. Among the MSS tumors, diffuse-type tumors had the lowest amounts of SNPs and deletions, which in turn was significantly increased with advanced age of the individual. The somatic mutation rate approached 10 SNVs per Mb, which is higher than for most solid cancers apart from lung cancer in smokers and melanoma. The study identified sixteen driver genes, whose mutations impart a survival advantage to the clone that harbors the so-called specific driver mutations in the genes, i.e. mutations though to underlie the malignant phenotype. These include well-known alterations in gastric cancer such as *ARID1A*, *CDH1*, *CTNNB1*, and *TP53*, as well as the less studied *MUC6*, *RNF43*, *CTNNA2*, *GLI3*, *ELF3*, *ZIC4*, *TGFBR2*, *ACVR2A*, *DCLK1*, *THBS1*, *SMAD4*, and *RHOA*.

Remarkably, mutations on TP53 have been previously acknowledged as one of the most common genetic aberrations in patients with gastric cancer<sup>135</sup>. It is a tumor suppressor that induces cell cycle arrest, apoptosis, and senescence in response to stress cues. The mutation rate in gastric cancer cohorts varies from 35% to 73% of patients, and normally these mutations impair the protein function, contributing to its role in cancer development<sup>135</sup>.

## **3. Somatic copy number aberrations**

Intestinal type gastric cancers display a large number of chromosomal alterations, gains or losses, in comparison to diffuse type gastric cancer and normal gastric tissue<sup>135</sup>. Notably, the study by Wang *et al*, identified disruptions in the copy number of two genes important genes in the treatment of gastric cancer: the human epidermal growth factor receptor (*ERBB2* or HER2) and MET<sup>134</sup>.

The HER2 is a proto-oncogene that encodes for a protein of the epidermal growth factor receptor family, which, as detailed previously, is part of the signaling pathways implicated in gastric carcinogenesis. It is tyrosine kinase receptor that transmits extracellular signals to initiate activity of MAP kinases, PI3K, PLC, PKC and STAT<sup>136</sup>. HER2 positivity in gastric cancer is very variable depending on the study, it has been reported to be upregulated in 4-53% of gastric tumors<sup>135</sup>. Contrary to the breast cancer scenario, where overexpression/amplification of HER2 is associated with increased aggressiveness, risk of distant metastases and poor prognosis in general, in gastric cancer the association is inconsistent, despite multiple studies addressing the subject<sup>136</sup>. However, a recent randomized clinical trial, the ToGA study, carried in 24 countries used standard chemotherapy regimens (cisplatin with either fluorouracil or capecitabine) combined with Trastuzumab, a monoclonal antibody directed to the extracellular domain of HER2 and

showed that the use of the Trastuzumab resulted in longer survival times in patients with HER2 positive gastric cancer, compared to the group treated with chemotherapy alone<sup>137</sup>. The results were improved when the HER2 positivity threshold was set to IHC score 2+ plus positive FISH or IHC score 3+. The exact timing of the expression of HER2 in gastric cancer is also debated, but a variation between histological subtypes have been established, being much more expressed in intestinal type tumors than in the diffuse type<sup>135</sup>. A higher expression has been reported in proximal tumors originating from the gastroesophageal junction than in the ones originated from mid- and distal stomach<sup>135</sup>.

cMET is a tyrosine kinase receptor for the hepatocyte growth factor (HGF) that is activated in various human cancer, including 10-53% of gastric carcinomas<sup>135,138</sup>. As cited before, it can be overactivated by amplification in the copy number of the gene, but also by mutations leading to constitutive activation, upregulated transcription, or plainly by the overexpression of HGF<sup>138</sup>. Aberrant signaling of the cMET pathway are correlated with advanced tumor stages and poor prognosis<sup>138</sup>. Interestingly, the activation of cMET is partly responsible for the acquisition of a mesenchymal-like phenotype and motility in epithelial cells infected with *H. pylori*<sup>139</sup>. The bacterial oncoprotein CagA was showed to bind c-MET, and could modulate cellular functions by deregulating c-MET receptor signaling.

#### **4. Epigenetic alterations**

Epigenetic regulation of gene expression has been showed to be an important phenomenon in gastric carcinogenesis. The main epigenetic mechanisms involved in gastric cancer pathogenesis are DNA-methylation and microRNAs<sup>135</sup>.

DNA-methylation occurs in CpG islands, sites with a high CG content that tend to cluster within the 5'-UTR regions upstream of gene promoters<sup>135</sup>. When these zones near the promoters are methylated, transcription factors are prevented of binding the DNA and usually gene expression as a consequence. Tumor suppressor genes, oncogenes, and genes involved in tumor progression and metastasis have been found to be regulated by DNA-methylation in gastric cancer<sup>140</sup>. Hypermethylation of the promoter of tumor suppressor genes such as *TP53*, *CDKN2A*, *MLH1*, and *CDH1*, as well as pro-apoptotic proteins, is often present in gastric cancer patient samples. Albeit less frequent, hypomethylation has also been described in gastric cancer, promoting derepression of target genes<sup>140</sup>. For example, the oncogene *MYC* was showed to be hypomethylated in gastric cancer with lymph node metastasis, and gastric cancer patient samples showed hypomethylation on the *ALDH2* promoter.

MicroRNAs are small (20-22 nucleotides) noncoding RNAs that can regulate the expression of target genes at the post-transcriptional level, and a single miRNA can target several messenger RNAs, affecting multiple signaling pathways<sup>140</sup>. MicroRNAs could be regulated by DNA-methylation as well. Downregulation via methylation of miRNA's promoters has been described to activate growth-promoting

pathways such as CDK6-VEGF and c-MYC. miR-9 downregulation via hypermethylation in gastric cancer has been found to increase proliferation, cell migration and invasion capacity. Target genes of miR-9 include NFκB, RAB34, Cyclin D1, and Ets1, so when the repression by miR-9 is lost, gastric carcinogenesis may be enhanced<sup>140-142</sup>. But independently of the mechanism, microRNAs regulation in general is associated with the pathogenesis of gastric cancer, promoting tumor growth (Let-7a, miR-375), tumor invasion and metastasis (miR21, miR-148a), and interacting with the tumor environment (miR-874)<sup>143</sup>.

## **v. Diagnosis**

One of the main challenges of gastric cancer diagnosis is that the disease is usually asymptomatic until it progresses to an advanced stage. The manifestations can include gas, reflux, biliary colic, and irritable bowel, and can be confused with dyspepsia and ulcer disease<sup>144</sup>. However, there are some features that could lead the clinician to suspicion, such as anorexia, dysphagia, weight loss, recurrent vomiting, and early satiety. Pain tends to be mild and located in the epigastric region, but progresses in intensity and duration as the disease advances.

Since the information obtained in the physical exam is limited in early stages, the diagnosis of gastric cancer and precancerous lesions normally relies in an upper GI endoscopy, and less frequently in a barium swallow tests. The latter has been widely replaced by endoscopy, especially given the great advancement in the field, but it remains very useful in the diagnosis of linitis plastica<sup>144</sup>. The use of endoscopy has a 90-96% accuracy in the diagnosis and detection of gastric cancer. One of the practical advantages of endoscopy is that any suspicious lesion can be biopsied to perform further pathologic analysis and confirm the diagnosis. The Sidney system recommends two biopsies from the corpus, two from the antrum, and one from the angular notch.

Macroscopic study of the stomach via white light endoscopy is a basic step for diagnosis of gastric cancer, and it allows the subclassification of early gastric cancer based on the characteristics of the lesions of type 0 in the Japanese system<sup>145</sup>. In recent years, technical innovations have made possible to go deeper in the observation of subtle morphological changes, difficult to detect using the white light endoscopy<sup>145</sup>. The so-called image enhanced endoscopy (IEE) includes the narrow band imaging, the blue light imaging and the i-scan. The use of IEE has improved the accuracy of early gastric cancer and increased the opportunity for endoscopic resection. Another alternative is the use of chromoendoscopy, in which a colored solution (frequently indigo carmin solution combined with acetic acid) is used to improve the visualization of superficial topography of the mucosa.

However, the method remains to be invasive and costly, and poor suited for mass screening, especially in regions with high gastric cancer risk and high prevalence of *H. pylori infection*. Therefore, the

search for non- or minimally-invasive diagnostic methods is very important. The given the high prevalence and mortality of gastric cancer in their country, the Japanese have lead the research on this field and a mass screening program has been implemented since the decade of 1960, initially with the use of X-ray examination<sup>146</sup>. The screening protocol has been carried out in other populations such as a high risk region in Costa Rica, where about 7000 individuals participated in an X-ray screening, followed by videoendoscopy, gastric biopsies, and treatment when pertinent<sup>147</sup>. During the study, the gastric cancer mortality was decreased by half, and the X-ray intervention exhibited 88% sensitivity, 80% specificity, and 3% predictive value for individuals with two screening visits, and case survival was 85% in the intervention group after 5 years, compared to 12-35% among the controls. The main problem for the permanent implementation and scaling up of the program was its high cost, which make it unsuitable for a non-rich country like Costa Rica.

Another approach is the search for serum biomarkers for gastric cancer. Pepsinogens (PG) are the proenzymes for pepsin, and are secreted in the gastric lumen: PG-I is secreted only by chief and mucous neck cells of the fundic glands, while PG-II is also produced by the cells in the pyloric glands<sup>148</sup>. About of PG can be found in serum and may function as a marker of the morphologic and functional status of the gastric mucosa. Thus, the total levels of serum pepsinogen, and more important, the PG-I:PG-II ratio can be use as predictors of gastric pathologies<sup>148</sup>. In recent years, a new method has been proposed, called the ABC method, which consists in the evaluation of gastric cancer risk based on the detection of *H. pylori* infection and serum pepsinogens<sup>149</sup>. The combined results place the patient in groups of low and high risk of gastric cancer, and those of the high-risk group are recommended to receive endoscopy. From 2011 to 2013 this program examined the blood of 17 000 patients, of which 3000 underwent endoscopic examination and 65 cases of gastric cancer were detected, 80% in early stages. The ABC program was established as an opportunistic screening, contrary to the organized campaigns of screening by the X-ray methods, and given the results, it could be a useful and cheaper tool to perform mass screening in high risk populations. Alternatively, a kit for serum biomarkers known as GastroPanel was developed in Finland in the late 1990's, and includes the detection of *H. pylori* status, serum PG-I and PG-II, and gastrin-17. A meta-analysis of 27 studies in different populations showed a sensitivities of 54,8% and 70.4% , and specificities of 76% and 92.4%, for atrophic gastritis of the antrum and the corpus, respectively<sup>150</sup>.

## **vi. Treatment**

Currently, the curative treatment of gastric cancer relays in the resection of the tumor. The procedure can be performed endoscopically when it is diagnosed in early stages (the invasion goes no deeper that the submucosa, regardless of the lymph node involvement) in patients with no or low risk of

lymph node metastasis, defined by endoscopic ultrasound, computed tomography and histological analysis<sup>151</sup>. The endoscopic resection could be performed either as mucosal resection (EMR) or submucosal dissection (ESD), depending on several criteria such as the size and the histology of the tumor, the ulceration and the depth of invasion, and the vascular involvement. These methods represent a minimally invasive, organ-preserving approach for the treatment of premalignancies or early stage cancers, usually with successful outcomes. When the patient has a cancer in advanced stage or an early gastric cancer that does not meet the criteria for endoscopic resection, a surgical total or subtotal gastrectomy, with D2 lymphadenectomy in the advanced cancers, must be performed<sup>152</sup>. These procedures may be done in an open surgery or laparoscopically, and the choice of the extent of the resection, the type of reconstruction of the gastrointestinal tract, and the method used, depend on the anatomic-pathologic features of the tumor and the patient's background and condition. No matter which procedure is used, a gastric resection is an aggressive intervention that leads to severe changes in the anatomy and physiology of the gastrointestinal tract and may have severe acute complications and long-term effects.

Studies show that the outcome of treatment can be improved if chemo- and/or radiotherapy accompany the surgical procedure. In Europe and the US, the predominant strategy consists on applying three to six cycles of a combined therapy (cisplatin and 5-fluorouracil and/or epirubicin) prior to the surgery, which provides a significant improvement in 5-year disease-free survival compared to surgery alone<sup>153</sup>. Radiotherapy before the surgery is not a usual practice in gastric cancer treatment<sup>154</sup>. In the postoperative setting, capecitabine or 5-FU monotherapy combined with radiation is the standard care<sup>153,154</sup>. For the unresectable locally advanced or metastatic gastric cancers the treatment is systemic therapy with a combination of a platinum and a fluoropyrimidine, with optional addition of anthracyclines or docetaxel<sup>155</sup>. These treatments have severe secondary effects, and even if they are the best available option, the overall treatment failure is high, with a 5-year recurrence or death rate over 60% in Western countries<sup>156</sup>.

There are few options of targeted treatment for gastric cancers. The major advance in the last years has been the introduction of Trastuzumab in the treatment of Her2 positive cases after the ToGA study<sup>137</sup>. Other monoclonal antibodies and pathway specific inhibitors have been tested in clinical trials, some of them with encouraging results, such as the ramucirumab (inhibitor of VEGFR-2), but there is still not enough evidence to include them in the routinely treatment schemes. Moreover, a recent meta-analysis of 11 targeted therapy trials reporting data about survival compared to the use of chemotherapy alone, found low quality evidence of a small effect of targeted therapies on mortality and progression free survival, even when separate in type of agent or tumor type<sup>157</sup>. This study highlights the need for further and deeper research for the improvement of targeted therapy in gastric cancer.

A significant challenge is the accurate identification of markers of response to a given treatment, which starts with a better understanding of the molecular events behind gastric carcinogenesis. The comprehension of the signaling pathways involved in carcinogenesis also contributes to the development of new treatments or even the use of already known molecules in an attempt to improve the overall outcome of the disease. For instance, the retinoid metabolism has been described to have a role in the carcinogenesis of hematologic and solid tumors and the all-trans retinoic acid (ATRA) exhibits a pro-differentiation role in acute promyelocytic leukemia. Based on the fact that gastric CSCs present a high ALDH activity and that some of the ALDH isoforms are involved in retinoid metabolism, a study by Nguyen *et al* showed all-trans retinoic acid (ATRA) has an inhibitory effect on gastric CSCs, making it a potential adjuvant for the treatment of gastric malignancies<sup>158</sup>. Part of the present work intends to characterize the role of the Hippo/YAP1 pathway in gastric CSCs to test if and how the inhibition of this pathway could contribute to the impairment of the carcinogenesis process in the stomach, aiming to the development of adjuvant therapies to gastric cancer.

## **vii. Models of study for human gastric cancer**

### **1. *In vivo* models**

Genetically engineered mouse models are convenient tools to study multiple diseases, and gastric cancer is not an exception. Current technologies have made it possible to manipulate the genetic background of small animals with minimal difficulty and to create strains adapted to the study of different aspects of the pathology. However, it must be kept in mind that these models show some limitations, such as modest gastric pathology, slow time course, and the absence of invasive or metastatic tumors, as well as inherent factors to the animal models such as high dependency on the genetic background, gender, diet and housing conditions<sup>159</sup>. Animal stress associated with manipulation, overcrowding, light cycles, temperature variations, and inadequate sanitation leading to infections with rodent pathogens have great influence in the outcome of experiments in murine models<sup>159</sup>. The compliance of ethical standards and considerations are of great importance in all the work performed involving animal facilities.

There are significant anatomical differences between the upper gastrointestinal tract of mice and humans<sup>160</sup>. The murine stomach mucosa is divided in two regions, the forestomach and the glandular stomach, while humans lack the first. The mouse glandular stomach is divided into three anatomical regions: the small cardia, the fundus, and the antrum, and the human is divided in four: cardia, fundus, body, and antrum. At the histological level, the gastric wall organization into four layers (mucosa, submucosa, muscularis propria, and serosa) and the basic cellular components are similar in both species. Humans have two types of gastric mucosa: fundic (body and fundus) and pyloric (cardia and pylorus), and

transition mucosa between the two types. In mice, the gastric pits are straight tubes similar to the human fundic mucosa. In the cardia, the glands are lined by abundant mucous cells, in the fundic region, they contain mucous neck cells, parietal cells, and chief cells, and in the pylorus the glands are composed only of mucous cells. A detailed comparison of both species is shown in table 5.

**Table 5. Comparative anatomy and histology of the murine and human stomach<sup>160</sup>.**

	Feature	Mouse	Human
<b>Gross</b>	Forestomach	Yes, keratinized squamous epithelium, thin walled right half	No
	Glandular stomach	Left half of the stomach, thick mucosa: cardiac, fundic, and pyloric region	Yes: cardias, fundus, corpus, and pylorus
	Rugal folds	Not as prominent	Prominent when stomach is empty
	Areae gastricae	Not described	Mucosal shallow grooves that don't flatten with distention
	Limiting ridge	Present projection between glandular and non-glandular stomach mucosa	No
<b>Tissue</b>	Mucosa	Keratinized squamous or glandular, simple straight glands; cell composition varies with region	Simple columnar epithelium
	Lamina propria	Yes	Same
	Submucosa	Yes	Same
	Muscularis mucosa	Yes	Same
	Muscular tunic	Inner oblique, middle circular, outer longitudinal	Same
	Serosa	Yes	Yes
	Squamous epithelium	Amount of keratin is diet- and fasting-dependent	No
<b>Cells</b>	Foveolar (cardiac) cells	Simple columnar, mucus-secreting, lines top portion of the mucosa	Same
	Parietal cells	Eosinophilic, acid-secreting, midportion of glands	Same
	Chief cells	Basophilic, zymogen-secreting, near the base of glands	Same
	Cardiac glands	Mucus glands near limiting ridge, smallest portion	Same
	Fundic glands	Parietal and chief cells, top portion lined with foveolar cells	Same
	Pyloric glands	Near duodenum, mucus cells only	Parietal cells, chief cells absent
	Enterochromaffin	Between basement membrane and chief cells	Diffusely scattered in epithelium

*a. Chemical carcinogenesis*

The use of carcinogenic compounds, especially nitrogen derivatives, has been used to induce carcinogenesis in mice. The induction of gastric adenocarcinoma is achieved after 5 weeks of treatment with N-methyl-N-nitrosurea (MNU) in drinking water, and the model is extensively used in the study of signaling pathways and transcription factors<sup>159</sup>. Its two main limitations are that the mechanism of induction of carcinogenesis by MNU is not known and that it lacks the inflammatory component present in an important proportion of gastric cancers.

*b. Genetically engineered mice*

The initial genetically modified mouse models of gastric cancer were based on oncogenes associated with transformation, but not specific of the stomach, such as the SV40 T antigen, which then was improved by using a stomach-specific promoter. However, this and other early models did not developed dysplasia, the malignancies did not progress following the precancerous lesions sequence, nor were they associated to chronic inflammation. Currently transgenic or knock-out mice, who overexpress or are deficient in growth factor or cytokines, or with mutations in tumor suppressor genes or oncogenes are the most used models for the study of gastric carcinogenesis. For example, the insulin-gastrin (INS-GAS) mice, where the human gastrin gene is under the control of the insulin promoter. Consequently, the serum level of gastrin is increased and the mice develop atrophy and hypochlorhydria, which progress to metaplasia and dysplasia and finally invasive gastric cancer in the corpus at 20 months of age. The infection with *H. felis* or *H. pylori* accelerates the process. Other examples of genetically altered mice models are listed in table 6.

**Table 6. Genetically altered murine model of gastric cancer. Modified from <sup>159</sup>**

Genotype	Cancer incidence (%)	Duration	Location	Phenotype
<b>MTH1<sup>-/-</sup></b>	13	18 months	Antrum	Adenocarcinoma, dysplasia, hyperplasia, lung and liver tumors
<b>TFF1<sup>-/-</sup></b>	30	5 months	Antrum	Intramucosal carcinoma, hyperplasia, NF-κB activation
<b>Smad4<sup>+/-</sup></b>	100	12-18 months	Corpus/ Antrum	Adenocarcinoma, dysplasia, hyperplasia, duodenal tumor
<b>GB-Smad4<sup>f/f</sup></b>	100	12-18 months	Antrum	Dysplasia, hyperplasia
<b>GAS<sup>-/-</sup></b>	60	12 months	Antrum	Dysplasia, metaplasia, atrophy, and increased susceptibility to MNU
<b>Gp130<sup>f/f</sup></b>	100	6 months	Antrum	Adenoma, decreased TFF1 expression
<b>IL1-β</b>	<70	12 months	Transition	Adenocarcinoma, dysplasia, metaplasia, atrophy, synergized with <i>H. felis</i>
<b>K19/K-ras</b>	37.5	16 months	Corpus	Dysplasia, metaplasia, atrophy
<b>Wnt1/C2me</b>	100	20 weeks	SCJ	Adenocarcinoma, dysplasia, metaplasia
<b>CDH1<sup>+/-</sup> + MNU</b>	45.8	40 weeks	Antrum	Signet-ring cell carcinoma, adenoma
<b>CDH1/p53</b>	100	12 months	Corpus	Poorly differentiated adenocarcinoma, signet-ring cell carcinoma
<b>RUNX3<sup>-/-</sup> + MNU</b>	71	52 weeks	Corpus/ Antrum	Adenocarcinoma, metaplasia, hyperplasia
<b>Villin-KLF4<sup>f/f</sup></b>	29	80 weeks	Antrum	Adenoma, susceptibility to MNU

### c. *Xenotransplantation in immunodeficient mice*

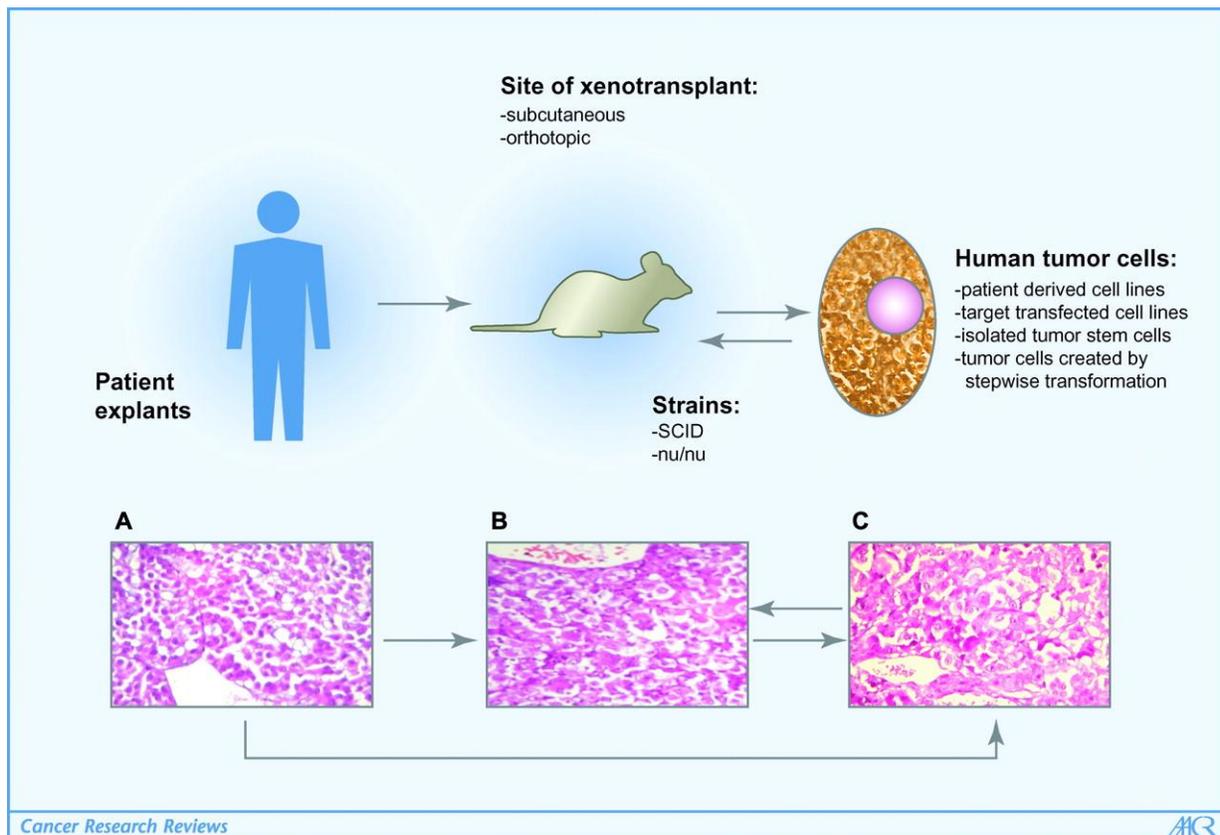
However useful the genetically modified mice models in understanding the basic oncogenic processes, they are still significant physiological differences between human and mice that affect the outcome of the research. Albeit not perfect, the xenotransplantation of human cancer cells in immunodeficient mice allows to overcome some of these drawbacks while keeping the practical convenience of the murine models. Strains of athymic mice (nu/nu) and other immunodeficient mouse strains (severe combined immunodeficiency, SCID) allow the grafting of explants of human tumors or cell lines to grow them as xenotransplants for the study of tumor cancer biology, notably the testing of anticancer drugs<sup>161</sup> (Figure 3). The model allows to test a wide variety of tumor types, the possibility of genetic or therapeutic manipulation before the implantation, and it is the method of choice for the identification of CSCs, that will be discussed later in this work. All the same, the model have disadvantages that must be taking in consideration, especially because the surroundings of the tumor remain to be the host's, blood and lymphatic supply and neovascularization as well as the tumor stroma are murine<sup>161</sup>.

Technically, the orthotopic engraftment is difficult, so the subcutaneous transplantation is widely used, introducing an artificial compartmentalization which renders the metastatic growth very infrequent and could induce architectural and histological changes in the tumor<sup>161</sup>.

At present, one of the most used strains of mice in xenografts for cancer studies is the NSG strain. Also known as NOD-*scid* gamma or NOD-*scid* IL2Rg<sup>null</sup>, in a NOD genetic background these mice carry two mutations: the *scid* or *Rag1*<sup>null</sup> or *Rag2*<sup>null</sup>, to induce immunodeficiency of the adaptive immune system, and the *IL2ry*<sup>null</sup>, to prevent cytokine signaling and cause a deficiency in NK cells<sup>162</sup>. Thus, they are highly immunodeficient and good recipients of human cancer cells.

For gastric cancer, subcutaneous patient derived xenografts (PDXs) have been developed from surgically-obtained tissues from gastric cancer patients and from gastroscopic biopsies, with moderate success in the establishment of tumors (24.2% and 34.1%, respectively)<sup>163,164</sup>. In these studies, the histological type and tumor cell percentages, as well as the ex vivo and overall procedure time were shown to be determinant in the achievement of the PDXs. Remarkably, the biological characteristics of the PDXs were stable over subsequent passages and highly consistent with the primary tumors, providing a very useful tool for the preclinical testing of anticancer drugs and tailoring of targeted therapy.

Orthotopic transplantation in the stomach has been performed in the stomach with cell line suspensions and intact tumor tissue, with a greater extent of metastasis in the latter, perhaps due to the intact histology and interaction of cancer cells with the co-implanted stroma<sup>165</sup>. The metastasis reflected the sites in the human pathology, for instance the liver and the peritoneum.



Cancer Research Reviews

AKR

**Figure 3. Xenograft murine models<sup>161</sup>.** Cell lines or patient explants can be grafted subcutaneously or orthotopically in immunodeficient mice.

## 2. *In vitro*

The wider use of *in vivo* models is still restricted by technical issues. The tumors can only be maintained in mice, which is costlier and requires a higher specialization than cultured cell lines<sup>166</sup>. There are also long latency periods from engraftment and variable success rates. Therefore, the use of cell lines cultured *in vitro* is relevant, especially in the initial steps of most current research and in the high scale screening of anticancer drug candidates. Commercially available gastric cancer cell lines commonly used are described in table 7.

**Table 7. Commercially available gastric epithelial cell lines.**

Cell line	Gender /Age	Origin	Gastric cancer subtype	Morphology	Genetic features
<b>AGS</b>	F/54	Stomach (primary tumor)	Intestinal	Epithelial adherent, non-cohesive	Hyperploid Mutations: CDH1 (homozygous), CTNNB1 (heterozygous, function gain), KRAS (heterozygous), PIK3CA (heterozygous)
<b>MKN 7</b>	H/39	Lymph node metastasis	Well differentiated tubular AC	Epithelial, cohesive	Mutations: TP53, APC (silent) Oncogenes: erb2 CDH1 WT, CTNNB1 WT, CD44 WT
<b>MKN 28</b>	F/70	Lymph node metastasis	Moderately differentiated tubular AC	Epithelial, cohesive Differentiation in cell polarity and microvilli with core filaments	Mutations: TP53, APC (non-sense) Oncogenes: no gross genetic alterations CDH1 WT, CTNNB1 WT, CD44 silenced by promoter hyper methylation
<b>MKN 45</b>	F/62	Liver metastasis	Poorly differentiated carcinoma	Multipotentiality for differentiation	TP53 WT, APC WT, CTNNB1 WT, CD44 WT Mutations: CDH1 deletion in exon6-intron 6 junction Oncogenes: c-met (amplification), low bcl-2 expression
<b>MKN 74</b>	M/37	Liver metastasis	Intestinal	Epithelial, cohesive	CDH1 WT, TP53 WT, CTNNB1 WT, CD44 Oncogenes: no gross genetic alterations
<b>NCI-N87</b>	M/?	Liver metastasis	Intestinal	Epithelial, cohesive Differentiation in cell polarity and microvilli	Near diploid CDH1 WT Oncogenes: myc, erb2 Mutations: TP53 (homozygous), SMAD4 (homozygous)

## **b. Stem cells and Cancer stem cells**

### **i. Normal stem cells**

Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation<sup>167</sup>. Self-renewal allows the maintenance of the stem cell pool, as the new cells retain the properties of the cell of origin. The differentiation process, on the contrary, generates heterogeneous, more specialized cells with characteristics specific to a particular organ or tissue.

During embryogenesis, the zygote produced by the fusion of an egg and a sperm is considered to be totipotent, having the capacity to give rise to the embryonic and extra-embryonic (trophoectodermal) tissues<sup>168</sup>. The embryonic tissue, composed by the inner cell mass of the blastocyst is formed by the pluripotent embryonic stem cells (ESCs), which can not only give origin to other ESCs through asymmetrical division but also to all the cells that form the endoderm, mesoderm, and ectoderm<sup>168</sup>.

As development goes further, the pluripotent stem cells become more committed to a particular lineage and their potential to generate new cells is restricted to the specific type of tissue where they reside<sup>168</sup>. These cells are known as multipotent stem cells, present in small quantities in adult tissues. These adult or somatic multipotent stem cells have been identified in practically every tissue in the body, such as the epidermis, neural tissue, gastrointestinal tract, liver, and mesenchyme. But perhaps the most studied, and the prototypical model for adult stem cells, are the hematopoietic stem cells (HSCs).

#### **1. Mechanisms of cell renewal**

Self-renewal is the division with maintenance of multipotency and undifferentiated state, and it is one of the most important properties of stem cells because it allows to maintain the pool of stem cells through life<sup>167</sup>. Yet, it is not an exclusive property of stem cells. Other types of progenitors such as lymphocytes and glial progenitors can also self-renew, but even if they share some mechanisms, the self-renewal of stem cells can be distinguished from those of the restricted progenitors because in the latter, the potential is limited<sup>169</sup>.

In the case of ESC, they possess indefinite self-renewal and pluripotency, conferred by a unique transcriptional and cell cycle regulation<sup>170</sup>. Studies on mouse, rat, and human ESCs allowed the identification of a core subset of transcription factors of “stemness”, which include Oct4, Sox2, and Nanog. These and several other factors act in cooperation to maintain pluripotency and serve as hubs for different signaling pathways. The main signaling pathways involved in self-renewal of ESCs include LIF/STAT3, Wnt/ $\beta$ -catenin, FGF/ERK, TGF/SMAD and PKC signaling.

A key feature of adult stem cells is to survive and retain their proliferative potential through the lifespan of the animal, but this fact does not necessarily mean an infinite capacity to divide or that the self-renewal is constant<sup>171</sup>. The frequency and timing of divisions are regulated to ensure the lifelong maintenance of the stem cell pool in a tissue, as reductions in their proliferative potential could cause tissue atrophy and premature aging, and in turn, mutations that stimulate stem cell division without a balancing differentiation could cause abnormal tissue development and malignancies<sup>171</sup>. The frequency of divisions depends on the nature of the tissue and the need for regeneration.

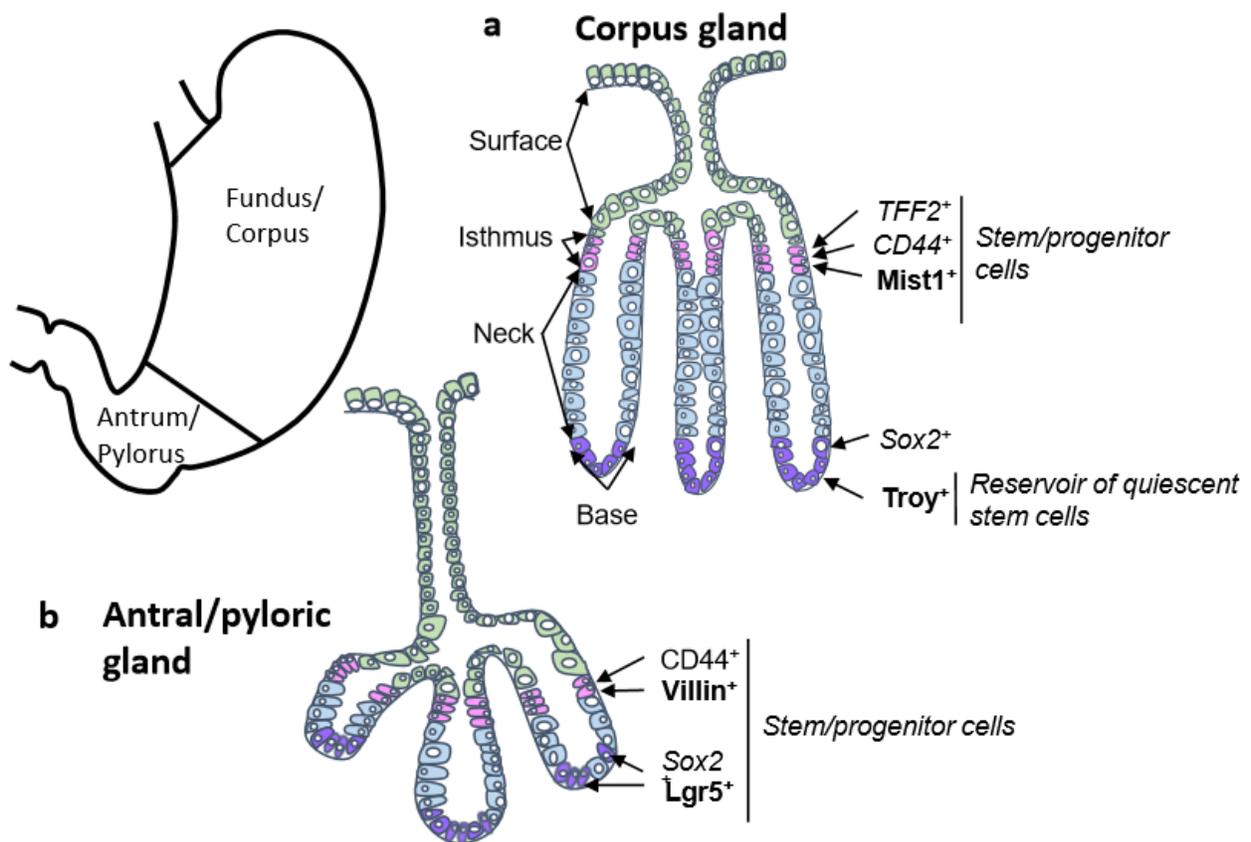
HSCs are required for the development, maintenance and regeneration of the hematopoietic system. In mice, approximately a billion of blood circulating cells are produced every day, and it has been shown that this is a finely regulated process mainly sustained by progenitors (HPCs) committed to the myeloid and lymphoid lineages rather than HSC<sup>171,172</sup>. These are not used in normal hematopoiesis and are preserved for hematopoiesis following special circumstances such as blood loss or infection. Aside from this emergence hematopoiesis, most HSC are in G0 phase and divide once per month, while the homeostasis is maintained by HPCs. HPCs also have the capacity of self-renewal, and interestingly, the granulocytic lineage-committed HPCs are short-term HPCs, differing from their lymphoid counterparts that are long-term HPCs, more similar to HSCs<sup>172</sup>. The regulation of HSCs occurs by two mechanisms: control of G0 phase and fate determination. These processes take place in the bone marrow and are regulated by the stem cell niche, composed by different types of cells that produce extracellular signals to stimulate stem cells<sup>172</sup>. CXCL12 is a cytokine produced by perivascular stromal cells, among others, and it has been described as the main responsible for maintaining the G0 state in HSCs upon recognition by the CXCR4. In contrast, interferons have been identified as activators of quiescent HSCs.

The case is different for intestinal stem cells (ISCs) as the intestinal mucosa is characterized by a very rapid turnover with a high rate of loss. This arise a constant necessity for new cells, which are generated by a population of multipotent ISCs that reside at the base of the crypts<sup>173</sup>. ISCs give rise to transit amplifying cells that expand through multiple cycles of cell division and progressively differentiate as they migrate upwards to the tip of the villi<sup>173</sup>. The Wnt/ $\beta$ -catenin and the Notch signaling pathways have been described as crucial for the preservation of the ISC pool and a recent study in a murine model identified Bmi-1 as a downstream target of both pathways, whose deficiency resulted in reduced proliferation and self-renewal of ISCs<sup>174</sup>.

## **2. Gastric stem cells**

The gastric mucosa, like its intestinal counterpart, is dynamic tissue with a rapid rate of self-renewal. As in other adult tissues, this physiological process is sustained by a subpopulation of multipotent stem cells. The epithelium that constitute the gastric mucosa is glandular, and each gland is considered to

be monoclonal and subdivided into four regions: foveolus, isthmus, neck and bottom (figure 4). The characteristics and type of cells present in the glands varies depending on the zone of the stomach and the function of the gland. In the corpus region, the glands are long and they contain zymogen-producing chief cells in the bottom, acid-producing parietal cells and hormone-secreting enteroendocrine cells scattered throughout the gland, proliferative undifferentiated cells in the isthmus, and mucous-secreting cells in the neck and the pit<sup>175</sup>. In the antrum, the glands are shorter and composed by mucous cells and enteroendocrine hormone-secreting cells that regulate the acid and enzyme production by the glands of the corpus. Both types of glands contain a discrete quantity of adult stem cells, but their localization varies, in the corpus, they can be found in the isthmus while in the antropyloric region glands, they are located at the bottom.



**Figure 4. Architecture of the gastric glands and localization of stem/progenitor cells of the stomach.** (a) Fundic/corpus gland. (b) Antral/pyloric gastric gland. Stem/progenitor markers are indicated in bold.

Putative progenitors/stem cells in the antrum and corpus of the murine stomach were first identified in the isthmus and neck regions, respectively, as a group of small undifferentiated and granule-free cells using 3H-thymidine radiolabeling by Leblond et al <sup>176,177</sup>. These cells give origin to the other granule cells of the gland in a bidirectional pattern of renewal, moving toward the pit and the bottom of

the gastric gland as they differentiate. Further evidence was provided when using a chemical mutagenesis-induced lineage tracing strategy, Bjerkens and Cheng showed that the adult gastric epithelium harbors functional multipotent stem cells<sup>178</sup>. Mice expressing  $\beta$ -galactosidase (LacZ) under control of the Rosa26 promoter were treated with N-ethyl-N-nitrosourea (mutagen), with inactivation of the LacZ allele in some cells as a result. If a progenitor cell has the mutation, all the daughter cells will inherit the nonfunctional allele and will not be labeled, allowing the identification of clones of related cells. With this, they demonstrated the existence of a long-term, self-renewing progenitor cell population in the adult gastric epithelium. With the development of more sophisticated lineage tracing techniques, specific markers for gastric stem cells have been identified, and currently, two co-existing subpopulations of gastric stem cells can be distinguished: a population of dividing gastric stem cells recruited under “homeostatic conditions”, which express CD44 or Lgr5 markers; and a rare population of quiescent cells recruited mainly upon tissue damage, expressing Villin, Troy and Mist as markers. Details on the markers of gastric stem cells are presented in table 8.

**Table 8. Markers of gastric stem cells identified by lineage tracing**

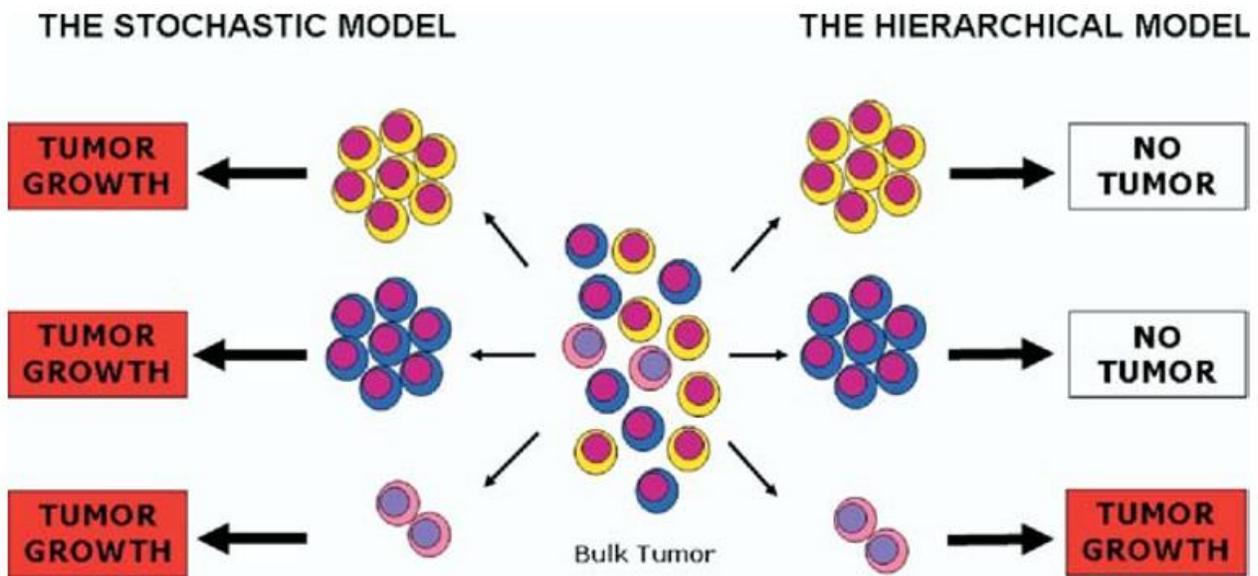
Marker	Location	Features of stem cell population
<b>Villin</b>	Bottom of antropyloric glands Isthmus in the corpus	Stem cell reservoir with a high proliferative potential
<b>Lgr5</b>	Bottom of pyloric glands	Multipotent stem cells responsible of the renewal of gastric epithelium
<b>Sox2</b>	Isthmus of glands of the corpus	Long-lived stem cells
<b>Tff2</b>	Isthmus of glands of the corpus	Short-lived progenitors
<b>Troy</b>	Base of corpus glands	Actively dividing population and small group of stem-like chief cells.
<b>Mist1</b>	Lower third of the glands in the corpus	Stem-like quiescent chief cells
<b>eR1</b>	Isthmus	Small population of terminally differentiated chief cells

## ii. Cancer stem cells

### 1. *Concept of cancer stem cell and the CSC model for tumor heterogeneity*

The individual cells that comprise a tumor display significant functional and morphologic heterogeneity, like those in normal tissue. The first model proposed to explain tumor heterogeneity is known as the clonal evolution or stochastic model, in which only a small number of cells within a tumor can initiate and sustain tumor growth, but all the tumor cells are biologically homogeneous and could potentially be responsible for these processes, given that they undergo the stochastic events that govern tumor initiating capacity<sup>179</sup> (Figure 5). These events could be intrinsic (adequate levels of critical

transcription factors for example) or extrinsic to the cells (microenvironment cues). The second and more accepted model is called the hierarchical or CSC model, which proposes that, in contrast to the stochastic model, only a specific subset of tumor cells possesses the capacity to regenerate the tumor<sup>179</sup>. Accordingly, it is then possible to identify and isolate the cells within a tumor that have the capacity to initiate tumors and sustain their growth. These cells are the so-called CSCs, defined by similar features to those of the normal SCs, their capacity of self-renewal and asymmetric division, the potential to develop into any type of cell in the tumor, and proliferative capacity to drive the continued expansion of the tumor population.

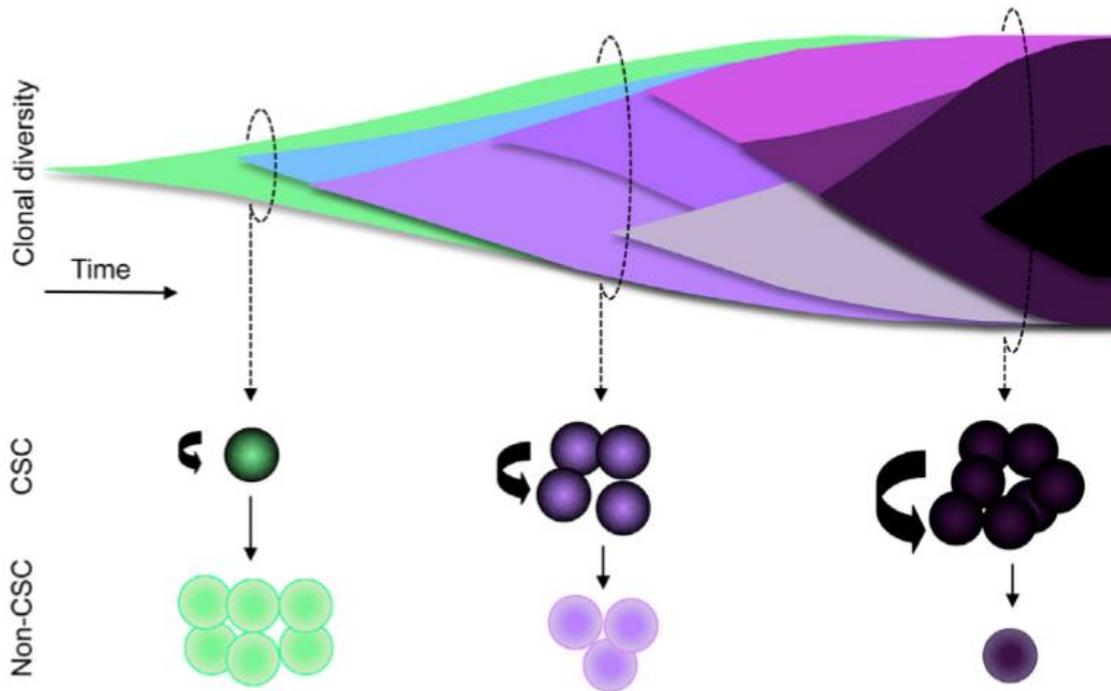


**Figure 5. Models explaining the heterogeneity of tumor cells<sup>179</sup>.** The stochastic model states all the cells within a tumor have the potential to regenerate a tumor, but it is a low probability stochastic event. Alternatively, the hierarchical (cancer stem cell) model postulates that a tumor possesses a subset of CSCs with the unique capacity of initiating and maintaining tumor growth.

Research in the cancer field has come to support the hierarchical model in the recent years, as not only the identification and isolation of CSCs has proven attainable in hematologic and solid tumors (thanks to the identification of specific surface markers), but also because some of the key challenges in oncology have been found to be directly associated with CSCs. This is the case of treatment failure and relapse of the disease, as another property of CSCs is the resistance to both chemo- and radiotherapy.

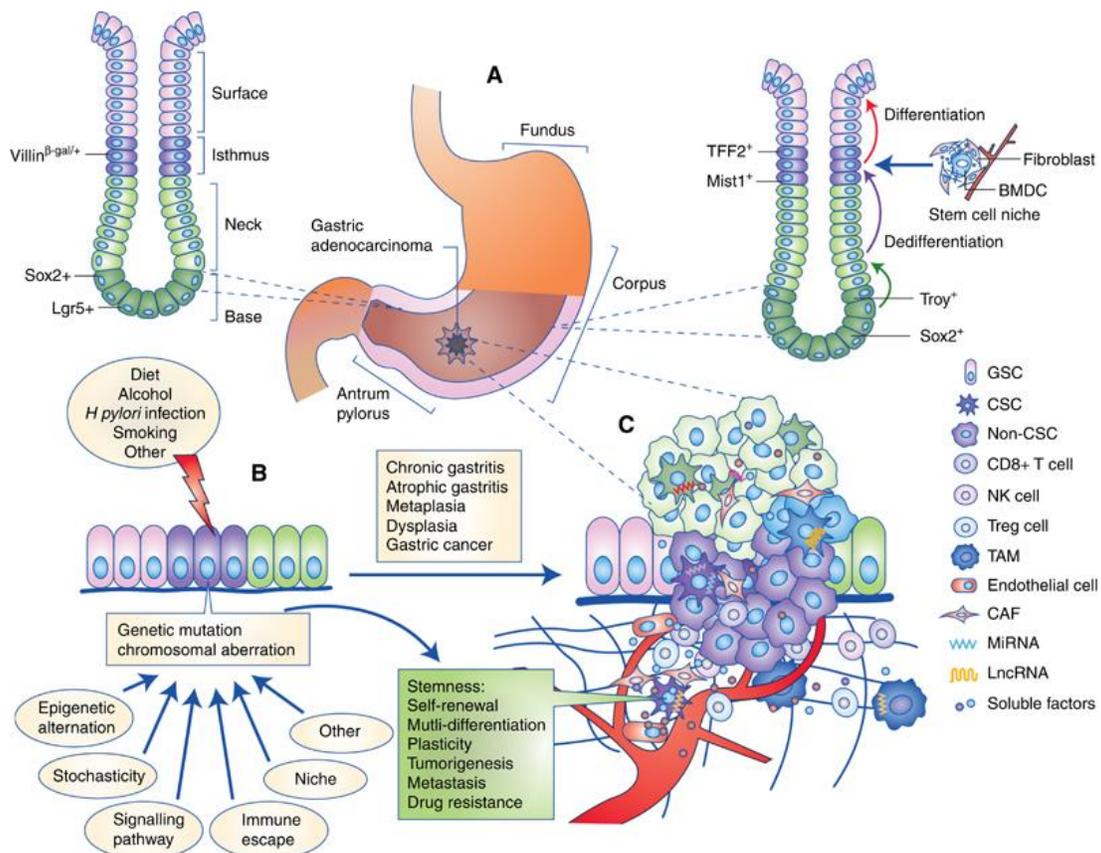
However, some studies using high throughput, single cell, and single nucleus sequencing developed in the last years have produced results that challenge the widely accepted hierarchical model and that fit better to the clonal evolution model. The identification of different genetic subclones suggests

that the clonal evolution model could also have a key role in tumor initiation, heterogeneity, progression, and metastasis. A comprehensive model that integrates both hierarchical and clonal evolution explanations of heterogeneity has been proposed by Kreso and Dick, in which favorable mutations accumulate in the rare CSC population within an early tumor as it develops, generating subclones in the CSCs, some of which achieve higher capacity for self-renewal<sup>180</sup>. Mutations could also occur and accumulate in cells without tumor-initiating capacities, eventually endowing them with stemness. The stemness state could also be achieved by dedifferentiation of these cells as a result of external stimuli, for example, EMT-like processes. The proposition is then of a model that considers the tumor-initiating cells not as static entities, but as ones capable of evolving, and this evolution process could be driven genetic determinants as well as epigenetics, gene expression stochasticity, the CSC niche, and the tumor microenvironment<sup>180,181</sup> (Figure 6).



**Figure 6. Unified model of clonal evolution and cancer stem cells<sup>180</sup>.** The acquisition of favorable mutations can result in clonal expansion of the founder cell. In parallel, another cell may also gain a favorable mutation and form a new subclone. Mutations can accumulate and subclones can evolve in parallel. CSCs are not static entities but can evolve as genetic changes influence CSC frequency and self-renewal potential.

Very recently, the unified model was proposed for gastric cancer by Song et al<sup>181</sup> (Figure 7). The existence of a functional hierarchy in GCSCs, the considerable variation in their function and phenotype, and the high diversity of driver mutations are features present in gastric cancer that support a model combining the stochastic and hierarchical theories, in which primitive GCSCs continually optimize their genome, improve the pattern of gene expression, and generate various subclones to adapt to the changing tumor microenvironment<sup>181</sup>. According to this model, the most primitive GCSCs are the result of the accumulation of cancer-related mutations in stem cells derived from epithelium, mesenchyme, or dedifferentiated mature cells, and these cells gradually acquire the properties of unlimited self-renewal and multilineage differentiation, creating the hierarchical structure of the tumor. At the same time, the GCSCs evolve into different subclones, and the dominant ones generate the respective hierarchical organization, while the less fit subclones disappear. Multiple factors such as the CSC niche, epigenetic alterations, dysregulations of signaling pathways, and tumor microenvironment are proposed to have a role driving the evolution of the GCCSs.



**Figure 7. Unified model for gastric cancer stem cells<sup>181</sup>.** GC stem cells originate from gastric stem cells, dedifferentiated epithelial cells or bone marrow-derived cells (A), and the accumulation of cancer-associated mutations and chromosomal aberrations in the initiating cells, induced by multiple factors, promotes the lesions leading to gastric adenocarcinoma (B). The formation, invasion and metastasis of GC is dynamically regulated by epigenetic, genetic, immune, and microenvironment-related determinants.

## **2. Evidence**

The first evidence supporting the CSC model was published by the group of John Dick in 1997<sup>182</sup>. Using a NOD/SCID mouse model and surface markers-based cell sorting, the authors established a hierarchy of stem cells in acute myeloid leukemia that resembled the organization observed in normal HSCs. A rare subpopulation of CD34<sup>+</sup>/CD38<sup>-</sup> cells with the ability to recreate the original disease in the immunodeficient mice after serial transplantation was identified. This experiment was a milestone in the CSC research, and was soon followed by studies in other types of cancer. In solid tumors, the first identification of CSCs was made by Al-Hajj in breast cancer<sup>183</sup>. Samples of breast tumors obtained surgically from patients were grafted in the mammary fat pads of NOD/SCID mice, and the cells of both xenograft-derived and primary tumors were analyzed for cells surface markers that allowed to distinguish a small subpopulation of CD44<sup>+</sup>CD24<sup>-/low</sup> cells capable of forming tumors when injected in immunodeficient mice in quantities as low as 100 cells, while the cells with other phenotypes were not. The existence of CSCs have been since then demonstrated in tumors in other organs such as brain, colon, pancreas, lung, and liver, amongst others<sup>179</sup>.

In gastric cancer, the existence of CSC was demonstrated by Takaishi et al in 2009. In this study, cells of six gastric cancer cell lines were sorted regarding the expression of six surface markers<sup>184</sup>. CD44<sup>+</sup> cells showed a high spheroid-forming capacity *in vitro* and tumor formation when grafted in skin and stomach of immunodeficient mice. These cells also exhibited a high resistance to chemotherapy *in vitro*. Furthermore, the knock-down of CD44, by the means of an shRNA, diminished the tumorigenic properties of the cells. The results were supported also by the presence of CD44 in immunostaining of dysplastic lesions following *H. felis* infection in INS/GAS mice and human tumor tissue. A study by Fukuda et al, identified a side population (SP) in gastric cancer cell lines using the Hoescht 33342 staining, which had increased *in vivo* tumorigenic properties and chemoresistance when compared to the cells of the main population (MP)<sup>185</sup>. The study by Takaishi et al, however, failed to do so and found no significant difference between the cells in the SP and those of the MP, regarding tumor-forming capacity.

## **3. Origin of cancer stem cells**

One of the main questions in the field of CSC is where do they come from. The answer to this question may have different and non-mutually exclusive answers, depending on the type of cancer or even the subpopulation of CSCs in question.

In hematologic malignancies, for example, it is considered that the transformation of normal hematopoietic stem cells gives rise to the leukemic stem cells. In some diseases, both normal and cancer stem cells share the same surface marker phenotype, like the case of acute myelocytic leukemia with the

CD34<sup>+</sup>CD38<sup>-</sup> phenotype<sup>182</sup>, and in B cell acute lymphoblastic leukemia where CSCs express B220 and CD19, which are markers of B-cells<sup>186</sup>. But there is also evidence that more committed progenitors can also be at the origin of leukemic CSCs by the acquisition of genetic alterations. The transduction of the oncogenes MOZ-TIF2, the retroviral overexpression of the HOXB6 gene, and the translocation of the MLL gene in myeloid progenitors have been demonstrated to confer leukemic SC properties such as self-renewal and maintenance of the disease in xenograft models<sup>187-189</sup>.

In solid tumors, the role of stem cells and progenitors has been studied in intestinal cancer, among others. In a study by Barker et al, Lgr5<sup>+</sup> intestinal stem cells were traced as intestinal cancer cell using mice a lineage tracing method, and the interbreeding of this strain with APC<sup>flox/flox</sup> mice, allowed to show that loss of APC in these stem cells lead to their malignant transformation and was responsible for the formation of adenomas in the mouse gut, while this was not the case of short-lived transit-amplifying cells<sup>190</sup>. A stem cell/progenitor hierarchy was maintained in early neoplastic lesions.

#### a. Gastric cancer stem cells

In gastric cancer, CSC are thought to originate from multiple cell types, including gastric stem cells, epithelial cells from the glands and bone marrow-derived cells (BMDCs).

##### i. Bone marrow derived cells (BMDCs)

The role of BMDCs has been approached using a *H. felis*/C57BL/6 model of gastric cancer, and the study found that upon chronic *H. felis* infection, the damaged glands were repopulated by BMDCs, and their progeny later developed metaplasia, dysplasia and intraepithelial neoplasia<sup>191</sup>. Furthermore, in the context of *H. pylori* infection in C56BL/6, using GFP<sup>+</sup> BMDCs from male mice grafted in female irradiated females and then infected with mouse adapted strains of the bacterium, Varon *et al* showed that 25% of the resulting dysplastic lesions included BMDCs and the bone marrow as a source of the cells was confirmed by detection of the Y chromosome<sup>192</sup>. Other roles for BMDCs in the gastric mucosa have been studies, such as contributing to the formation of the stem cell niche, repairing the damage caused by chronic inflammation, and promoting the proliferation and metastasis of gastric cancer cells<sup>181</sup>.

##### ii. Gastric stem cells (GSC)

The origin of GCSCs from GSCs have been examined by Barker *et al* using a similar strategy to the one in the previous study in the intestine, and the authors showed that loss of APC accelerated the expansion of transformed Lgr5<sup>+</sup> GSCs and the formation of adenomas in the pyloric region<sup>193</sup>. Moreover, a recent study showed that the deletion of Smad4 and PTEN in Lgr5<sup>+</sup> GSCs resulted in intestinal type gastric

cancer<sup>194</sup>. In other GSCs, such as Villin<sup>+</sup> cell, the depletion of the tumor suppressor *klf4* induced malignant transformation and tumor formation in the antrum<sup>195</sup>.

### iii. *Dedifferentiated epithelial cells*

The third possible origin of GCSCs are dedifferentiated epithelial cells. By studying the chronology of cytokeratin-7 (CK7) expression the precancerous cascade in the Mongolian gerbil model, highly expressing CK7 dedifferentiated cells were associated to metaplastic and neoplastic changes in the precancerous sequence. These cells exhibited a low proliferation and beta-catenin accumulations, like stem cells, suggesting that dedifferentiation of cells may be associated to stemness in gastric cancer<sup>196</sup>. Transdifferentiation of mature chief cells was demonstrated to be at the origin of drug- and *H. felis*-induced metaplasia in a mice model, using lineage tracing techniques<sup>197</sup>. The dedifferentiation and acquisition of stemness properties was directly linked to inflammation, as it was not present in the metaplasia model induced in the absence of inflammatory mediators with the DMP-777 drug in contrast to the induction with L-635 and *H. felis* infection. Further evidence of the role of dedifferentiation of gastric epithelial cells in the emergence of GCSCs was provided by Bessede *et al*, in a model of gastric epithelial cell lines infected with *H. pylori*, where dedifferentiation of cells by EMT was shown to be at the origin of a population of cells with GCSCs properties<sup>131</sup>.

## 4. **Markers of CSCs**

### a. *General*

Cell surface markers can be used to identify CSCs. Using technologies such as FACS the cells in a tumor can be separated in subpopulations based on the expression of cell surface proteins, and their stemness characteristics can be verified experimentally to determine which markers distinguish CSCs from the bulk of cells. The isolation of CSCs with the appropriate markers is of vital importance to the study of their biological properties, and it has been the subject of numerous studies aiming to identify those markers have been carried out. The cell surface markers for hematologic CSCs and solid tumors are detailed in table 9.

**Table 9. CSC markers in hematological malignancies and solid tumors.** Modified from<sup>198</sup>

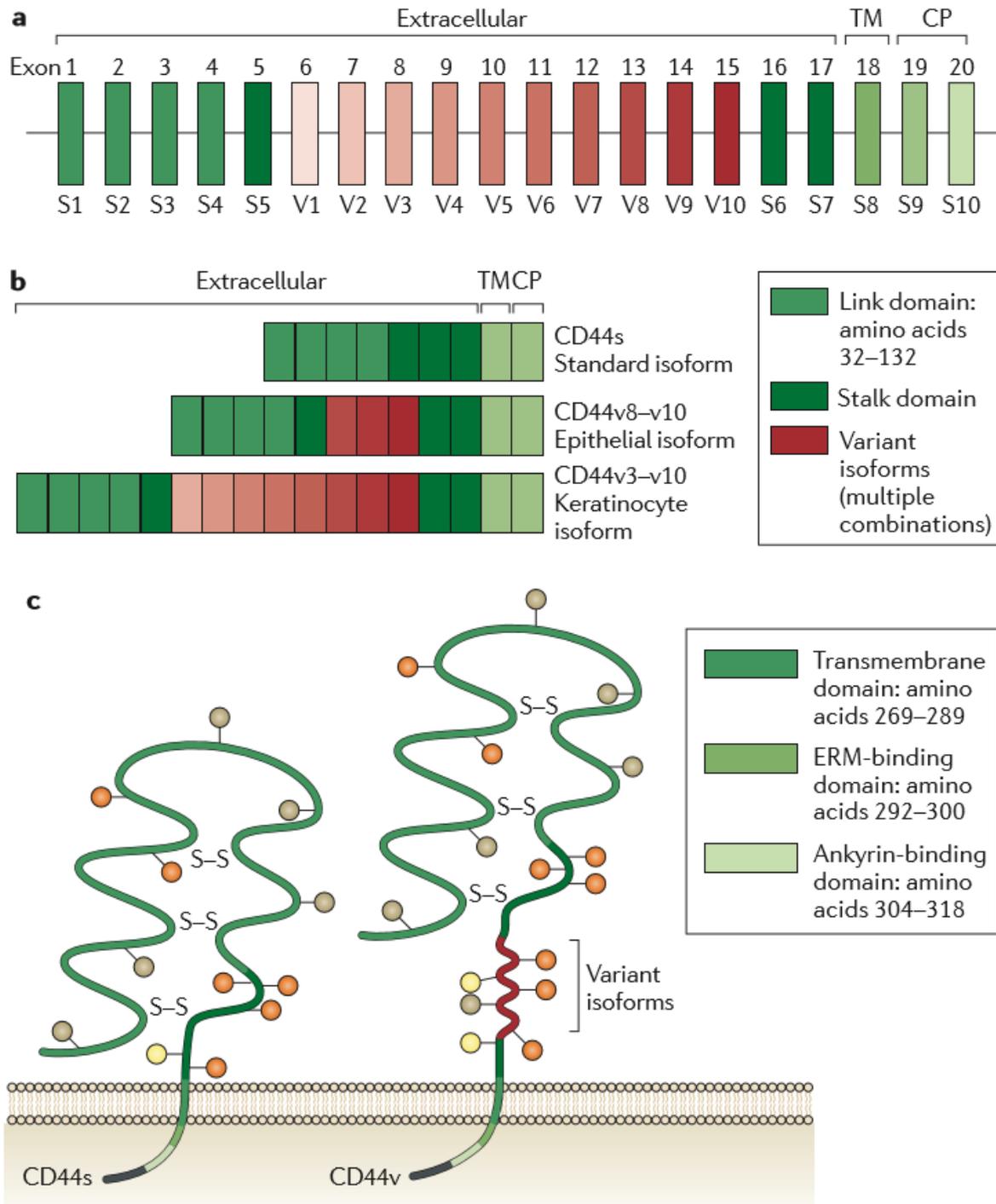
Hematological malignancies		Solid tumors	
<i>Tumor type</i>	<i>Cell surface markers</i>	<i>Tumor type</i>	<i>Cell surface markers</i>
<b>Acute myelocytic leukemia</b>	CD34 <sup>+</sup> CD38 <sup>-</sup>	<b>Breast</b>	CD44 <sup>+</sup> CD24 <sup>-</sup> /low Lineage <sup>-</sup> ESA <sup>+</sup>
		<b>Central nervous system</b>	CD133 <sup>+</sup>
<b>B-cell acute lymphocytic leukemia</b>	CD34 <sup>+</sup> CD10 <sup>-</sup> /CD34 <sup>+</sup> CD19 <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD19 <sup>+</sup>	<b>Colon</b>	CD133 <sup>+</sup> ESA <sup>high</sup> CD44 <sup>+</sup> Lineage <sup>-</sup> (CD166 <sup>+</sup> )
		<b>Ewing's sarcoma</b>	CD133 <sup>+</sup>
<b>Multiple Myeloma</b>	CD34 <sup>-</sup> CD138 <sup>-</sup>	<b>Head and neck</b>	CD44 <sup>+</sup> Lineage <sup>-</sup>
		<b>Melanoma</b>	ABC5 <sup>+</sup>
<b>T-cell acute lymphocytic leukemia</b>	CD34 <sup>+</sup> CD4 <sup>-</sup> /CD34 <sup>+</sup> CD7 <sup>-</sup>	<b>Liver</b>	CD90 <sup>+</sup> CD45 <sup>-</sup> (CD44 <sup>+</sup> )
		<b>Ovarian</b>	CD44 <sup>+</sup> CD117 <sup>+</sup>
		<b>Pancreas</b>	CD44 <sup>+</sup> CD24 <sup>+</sup> ESA <sup>+</sup> CD133 <sup>+</sup>

*b. CD44 (Cluster of differentiation 44)*

*i. Structure and function*

CD44 is a transmembrane glycoprotein that functions as a receptor for hyaluronic acid, a major component of the extracellular matrix, and also as a co-receptor for many growth factors and cytokines<sup>199</sup>. CD44 also binds other molecules, ECM-related (collagen, fibronectin, laminin, and chondroitin sulfate) or not (serglycin, osteopontin, and mucosal addressin)<sup>200</sup>. It mediates cell-cell and cell-ECM interaction, cell traffic and homing, uptake and intracellular degradation of HA, and signal transmission involved in hematopoiesis and apoptosis.

It is encoded by the homonymous gene, located in chromosome 11 in humans<sup>199</sup>. The *CD44* gene has 20 exons, and alternative splicing gives origin to various isoforms or variants, allowing a high heterogeneity of the protein. The cytoplasmic and the transmembrane portions of the protein are coded by the exons 18-20, while the remaining exons represent the extracellular domains<sup>201</sup>. The exons 1 to 5 and 16 to 20 are always present, hence they are considered as the constant exons and the resulting protein is known as the standard form (CD44s, 85 kDa). Exons from 6 to 15 are variably present in the variant forms (CD44v)<sup>201</sup> (Figure 8). Several combinations exist, 12 of which are commonly found, and their distribution is variable amongst tissues<sup>202</sup>. While the CD44s is highly expressed in hematopoietic cells, the variants are more restricted. For example, the epithelial isoform or CD44E, includes exons 8 to 10, and the isoform found in keratinocytes includes exons 3 to 10. Other combinations have been found to be even more restricted to malignant cells in different types of epithelial cancers, so the study of CD44 isoforms and their use as specific markers for cancer stem cells is a very active and promising field of research. Additional variability is present due to different patterns of N-glycosylation, O-glycosylation, and glycosaminoglycanation<sup>200</sup>.



**Figure 8. CD44 gene and protein structure**<sup>123</sup>. CD44 consists of several exons, some of which are constant region exons that are used in every CD44 mRNA and protein (green bars) and others are variant exons (red bars) that are used in the CD44 variant proteins and are selected by alternative splicing (a). Examples of alternatively spliced CD44 proteins (b). CD44 protein is composed of an extracellular link domain, a stalk-like region in the extracellular domain close to the transmembrane region, where the variant exon products (red) are inserted, the transmembrane region and the cytoplasmic tail. There are multiple sites for N-glycosylation (brown circles), O-glycosylation (orange circles), and glycosaminoglycan-binding sites (yellow circles)

## *ii. Role in cancer*

CD44 is expressed in many cancer cell types, and besides from being used as a surface marker, studies show that it plays roles in tumor initiation and growth, cell migration, and metastatic dissemination in gastric tumors and gliomas, amongst others<sup>199</sup>. These are functional characteristics that associate CD44 with stemness in cancer cells, and in fact, it is one of the molecules most commonly identified as a CSC marker (alone or in combination with other cell surface proteins). The recognition of ECM components by CD44 can activate signaling pathways associated with stemness, and its role as a co-receptor have been described for many tumor-associated growth factors such as EGF, FGF, HGF, VEGF, and TGF- $\beta$ <sup>199</sup>. In the light of the currently available evidence, CD44 seems to be a crucial mediator of the interaction of CSCs with their surroundings, and therefore, aside from the role of CSCs marker, it has become a target for cancer therapy<sup>201</sup>.

## **5. Methods of identification and isolation of CSCs**

### *a. Colony and sphere formation assays*

The evaluation of the clonogenic potential of CSC evidences their capacity of self-renewal and multidifferentiation, as only CSC are able to originate colonies or spheres from a single cell. The colony formation capacity is tested by plating the cells to grow in the presence of “feeder” cells or a conditioned medium, either of which has the role of providing growth factors. The usual method consists in growing the cells in a layer of soft agar, which prevents cells to adhere to the culture plate<sup>203</sup>. Non-transformed cells have dependence on contact with the ECM, and if they don’t have it, they are not able to survive. This allows to infer that each colony is originated in a CSC, and therefore, permits a quantitative evaluation of cells with self-renewal capacities.

The same rationale is behind the sphere formation assay, in which the cells are seeded in low density in a defined serum-free medium, usually supplemented with growth factors (EGF, FGF, and insulin) and nutrients and in non-adherent conditions. Only cells that have lost the adhesion dependence and have self-renewal properties are able to grow in these conditions.

These techniques are widely used in the CSC research field, it is accepted as a reference in vitro method for the characterization of CSCs, but there are some limitations that must be taken in account when interpreting the results. Some cell lines have a ubiquitous capacity of growing in non-adherent serum-free conditions, and high densities of cells may promote the formation of aggregates that may be confuse with real spheres, and also an inability to detect quiescent CSCs<sup>204</sup>. However, a careful method and sequential dissociations and passages may be enough to overcome some of these criticisms.

*b. Side-population assay*

This is a flow cytometry-based method that relies on the capacity of some cells to exclude dyes such as Hoechst 33342 and rhodamine, thanks to the abundance of ABC-transporter proteins, also associated with drug resistance, such as ABCG2. The exclusion of the dyes places these cells in a side group, negative for the staining in the cytometry plot, and separated from the bulk of tumor cells that are not capable of excluding the dye and are positive. This “side population” (SP) is enriched CSCs, as was demonstrated in a study by Hirschmann-Jax *et al*, which isolated and evaluated the properties of side populations in primary tumor cells of neuroblastoma patients and cell lines from solid tumors<sup>205</sup>. The authors found that the cells from the SP were capable of self-renewal and asymmetric division. Furthermore, the cells in the SP expressed high levels of transporter proteins such as ABCG2 and ABCA3, reflected also in the capacity to expel cytotoxic drugs, and a consequently increased drug resistance, another feature of CSCs.

*c. Aldehyde dehydrogenase (ALDH) activity assay*

Aldehyde dehydrogenase catalyzes the oxidation of retinaldehyde to retinoic acid, which regulates gene expression in complex with the RAR/RXR receptors. Normal and cancer SC show a high ALDH activity, which is thought to have a key role in protecting the cells from oxidative stress and cytotoxic agents. In a flow cytometry-based assay, a substrate of ALDH, BODIPY-aminoacetaldehyde (BAAA), is added to the cells and then converted by the ALDH in BODIPY-aminoacetate (BAA-), which is fluorescent and allows the cells to be classified as ALDH<sup>high</sup> (SCs) or ALDH<sup>low</sup>. The presence of ALDH can also be detected by immunohistochemistry in tumor tissues. The ALDH activity can be combined with cell surface markers, such as CD44, in flow cytometry to have a more specific identification of CSCs.<sup>206</sup>

*d. Isolation based on marker expression*

As detailed above, numerous cell surface proteins have been identified as markers for CSCs in different types of cancer. Based on the differential expression of these markers between the CSC and the other cells in the tumor, CSCs can be isolated using fluoresce- or magnetic-activated cell sorting platforms (FACS and MACS, respectively). The use of FACS has the possibility of performing staining with different fluorochromes, and hence, the sorting of cells based in expression of multiple markers. The determination of phenotypes or combinations of surface markers increases the specificity of the selection and enhances the isolation of CSCs. In the case of MACS, the antibodies directed against the surface markers are coupled to micro-beads, which only allows the separation based on one marker.

e. *In vivo xenotransplantation*

The capacity of forming new tumors *in vivo* is a hallmark of CSCs. The evaluation of tumor-initiation capacity of cell when grafted in highly immunodeficient mice (NSG strain for example, detailed above) is the gold standard for identification of CSCs. This approach also allows the estimation of the proportion of CSCs present in a tumor, using the limiting-dilution method, in which the minimal number of cells required to originate a tumor when injected in mice is determined. Given that only CSCs have the ability to initiate a tumor, the number of CSC in the total cell number can be calculated from these data. The method has been used to study CSCs of multiple solid cancers, gastric included. In a recent study by Nguyen *et al*, the frequency of tumor-initiating cells was calculated between 1/29 and 1/1020 in CD44<sup>+</sup> cells for three cases of gastric adenocarcinoma (two intestinal-type and one diffuse-type) using this method<sup>206</sup>.

## c. *Helicobacter pylori*

### i. History

Due to the acidic nature of the gastric environment, it was commonly assumed that no bacterium could survive in this anatomic site. Nevertheless, the presence of spiral bacteria in the gastric mucosa was described as early as the 19<sup>th</sup> century by the Italian pathologist Giulio Bizzozero in the stomach of dogs<sup>207</sup>. This was later confirmed by Kasai and Kobayashi, who also described the colonization in other animals and the bacterial reaction to Salvarsan and other compounds<sup>208</sup>. Other investigation showed the presence of a urease activity in the stomach of carnivorous animals<sup>209</sup> and humans, which was sensitive to antibiotics and absence in gnotobiotic animals<sup>207</sup>. It was finally in 1984, when Warren and Marshall cultured and identified a curved gram-negative bacillus present in human gastric biopsies, and confirmed its association with antral gastritis<sup>210</sup>. It was initially placed as a member of the *Campylobacter* genus, but after a deeper study it was classified as *Helicobacter pylori* in the newly-created *Helicobacter* genus, alongside with a related bacterium of ferrets, *Helicobacter mustelae*<sup>211</sup>. After numerous studies linking the presence of *H. pylori* with dyspeptic conditions<sup>212-215</sup> and gastric cancer<sup>216-218</sup>, it was classified as a Type I carcinogen by the World Health Organization in 1994<sup>219</sup>. Thanks to their discovery, Warren and Marshall were awarded with the Nobel Prize in Physiology and Medicine in 2005.

### ii. Epidemiology

*H. pylori* infects approximately half of the world's population<sup>220</sup>. The infection is more prevalent in Central and South American and Asian countries where it is present in 70-90% of the older adult population, in comparison with 20-50% in Northwestern European countries, Japan and the United States<sup>221</sup>. Some authors suggest that prevalence increases with age<sup>222</sup>, however, it is widely accepted that this is due to a cohort effect and that the seropositivity percentage in the adult population is reached around the age of 15<sup>223-225</sup>. The widespread antibiotic use and the improvements in the living conditions of the population have caused a decrease in the prevalence of infection in industrialized countries such as Germany and Australia, where the higher infection rates are found in regions with lower socioeconomic indicators and their amelioration correlates with lower prevalence<sup>226</sup>. In more heterogeneous countries like the United States, the percentage of *H. pylori*-positive individuals amongst white, non-Hispanic people has decreased in a period of ten years, unlike the case of Hispanic and African American populations, in which rates remained stable<sup>226</sup>. The decreasing trend seen in the prevalence of the infection maybe related with factors such as the index of human development, access to safe drinking water, adequate

sanitary conditions, and good healthcare<sup>222</sup>. The massive use of antibiotics may also play a role in this decrease<sup>220</sup>.

When a human acquires *H. pylori*, a persistent infection is established, ending only by the eradication with antibiotic treatment. The infection usually takes place during childhood, and the reinfection in the adult after eradication is rare<sup>222,227</sup>. Even so, the colonization has no deleterious consequences in the host. Only a small percentage of cases is associated with severe pathologies (10-15 %), such as chronic atrophic gastritis, peptic ulcers and gastric malignancies (1-2 %), notably gastric adenocarcinoma and MALT lymphoma<sup>228</sup>.

There are multiple transmission routes proposed for the *H. pylori* infection. It is accepted that it happens directly between humans, either by oral-oral, fecal-oral, or gastro-oral, none of them being mutually exclusive<sup>220,227,229</sup>. The transmission takes place preferentially inside the family nucleus, where close contact and hygiene conditions are the most important determinants<sup>230</sup>. Studies carried in different countries have shown that children from infected mothers have up to a 20-fold increased risk of infection compared with those of non-infected mothers, and the same case is observed in the case of infected fathers in countries where the relation between father and child is close<sup>227,230</sup>. This associations were initially evidenced by epidemiological studies, and have been supported by a MLST analysis showing a similar pattern of transmission in Japan<sup>231</sup>.

Other transmission ways such as iatrogenic, water- and food-borne have also been proposed, but they are less likely due to the characteristics of the bacterium<sup>227,232-236</sup>. Even so, all those routes could be co-existing, and the predominant ones will depend on the particular conditions. An international study showed that in rural settings there could be a more important waterborne transmission, as opposed to urban communities where higher sanitary conditions allow a predominance in the intrafamilial transmission<sup>237</sup>.

### iii. Biology

According to the Bergey's Manual of Systematic Bacteriology, the genus *Helicobacter* belongs to the Helicobacteriaceae family, in the Campylobacterales order of the  $\epsilon$ -Proteobacteria class<sup>238</sup>. These are microaerophilic gram negative helical or curved bacilli, which present characteristics such as motility by sheathed flagella, presence of a glycocalyx or capsule-like structure when cultured in liquid media, and a 24-48% G+C content on chromosomal DNA. The genus comprises 36 validated species<sup>239</sup>, including many pathogens to humans and other animals, of which *H. pylori* is the type species.

*H. pylori* is an S-shaped bacillus of approximately 0.5 to 5  $\mu\text{m}$  with 5-8 polar flagella<sup>240</sup>. It produces circular small translucent colonies when cultured in blood agar in a microaerophilic atmosphere

(3-7%)<sup>238,241</sup>. In old cultures, there is a predominance of coccoid forms of diminished viability<sup>240</sup>. *H. pylori* has a respiratory metabolism with glucose as the only source of carbohydrates, and grows in narrow ranges of temperature (33-40°C) and pH (6.9-8.0)<sup>238,241</sup>. Its main biochemical features are a strong activity of the urease enzyme and positive reactions to the oxidase and catalase tests. The bacterium is negative for most of the conventional tests of identification, It does not ferment or oxidizes carbohydrates, does not hydrolyze hippurate, does not reduce nitrates nor forms indol<sup>241</sup>.

#### iv. Virulence factors

##### 1. Colonization

###### a. Adaptation to acid environment survival: Urease

Common bacterial mechanisms of acid resistance such as proton pump activation, decarboxylases, and membrane lipid modification are not sufficient for long term survival or very low pH conditions<sup>242</sup>. Remarkably, the genus *Helicobacter* presents genes that allow further adaptation to the acid environment, and not just the aforementioned resistance. The urease, which catalyzes the conversion from urea to ammonium and carbon dioxide and therefore contributes to neutralization of the bacterium's surroundings, was the first protein identified with a role in adaptation to acid environment in *H. pylori* and it is considered a virulence factor<sup>243</sup>. It is composed by four trimers of heterodimers UreA-UreB with 24 nickel ions<sup>244</sup>, and it has been proven to be essential to the survival of *H. pylori* in the gastric mucosa<sup>245,246</sup>. Other potential roles for the urease have also been described such as inhibition of the antibacterial activity of phagocytes such as macrophages and neutrophils<sup>247</sup> and the induction of apoptosis of gastric epithelial cells<sup>248</sup>. The presence of the urease in all strains of *H. pylori* also makes its detection the gold standard for non-invasive diagnostic of the infection<sup>249</sup> and even a potential target for treatment<sup>250,251</sup>.

The effect of acid in the transcription regulation during chronic infection was studied by comparing the transcriptomes *in vivo* e *in vitro*, and three groups of genes were particularly increased. As expected, the transcription of the gene cluster *ure* (*ureA*, *ureB* and *ureI*) as well as *rocF*. The other two groups were genes related to motility and chemotaxis and those associated with virulence, notably *cagA*<sup>252</sup>. The urease-coding genes in *H. pylori* are organized in the cluster *ureABIEFGH*, formed by two operons: *ureAB* and *ureIEFGH*. The later also presents post-transcriptional regulation by degradation of mRNA in response to pH<sup>253</sup>. The first operon encodes for the UreA and UreB subunits of the enzyme whilst the second encodes for the urea channel UreI and other accessory proteins required for the maturation and the assemblage of the Ni<sup>2+</sup> in the apoenzyme<sup>254</sup>.

Some other genes whose expression is controlled by acid have also been described, for example *wbcJ*, which codes for a protein involved in LPS synthesis<sup>255</sup>. Mutant strains defective for *wbcJ* present

structural alterations of the LPS and a decreased viability in acidic conditions. Wen *et al.* in 2003 revealed more than 300 genes which have their expression regulated by stomach acid, with functions not only in the pH homeostasis but also as diverse as transcription regulation, motility, cellular structure and pathogenicity<sup>242</sup>.

In *H. pylori*, the detection of pH is by the means of a two-component system formed by the histidine kinase type receptor ArsS and its response regulator ArsR, which stimulates the transcription of multiple genes<sup>256</sup>. The ArsS-ArsR system controls approximately 109 genes, including the ure cluster and the amylases AmiE and AmiF<sup>257</sup>. Other elements regulated by the system are related to acetone metabolism, resistance to oxidative stress and “quorum-sensing”<sup>258</sup>.

#### *b. Bacterial motility and chemotaxis*

Asides from the acidic environment, the motility- and chemotaxis-related genes were the second most important categories identified by a study of essential genes for gastric colonization in the Mongolian gerbil and in murine models<sup>259,260</sup>.

A study with motile and non-motile variants of *H. pylori* inoculated in gnotobiotic piglets was one of the firsts to link motility to the colonization capacity, as non-motile forms were less able to successfully colonize the animals<sup>261</sup>, and since then, multiple studies have confirmed the bacteria capacity to swim through the mucus layer towards the epithelial surface as a major colonization factor<sup>262,263</sup>. The main factor of bacterial motility are the flagella<sup>264</sup>, in the case of *H. pylori*, they are lophotrichous and individually sheathed, the number varies from two to six, they have club-shaped thickening at the ends, and they are mainly constituted by two types of flagellines, FlaA and FlaB<sup>265-267</sup>. The sheath function is most likely the protection of the flagellum from the gastric acidity and mechanical damage<sup>268</sup> and also adhesion, as it carries the adhesin HpaA<sup>269</sup>. Another determinant for the motility of *H. pylori* is the helical shape of the cells. A structural study showed that *H. pylori*'s periplasm is cage-like with 18-fold symmetry that provides the necessary torque needed to swim through a high viscosity environment as the mucus layer in a corkscrew-like movement<sup>270,271</sup>, and the genes involved in the helical morphology of *H. pylori* have identified, comprising the *csd* family which encodes for peptidoglycan peptidases<sup>272-274</sup>, and *hp0506*, whose product, HpdA is a M23B family metalloprotease<sup>275</sup>.

In order to swim away from the acid and movement of the stomach lumen to the more favorable conditions in the mucus layer, *H. pylori* is guided away from low pH by a complex chemotactic machinery<sup>276</sup>. Chemotaxis is mainly directed by a two-component system, composed by the histidine kinase CheA (*HP0392*), the transducer CheY (*HP1067*) and other accessory proteins (CheW, CheZ and multiple proteins with CheY-like domains)<sup>277-279</sup>. In addition, four chemoreceptors have been described, TlpA, TlpB, TlpC and TlpD<sup>280-283</sup>. Together, these proteins account for the recognition of a large quantity of

signal such as arginine, bicarbonate, urea, metals and acid<sup>284-286</sup>. The gradient of this latter is a very important factor to the spatial localization of *H. pylori* in the mucus layer<sup>287</sup>. The other signals likely diffuse from the blood through the epithelial cells thus helping the bacterium to get near them<sup>276</sup>. TlpB is also involved in the recognition of the quorum sensing molecule AI-2 as a repellent<sup>288</sup>.

Even if they have been widely studied, the motility and chemotaxis remain to be elucidated in further detail. Some of the more recent findings are a chemotaxis-related preferential localization of *H. pylori* in injury sites<sup>263</sup>, and new putative chemotaxis arrays at the flagellar pole<sup>271</sup>.

### c. *Adhesion: Outer membrane proteins*

In order to colonize epithelial cells, especially those subject to constant clearance mechanisms, bacterial pathogens have developed surface proteins which allow them to recognize and attach to molecules on these cells<sup>289</sup>. *H. pylori* is no exception, and the presence of adherent bacteria (even if the percentage is small) is very important to the establishment and persistence of the infection<sup>290</sup>. There are at least 63 outer membrane proteins (OMP) or putative OMPs described for *H. pylori*<sup>291</sup>. Amongst them, the “major OMP” or Hop family is the largest and includes proteins such as BabA, SabA, HopZ, OipA, and AlpA/B<sup>292,293</sup>. For BabA and SabA, the ligands in the host’s cells are fucosylated and sialylated glycans of the Lewis and ABO antigen groups, while for the others, the ligands are still unknown<sup>294,295</sup>. These and other adhesines like HomB or CagL (binds to integrin  $\alpha 1\beta 5$ ) are also involved in bacterial pathogenicity and the outcome of infection<sup>296-298</sup>.

## 2. *CagA and cagPAI*

The Cytotoxin associated gene A (CagA) is the main and most studied virulence factor of *H. pylori*. It is a 128 KDa protein whose expression is strongly associated with a higher virulence of the bacterium and therefore with the infection-related pathologies such as peptic ulcer, atrophic gastritis and gastric adenocarcinoma<sup>299-301</sup>. Once injected in the host cell by a type IV secretion system, CagA is phosphorylated by kinases of the Src and Abl families<sup>302-305</sup>. This phosphorylated form specifically binds to and deregulates the tyrosine phosphatase SHP-2, and initiates changes in the host cell signaling<sup>306,307</sup>. SHP-2 upregulates growth factor signals and is required for the activation of the Ras-Erk MAP kinases cascade<sup>308,309</sup>. SHP-2 inhibits the focal adhesion kinases (FAK), which regulates the turnover of the focal adhesion spots<sup>310</sup>. Phosphorylated CagA also bind to Csk and mediated the inhibition of Src family kinases, in a negative regulation loop that seems to limit the bacterial damage to the epithelial cells and favors the persistence of infection<sup>311</sup>. The Src family kinases also phosphorylate other proteins such as vinculin, ezrin and cortactin, and their inhibition leads to alterations in the cytoskeleton. Notably, the dephosphorylation of

cortactin induced by CagA, as well as the inhibition of the PAR1-kinase activity, have been described during the infection<sup>312-314</sup>. All these phenomena contribute to the acquisition of the so-called “hummingbird” phenotype upon infection, which is a change in the cellular morphology to an elongated, mesenchymal-like shape associated with actin cytoskeleton rearrangements and increased motility.

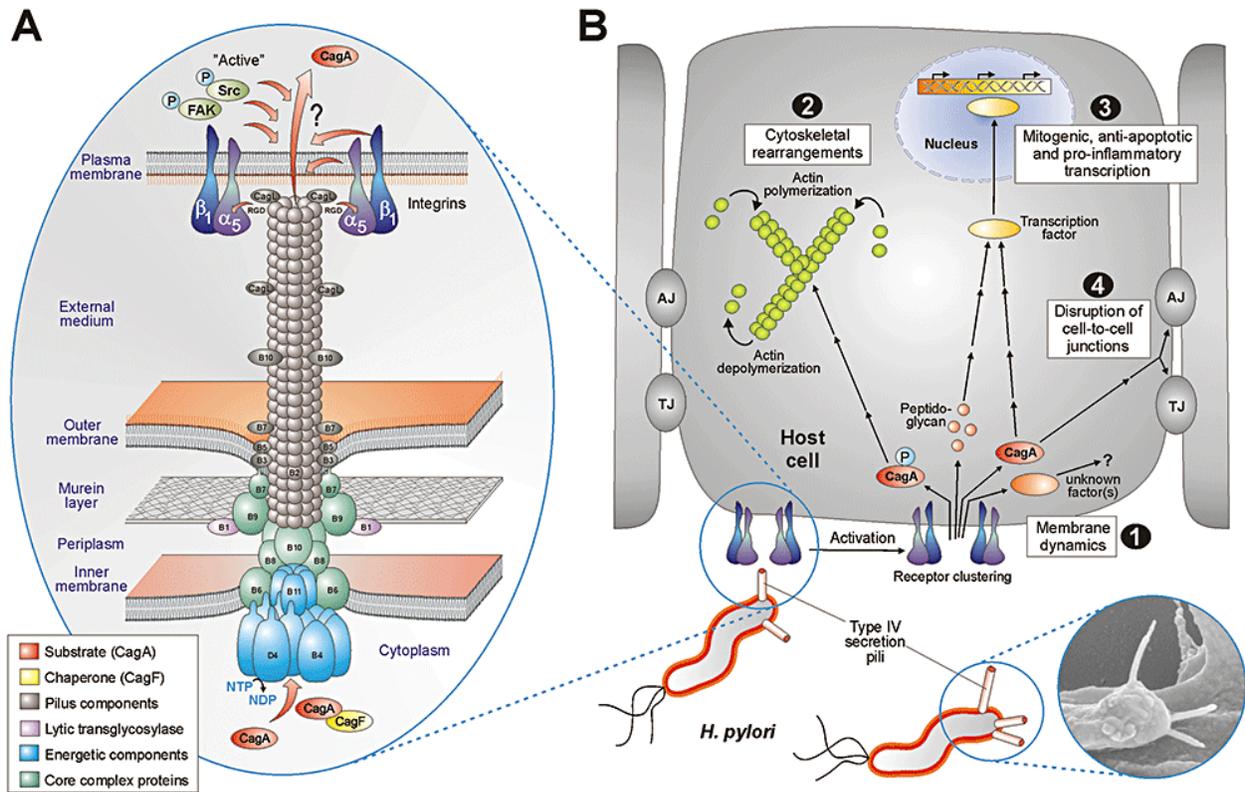
CagA phosphorylation occurs in the carboxy-terminal end, in tyrosine phosphorylation motifs of the sequence Glu-Pro-Ile-Tyr-Ala (EPIYA)<sup>315</sup>. This region is polymorphic, and the variations are especially evident when comparing Western and East Asian strains, both regarding the number of phosphorylation motifs and the sequences flanking them<sup>315,316</sup>. Regarding the surrounding sequences, four types of EPIYA motifs are distinguished: EPIYA-A, -B, -C and -D<sup>317,318</sup>. EPIYA-A and -B are present in the CagA of all the positive strains, while the EPIYA-D is typical of the East Asian forms of CagA and the EPIYA-C can be present up to three times in the CagA from Western isolates. The variations alter the phosphorylation degree of CagA and its biological activity, and in consequence, the clinical outcome of the infection<sup>319-321</sup>.

CagA also exhibits activities which are independent of the tyrosine phosphorylation. CagA is able to bind to the hepatocyte growth factor receptor (HGFR or c-Met) and to the growth factor receptor bound protein 2 (Grb2), to stimulate cell growth<sup>322,323</sup>. Injected CagA targets the intercellular junctions of host cells leading to a disruption of epithelial junction-mediated functions and dysplastic changes in epithelial cell morphology, and both alterations have been shown to play a role in carcinogenesis<sup>324,325</sup>.

By studying the region upstream the *cagA* gene, Censini et al, described an “alien” DNA insertion on the glutamate racemase gene<sup>326,327</sup>. Of approximately 40 kb long, it has the features of a pathogenicity island, such as a different content of G+C (35% compared to 38-45% in the genomic DNA), flanking direct repeats, and IS elements. It contains approximately 30 genes which encode for a type IV secretion system (T4SS), homologous to the VirB system of the plant pathogen *Agrobacterium tumefaciens* and the Ptl system of *Bordetella pertussis*<sup>328,329</sup>. The *cagPAI* can be found as a continuous unit or divided in two segments, *cagI* and *cagII*, by the insertion sequence IS605, or by chromosomal DNA<sup>326,330</sup>. A percentage of *H. pylori* strains does not possess the *cagPAI* or it is incomplete and not functional, and the presence of a complete and functional *cagPAI* is directly associated with gastric pathologies<sup>330,331</sup>. The *cagA* gene is commonly used as a marker for the entire *cag* locus, although the presence of *cagA* does not necessarily signify the presence of an intact *cag* island<sup>332,333</sup>. Other genes such as *cagE*, *cagG*, and *cagM* have been also considered to be good markers for the presence of *cagPAI*<sup>334,335</sup>. Transcriptionally, the *cagPAI* is organized in at least eleven operons and five monocistronic genes and this organization seems to be conserved between different strains<sup>336</sup>.

This secretion system allows the translocation of the CagA protein and of the peptidoglycan from the bacterial wall to the cytosol of the epithelial cells of the gastric mucosa<sup>337</sup>, and consequently, the presence of a functional secretion system is necessary for CagA to exert its effects. But also, the sole

presence of the *cagPAI* induces the activation of NF- $\kappa$ B, and AP-1, which are required for the proinflammatory response, in a CagA-independent manner<sup>326,338-340</sup>. The activation of these factors is responsible for the production of the chemokine IL-8 in the gastric mucosa seen during the infection<sup>340</sup>. IL-8 is the main chemotactic signal for neutrophils, therefore its levels are directly related to the strength of the inflammatory response and severity of the gastritis<sup>341,342</sup>. This chemokine is also related with the angiogenic process in gastric carcinogenesis<sup>343</sup>. Additionally, it has been reported that *cagPAI*<sup>+</sup> strains induced IL-10 secretion in human neutrophils<sup>344</sup>.



**Figure 9. *cagPAI* T4SS structure and CagA effects on the host cells<sup>345</sup>.** Hypothetical model of the *cagPAI* T4SS machinery is shown in A. Upon injection in the host cell cytoplasm, CagA affects membrane dynamics, actin cytoskeleton, nuclear signaling and cell-to-cell junctions, among others. AJ, adherens junction; TJ, tight junction.

Together, these alterations in cellular functions induced by CagA and the T4SS at multiple levels, provide an explanation for the association of *cagPAI*<sup>+</sup> strains with more severe pathologies, including gastric adenocarcinoma, and justify the denomination of CagA as the first bacterial oncoprotein<sup>346</sup>.

### 3. *VacA*

The vacuolating toxin (VacA) is a secreted protein of 87 kDa which was initially described for its capacity of inducing vacuolization in gastric epithelial cells *in vitro*<sup>24,347,348</sup>. It displays multiple effects on the host cell and is associated with gastroduodenal ulcer and gastric adenocarcinoma, hence it is considered a major virulence factor of *H. pylori*<sup>349</sup>. VacA protein is secreted by the bacteria either as monomers or as oligomers of six or seven units of the 88kDa protein. In its mature form, VacA is an AB toxin with N-terminal (p34) and C-terminal (p58) peptides separated by a protease sensitive loop<sup>349</sup>. At low pH, the oligomers dissociate into monomers that bind to epithelial cells, and the p58 binds to the host cell receptors and forms channels selectively permeable for anions and small neutral molecules such as urea<sup>350-353</sup>. Membrane-bound VacA can be endocytosed, and accumulates and forms pores in membrane-delimited vacuoles which exhibit markers of late endosomes or lysosomes<sup>354</sup>. The influx of anions alters the permeability of the vacuoles, leading to water flux and vesicle swelling, thus forming the large vacuoles typical of VacA activity. VacA can also be translocated across the plasma membrane into the cytosol where it exerts its action<sup>355,356</sup>. The p34 domain is considered to be the enzymatically active part of VacA, which becomes exposed to the host cell cytosol and influences the regulation of the endosomal fusion<sup>357</sup>. Host cell vacuolation, which *in vivo* is not as evident as *in vitro*, may slowly lead to the eventual death of primary human gastric epithelial cells<sup>358</sup>. *In vivo*, animal models evidence the process by a loss of epithelial cells<sup>359</sup>.

Besides vacuolation, other effects have been attributed to VacA. One of them is the capacity to induce apoptosis both *in vitro* and *in vivo*<sup>360,361</sup>. Intracellular expression of VacA or the application of the purified toxin in HeLa cells has been reported to induce the release of cytochrome C from the mitochondrial intermembrane space, suggesting that VacA induces apoptosis by activating a mitochondrial-dependent pathway<sup>362</sup>. This process, as well as the vacuolation, are dependent in the formation of anion-selective channels<sup>363</sup>.

A VacA immunosuppressive activity on T lymphocytes has been described, as VacA could block the proliferation of these cells by mechanisms both dependent and independent of the toxin's pore-forming capacity. In the first scenario, the formation of anion-selective channels causes membrane depolarization and prevents Ca<sup>2+</sup> influx from the extracellular milieu, therefore inhibiting the nuclear translocation of the NFAT transcription factor which is key for the expression of cytokines necessary to activate the cell<sup>364,365</sup>. On the other hand, independently of the channel formation, VacA induces the indirect activation of the GTPase Rac and to a reorganization of the actin cytoskeleton which could inhibit T cell activation and division<sup>364</sup>. VacA also exerts a local immunosuppressive activity by interfering with antigen presentation in B cells and phagocytes, through inhibition of the proteolytic processing of antigens and their presentation in the MHC<sup>228,366</sup>.

VacA seems to be implicated in the loosening of tight junctions in monolayers of polarized epithelial cells, increasing the permeability of the host cells<sup>367</sup>. This contributes to the capacity of *H. pylori* to colonize and persist in the human gastric mucosa, allowing the passage of important nutrients, as it increases the passage of small molecules and anions and the release of important substrates such as urea<sup>351,367</sup>. An additional increase in mucosal permeability is also present due to the induction of proinflammatory cytokines (IL-6, TNF- $\alpha$ ) in mast cells by VacA<sup>368</sup>. A balance between the moderate induction of inflammation in one side, and immunosuppressive effects on the other, may place VacA as a key factor for the maintenance of the long-term colonization of the gastric mucosa by *H. pylori*.

The *vacA* gene, which codes for VacA, is present in all the *H. pylori* isolates, but there are variations in expression and structure amongst them which give place to polymorphisms resulting in different levels of cytotoxicity<sup>228,369-371</sup>. These variations are mostly in the signal region (s), where two alleles, *s1* and *s2*, can be found, and the intermediate region (m), with the alleles *m1* and *m2*<sup>369,370</sup>. All combinations of alleles are common and possible, except *s2m1* which is very rare. Strains carrying the *s1m1* genotype have a greater VacA activity *in vitro* than those carrying *s1m2*, while the *s2m2* lacks activity<sup>369</sup>. The *s1* type produces a fully active cytotoxin, whereas type *s2* produces a toxin with a hydrophilic N-terminal extension that blocks vacuolating and pore-forming activities<sup>372</sup>. Variations in the m region are associated to a decreased activity because this region is part of the p58 subunit and therefore defines the cellular specificity of the toxin<sup>373</sup>. In cells that are recognized by the *m2* form of the toxin, like the rabbit kidney line RK3, the activity is similar to that observed in other cells that bind to the *m1* form, provided that the s region genotype is the same<sup>374</sup>. Therefore strains possessing the *s1m1* genotype of *vacA* show an enhanced cytotoxic activity compared to *s1m2* and *s2m2* bearing ones, and are epidemiologically associated with peptic ulcer disease and gastric adenocarcinoma<sup>375</sup>. Other variable regions for VacA have been described, the intermediate region (i) and the deletion region (d)<sup>376,377</sup>.

#### **4. LPS**

The bacterial lipopolysaccharide (LPS) is an essential glycolipid of the outer membrane of Gram-negative bacteria. The classical LPS is formed by three parts: the lipid A, a hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane, the core oligosaccharide, which maintains the integrity of the outer membrane, and the O antigen polysaccharide, which consists of a polymer made of repeating oligosaccharide units in direct contact with the external milieu<sup>378</sup>. The LPS is also known as bacterial endotoxin, because it triggers a potent inflammatory response both *in vitro* and *in vivo*, the latter characterized by a systemic production of inflammatory cytokines, leading to tissue damage, alterations in body temperature, and lethality<sup>379</sup>. However, the LPS of *H. pylori* has been reported for a decreased toxicity, as higher quantities are needed to elicit a host response in comparison with the *Escherichia coli*

LPS, due to modifications in the lipid A which impair its recognition by the host receptors<sup>380</sup>. In addition to these changes, the O-antigen contains different human Lewis antigens (Le<sup>x</sup>, Le<sup>y</sup>, Le<sup>a</sup>, Le<sup>b</sup>) which are also expressed in gastric epithelial cells, thus providing a strategy to escape the host's immune response through molecular mimicry<sup>381-383</sup>. The Lewis antigens in the bacterium may also play a role in adherence to gastric epithelial cells, and their capacity to undergo phase variation would provide a dynamic adherent phenotype<sup>384</sup>.

#### **v. Pathogenesis of gastric disease: Gastritis and precancerous lesions of the Correa cascade**

In non-pathological conditions, the gastric mucosa lacks myeloid and lymphoid tissues<sup>385</sup>. Gastritis is characterized by the infiltration of the lamina propria with polymorphonuclear and mononuclear leucocytes, and the presence of *H. pylori* is the main inductor<sup>28</sup>. Pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-8, and IL-6 are increased in the gastric mucosa of infected subjects<sup>337</sup>. The sustained inflammatory process directly and indirectly activates different branches of the immune system, and together with bacterial factors (mainly CagA and VacA) contribute to substantial changes in the architecture of the gastric mucosa. *H. pylori* stimulates the gastric epithelium, inducing the activation of dendritic cells and macrophages, as well as a Th1-predominant adaptive response<sup>337</sup>. The resulting immune response is incapable of eradicating the bacterium and the persisting stimulus leads to a chronic inflammation process and mucosal damage.

Multifocal atrophic gastritis is the first stage of the sequence of lesions that lead to intestinal-subtype gastric adenocarcinoma. It initiates as a chronic superficial pangastritis involving the antrum's pyloric mucosa, the body's oxyntic mucosa, and stomach fundus in a spotted pattern of multiple foci<sup>386</sup>. This superficial gastritis turns to a chronic atrophy with loss of the glandular structures and their replacement with metaplastic pyloric or intestinal glands and fibrosis of the lamina propria<sup>28,386</sup>.

Approximately half of *H. pylori*-positive cases of chronic superficial gastritis will evolve to gastric chronic atrophy<sup>387</sup>, depending on the environmental conditions, bacterial virulence factors, and host's genetic background. Bacterial contact with epithelial cells also deregulates oncogenic signaling pathways that, along with chronic inflammation, induce genetic and epigenetic changes. For example, IL-6 production in response to infection was shown to induce STAT3 activation and stimulate ERK1/2 signaling in *H. pylori*-associated gastritis and during infection with *H. pylori* CagA<sup>+</sup>, and this aberrant activation could be one of the early events in tumor initiation and progression<sup>388</sup>. Another example is the *H. pylori*-induced hypermethylation of the promoters of genes such as *p16*, *CDH1*, *DAPK*, *APC*, *MLH1*, and *COX2*, which decrease when the bacterium is eradicated<sup>389</sup>.

The development of atrophy determines the course of the *H. pylori* infection, because the infected individuals that do not present it, have an increased probability of developing duodenal ulcer, end in the other hand, those who do develop atrophy are at greater risk of gastric adenocarcinoma<sup>387</sup>. As atrophic gastritis is the initial step on the Correa cascade of precancerous lesions leading to intestinal subtype gastric adenocarcinoma, its presence induces a five-fold increase in the risk of developing this type of cancer<sup>33,390</sup>. If atrophy constitutes or not the point of no return in the way to gastric cancer is still a subject of debate. However, additional alterations in signaling pathways have been described in metaplasia and dysplasia, which could be associated to malignant transformation<sup>388,389</sup>. The transition from dysplasia to adenocarcinoma is not only explained by the accumulation of mutations in transformed cells but it requires the penetration of neoplastic cells in the surrounding stroma, therefore, the acquisition of ECM degrading and migration capacities<sup>28</sup>, which can result from aberrant activation of signaling pathways that confer CSC-properties through an EMT<sup>131</sup>.

## vi. Models of study

### 1. *In vitro* models

#### a. Cell lines

As *H. pylori* naturally affects the stomach, the most used *in vitro* model for study of the infection are human epithelial cells from the gastric mucosa. Unfortunately, the primary culture of these type of cells is not attainable in the long term because cells loss their cohesive properties and lack the ability to renew themselves. As a consequence, *in vitro* studies of *H. pylori* infection are often carried in immortalized cell lines or, more frequently, in cell lines derived from gastric adenocarcinomas. Even if they are already transformed, these cell lines mimic the properties of a rapidly-changing, proliferative epithelium such as the gastric epithelium which is the niche of the bacterium. Therefore, they represent a convenient and accepted model for the study of the molecular aspects of the host-pathogen interaction. The main characteristics of commonly used gastric epithelial cell lines are summarized in table 7.

#### b. Organoids

One of the main flaws of the conventional tumor cell culture in two dimensions (2D) is the absence of a higher three-dimensional organization (3D), which could be important in the outcome of experiments performed in this model. As an alternative strategy, culture systems that allow the gastric cell lines to grow on 3D, using extracellular matrix proteins, nanofibers, co-culture with fibroblasts, or non-adherent culture in a serum-free medium, have been developed<sup>391-393</sup>. Even if these approaches have proved very useful, they do not overcome the lack of heterogeneity that cell lines show when compared

to the human stomach, much more complex than a monolayer of a single type of cells, nor they give answer to the common problems of the cell lines such as genetic and epigenetic changes in tumor suppressor genes and oncogenes, accumulated mutations due to years of culture, and even infection with viruses which could modify the cell behavior towards infection<sup>394</sup>. Different groups have approached this issue with strategies, such as de novo generation of human gastric organoids in vitro through the directed differentiation of pluripotent stem cells or from surgical samples of human stomach including healthy and tumor tissue and sorted stem cells<sup>395,396</sup>. One of many technical challenges of these methods for the study of *H. pylori* infection is that, as the bacterium can only interact with the apical surface of the epithelial cells, the infection has to be made by microinjection of the bacteria in the lumen of each organoid. As an alternative procedure, gastric organoids have been sheathed to establish a 2D culture of polarized gastric epithelial cells which are more suitable for infection and could be adapted to other experimental conditions<sup>397</sup>.

## **2. In vivo models**

### **a. Murine models**

After infection with *H. pylori*, precancerous lesions can be observed in both C57BL/6 and BALB/c mice, but the infection does not cause the appearance of any type of gastric adenocarcinoma, only MALT lymphomas<sup>398,399</sup>. B6129 mice, derived from the mating of C57BL/6 with 129S6/SvEv, can develop dysplastic lesions but not adenocarcinomas upon infection<sup>400</sup>. In order to have a more suitable model for gastric adenocarcinoma, different strategies of transgenic mice have been developed. Mice knocked-out for TGF- $\beta$ , p27 or TFF2 and infected with *H. pylori* are able to develop dysplastic lesions and gastric adenocarcinoma<sup>401-403</sup>. Also the overexpression of gastrin (the INS-GAS model), and to a lesser extent, of IL-1 $\beta$ , allow the development of gastric adenocarcinomas in mice infected with *H. pylori*<sup>404,405</sup>.

### **b. The Mongolian gerbil model**

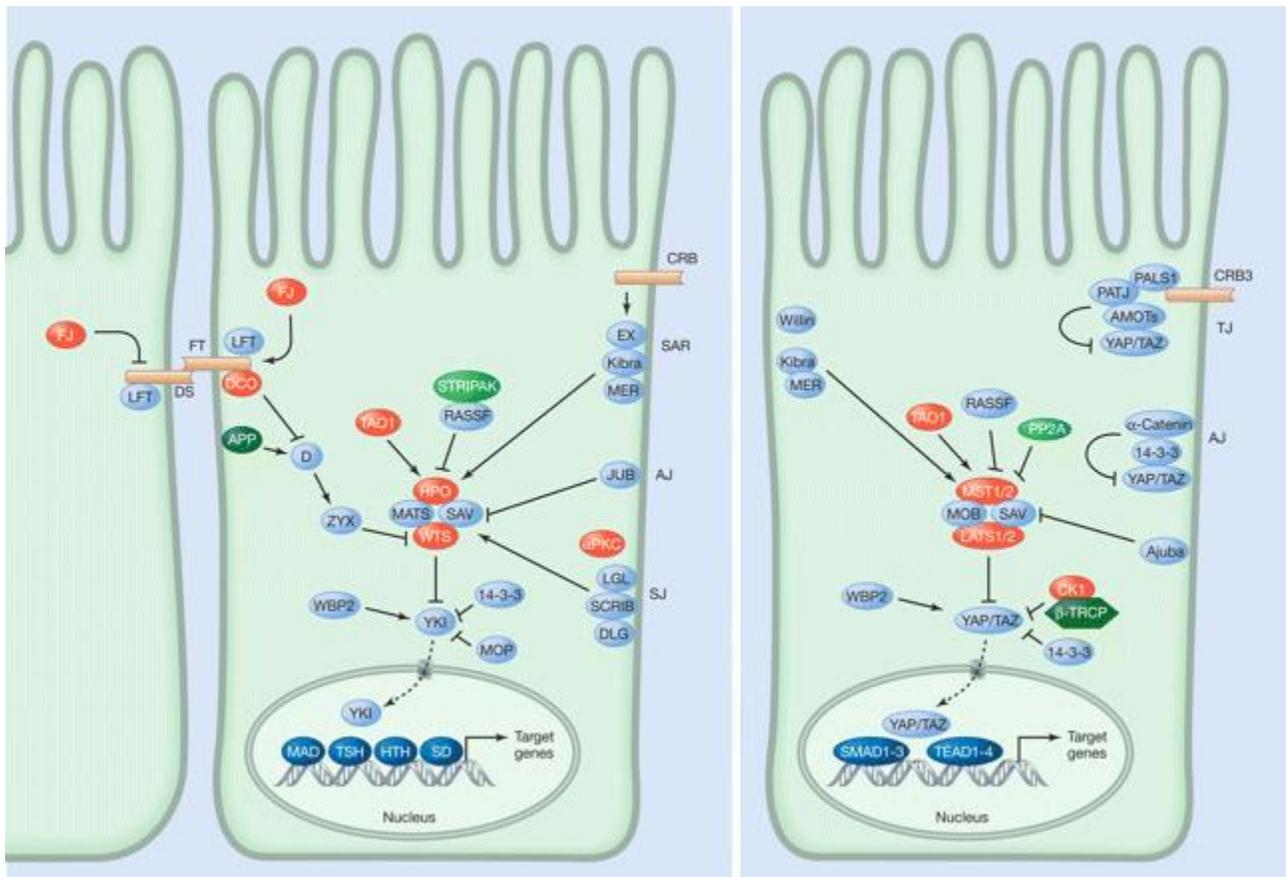
The Mongolian gerbil model has been so far, the only successful animal model for gastric adenocarcinoma following infection with *H. pylori*. The first description of *H. pylori*'s capacity to infect this animal dates from 1991, but the follow-up was for only two months, which made it impossible to observe any lesions<sup>406</sup>. Then, the period of observation was increased to six months, allowing the detection of ulcers and intestinal metaplasia<sup>407,408</sup>. And finally, after a longer period of observation, Watanabe *et al* described the appearance of neoplastic gastric lesions in gerbils infected with *H. pylori* after 62 weeks<sup>409</sup>. Since then, several studies have used the model with similar results, the developing of gastric adenocarcinoma after 62 to 90 weeks of infection<sup>410-413</sup>. Some studies have found earlier apparition of

lesions (8 to 12 weeks), most likely linked to the use of a more virulent strain of the bacterium<sup>56,414,415</sup>. On the other hand, another study failed to find the neoplastic lesions in the gerbil even after a long follow up period<sup>416</sup>. The different results among studies may be explained partially by the genetic background of the gerbils, their microbiota, and the anatomic-pathologic assessment performed<sup>416</sup>. Another important fact concerning the model, is the absence of metastases and mortality of the gerbils due to the gastric adenocarcinoma, which points to important differences between the evolution of the pathology in the gerbil and in the human being.

## d. Hippo pathway

### i. General description

The Salvador-Warts-Hippo pathway was first described as a size-controlling pathway in the epithelial precursors in *Drosophila melanogaster*, where the main components of the pathway were isolated in genetic mosaic screens for the detection of genes associated with cell growth and proliferation, by studying the mutations present in flies with organ size abnormalities<sup>417</sup>. It is a highly evolutionarily conserved pathway, composed by a tumor suppressor core consisting of a module of two kinases, MST (Hippo or HPO in *D. melanogaster*) and LATS (Warts or WTS in *D. melanogaster*), and their regulatory proteins SAV (Salvador) and MOB (MATS)<sup>418</sup>. The second component includes the output elements, which are the targets of phosphorylation by the kinase module. In the fly, it is the transcriptional coactivator YKI (Yorkie), homolog to the YAP1 and TAZ proteins in mammals. The Hippo pathway components in mammals and their homologs in *D. melanogaster* are listed in table 10 and showed in figure 10.



**Figure 10. Hippo pathway.** Component of the Hippo signaling pathway in *Drosophila* (left) and mammals (right). Modified from <sup>418</sup>

**Table 10. Hippo pathway components in *Drosophila* and human homologs<sup>418</sup>.**

	<i>Drosophila melanogaster</i>	Human
<b>Upstream</b>	Fat (FT)	FAT1—FAT4
	Dachsous (DS)	DCHS1 and DCHS2
	Discs overgrown (DCO)	CK1ε and CK1δ
	Lowfat (LFT)	LIX1 and LIX1L
	Four-jointed (FJ)	FJX1
	Dachs (D)	?
	Approximated (APP)	ZDHHC9, ZDHHC14 and ZDHHC18
	Zyxin (ZYG)	Zyxin, LPP and TRIP6
	Merlin (MER)	Merlin (also known as NF2)
	Expanded (EX)	Willin/FRMD6 and FRMD1
	Kibra	Kibra
	Crumbs (CRB)	CRB1—CRB3
	Lethal giant larvae (LGL)	LGL1 and LGL2
	Discs large (DLG)	DLG1—DLG4
	Scribble (SCRIB)	SCRIB
	aPKC	aPKCι and aPKCζ
	STRIPAK (PP2A)	PP2A (STRIPAK)
	RASSF	RASSF1-RASSF6
	Myopic (MOP)	HD-PTP
	JUB	Ajuba, LIMD1, WTIP
TAO1	TAO1—TAO3	
<b>Kinase core</b>	Hippo (HPO)	MST1 and MST2
	Salvador (SAV)	SAV1
	Mats (MTS)	MOBKL1A and MOBKL1AB
	Warts (WTS)	LATS1 and LATS2
<b>Downstream</b>	Yorkie (YKI)	YAP1 and TAZ
	WBP2	WBP2
	Scalloped (SD)	TEAD1—TEAD4
	MAD	SMADs
	TSH	TSHZ1—TSHZ3
	HTH	MEIS1—MEIS3

Upon phosphorylation by LATS, YAP1 and TAZ are bound to 14-3-3 proteins in cell-cell junctions or undergo proteasomal degradation. This sequestration limits their activity by preventing the nuclear accumulation. Active nuclear YAP1 and TAZ function together with TEAD family transcription factors to promote tissue growth by increasing the expression of genes that positively regulate cell growth and inhibit apoptosis<sup>418</sup>. YAP1 and TAZ can also work together with other transcription factors such as SMAD-family, TBX5, RUNT1/2, and p73<sup>419</sup>. Their capacity to regulate transcription of genes regulation cell survival and proliferation make YAP1 and TAZ, oncogenes.

## ii. Upstream regulators

The Hippo pathway does not have dedicated extracellular ligand/receptor complexes like other signaling pathways. Instead, the pathway integrates multiple extracellular signals including cell polarity, contact inhibition, stress, mechanotransduction, and cell attachments, as well as response to hormones and growth factors. Some of the inputs that mediate the response to these processes are described below.

### 1. *FERM- and WW-containing proteins upstream MST*

Both FERM domains and WW domains are modular domains that facilitate protein-protein interactions, and their presence is a common feature amongst the various proteins that regulate the Hippo pathway. WW domains are small modules of 35 to 40 amino acid residues, which bind to proline rich binding motifs (PPxY), and include two conserved tryptophan (W) residues, therefore the name of the domain. The FERM (4.1/ezrin/radixin/moesin) domains are common in proteins that interact both with the cytoskeleton and the plasma membrane<sup>420</sup>. They are cysteine-rich hydrophobic protein modules of around 300 amino acid residues, able to bind a broad range of biochemically different molecules, such as phosphoinositols, glycoporphins, receptors including CD44, ICAMs, actin, and other FERM-containing proteins<sup>420</sup>.

### 2. *Merlin and angiomotins*

Merlin is a FERM domain-containing, 69 kDa protein encoded by the NF2 (neurofibromin 2), which is a tumor suppressor whose mutations have been described as a cause in neurofibromatosis type 2 and as a predisposition to various tumors of the nervous system<sup>421</sup>. Merlin is normally located in cell junctions, in association with  $\alpha$ -catenin or angiomotins. It is implicated in the contact-depending inhibition of cell proliferation by coupling signals from the cell junctions to growth regulatory signaling pathways, including Hippo. Although the exact mechanism remains to be confirmed, the current evidence suggests that Merlin may promote the Hippo signaling and YAP1 inactivation by assembling platforms where the LATS1/2 kinases phosphorylate and inactivate YAP1<sup>421</sup>.

Merlin-interacting proteins angiomotin (Amot) and angiomotin-like 1/2 (Amotl1/2) are tight junction proteins described for their roles in angiogenesis during development. Angiomotins have been identified as binding partners and negative regulators of YAP1, as they favor YAP1 cytoplasmic localization and have been describe to promote its phosphorylation and inhibit the expression of the YAP1-target genes CTGF and CYR61<sup>421</sup>.

### **3. Willin/FRMD6**

Willin is a 71 kDa protein with a FERM domain, the human homologue of the Expanded protein of *D. melanogaster*, and is widely expressed in both neuronal and non-neuronal tissues<sup>420</sup>. Intracellularly, it is located at the plasma membrane, possibly playing a role in contact inhibition and adhesion. Willin frequently colocalizes with Merlin and interacts with YAP1. A recent study showed that Willin alone is sufficient to activate the Hippo pathway and antagonize YAP1-induced phenotype<sup>422</sup>.

### **4. KIBRA**

KIBRA is a 125 kDa protein which contains two amino-terminal WW domains, first identified in a yeast screening as a partner for the modulator of the post-synaptic cytoskeleton Dendrin<sup>423</sup>, and was then shown to be a component of the Hippo signaling pathway<sup>424</sup>. KIBRA binds to Merlin, independently of its WW domains, and may contribute to the stabilization of the multiprotein complex and enable the transmission of signal to activate the Hippo cascade<sup>424</sup>. A Merlin-independent mechanism has been proposed for Hippo regulation by KIBRA. Via its WW domains, KIBRA can interact directly with LATS1/2 and increase both its proteins levels and phosphorylation, at least in part by inhibiting ubiquitination and the proteasomal degradation of LATS2, which can ultimately lead to an activation of the pathway and YAP1 phosphorylation<sup>123</sup>.

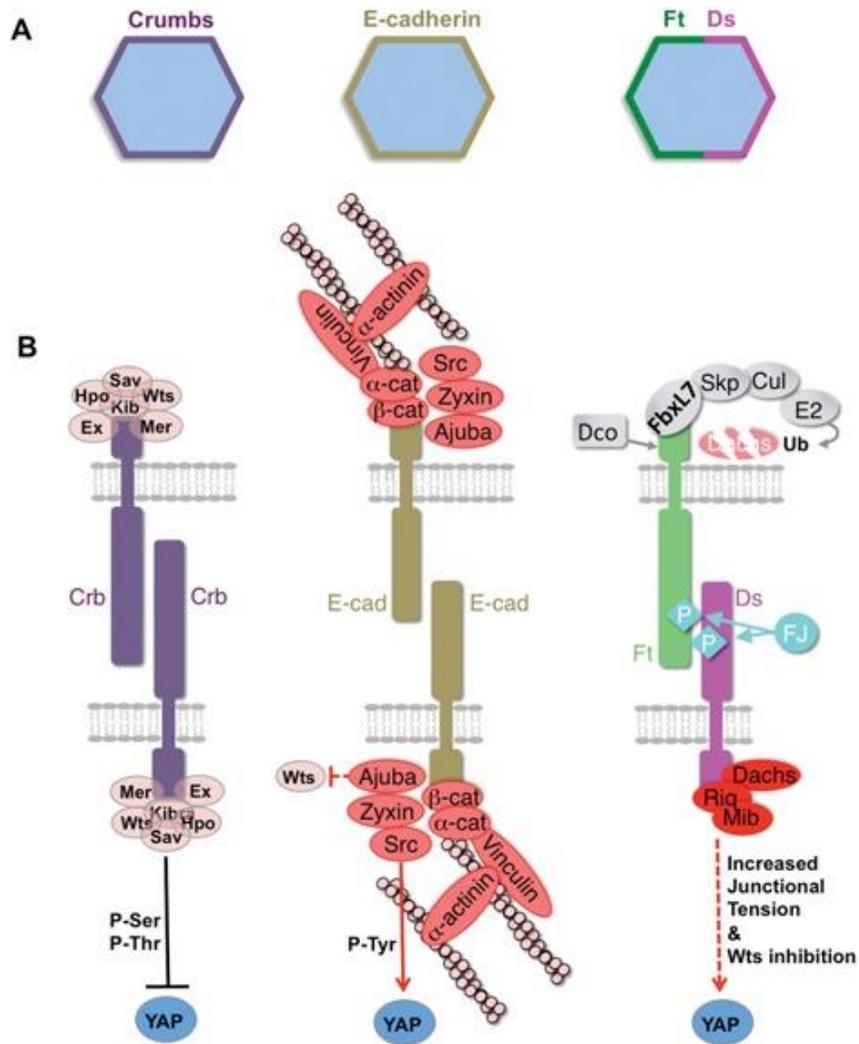
### **5. Cell polarity and adhesion**

The modulation of the Hippo pathway, and concretely of YAP1 activity, is caused by either the increased activity and the availability of the Hippo kinases towards YAP1 or sequestration of YAP1 at cell junctions<sup>425</sup>. Various systems of cell polarity have been found to be involved in Hippo regulation, the apical and basolateral polarity complexes in epithelial cells, and the planar polarity regulation, whose participation is mainly restricted to the context of development of specialized structures in *Drosophila* and therefor will not be detailed in this work (figure 11).

#### ***a. Apical cell polarity***

The Crumbs (Crb) protein is the main regulator of apical cell polarity in epithelial cells. It is a transmembrane protein that share a conserved structure consisting of a large extracellular region linked to a short intracellular tail containing FERM- and PDZ-binding domains<sup>426</sup>. Crumbs recruit a protein scaffold that comprises the proteins Stardust, aPKC, Moesin, Yurt, and Par6<sup>427</sup>. Crumbs can bind to the FERM-domain of Willin and therefore, modulate the Hippo pathway<sup>427</sup>. Besides, the direct binding of YAP1 and

TAZ by the Crumbs complex has also been described in high density-cultured epithelial cells<sup>428</sup>. Furthermore, in a developing lung model where it was shown that changes in cell polarity dictate airway epithelial differentiation, and Crumbs is required for this process, because it promotes the interaction between YAP1 and LATS1/2, consequently inducing YAP1 phosphorylation and cytoplasmic retention<sup>429</sup>.



**Figure 11. Cell polarity systems involved with Hippo regulation<sup>430</sup>.** The apical and polarity complex (left) and cell adhesion proteins (center) in epithelial cells, and the planar polarity complex (right) are upstream signals for the Hippo pathway.

*b. Basolateral cells polarity*

The scribble homologue (SCRIB)-lethal giant larvae homologue (LGL)-disc-large homologue (DLG) complex defines the basolateral plasma membrane domain, and it is localized with adherens junctions<sup>431</sup>.

The SCRIB-LGL-DLG complex is considered a tumor suppressor, as modifications of the expression of its components promotes the acquisition of invasiveness in various cancers such as colon cancer and hepatocellular carcinoma<sup>431</sup>. The Lgl protein has been shown to regulate Hippo signaling in *Drosophila*. However, unlike the regulation by Crumbs, the regulation is not related with the localization of the MER/KIBRA/Ex complex but directly with Hpo<sup>428</sup>. Furthermore, a correct localization of Scribble is required for the Hippo pathway activity and a Scribble knock-down leads to an increase of TEAD-reporter activity and a decrease in YAP1-phosphorylation<sup>432</sup>. Co-immunoprecipitation experiments suggest the existence of a Hippo regulatory complex formed by MST1, LATS1, Scribble, and the Scribble regulators LKB1 and MARK (PAR family)<sup>432</sup>.

### c. *Cell adhesion proteins*

Cell adhesion proteins are intimately associated with cell polarity. The tight junctions (TJ) not only define the limits of polarized membrane domains, as they divide the apical from the basolateral segments of the plasma membrane, but also these and the adherens junctions (AJ) bind to polarity complex proteins and can modulate the regulation they exert on Hippo signaling<sup>433</sup>. Together, adhesion proteins function to sense the integrity of the epithelium, and it has been shown that disruptions and discontinuities in the epithelial cell layers induce the nuclear activity of YAP1 and TAZ<sup>434</sup>. Moreover, during development, even in non-polarized cells, proteins of cell adhesion such as E-cadherin, participate in the regulation of YAP1 activity.

TJ and AJ proteins sequester YAP1 and TAZ by interaction with several adhesion proteins. The TJ protein  $\alpha$ -catenin interacts strongly with phosphorylated YAP1, in a binding mediated by 14-3-3 proteins, sequestering YAP1 and preventing its dephosphorylation and nuclear translocation<sup>434</sup>. The interaction of cell adhesion proteins with YAP1, TAZ, and with regulators of Hippo signaling such as the Mer-KIBRA-Willin has been described for PTPN14, LKB1, and ZO-1/2<sup>434</sup>.

E-cadherin, is a very important molecule of cell-cell adhesion in epithelial cells because its homophilic binding allows the formation of the epithelial junctional complex. Loss of E-cadherin promotes tumor progression and metastasis, and it's a hallmark of many epithelial cells cancers as well as a feature of EMT. Increased expression of E-cadherin in cancer cells blocks invasion abilities and tumor progression by increasing the cell adhesion and the  $\beta$ -catenin activity. A study by Kim et al, recently showed that the Hippo signaling is required for the inhibition of proliferation mediated by E-cadherin, as depletion of LATS, Merlin, and KIBRA or over expression of YAP1 blocked this inhibition<sup>435</sup>. Furthermore, the authors of this study found that the E-cadherin- $\beta$ -catenin complex regulates the phosphorylation status and nuclear localization of YAP1 in MCF10A cells cultured in high density.

## 6. **Cytoskeleton, extracellular matrix and mechanical forces**

The extracellular matrix, the neighboring cells, and even its own cytoskeleton, exert mechanical forces over the cell. Cells can sense these mechanical cues and respond by undergoing different processes such as proliferation, differentiation, apoptosis, and migration, and the cytoskeleton is a key element in this response<sup>434</sup>. The rearrangement of actin cytoskeleton is a key element in the YAP1/TAZ-mediated response to cell geometry, stiffness of the ECM, and attachment to a surface. Moreover the stabilization of F-actin using the drug jasplakinolide, was shown to induce the expression of YAP1/TEAD target genes CTGF and CYR61 and the effect of the drug was inhibited when YAP1 was knocked-down<sup>436</sup>. The RhoA kinase has been demonstrated to play a crucial role in the YAP1 regulation by actin, as its overexpression induces dephosphorylation of YAP1, and both RhoA activity and actin polymerization are required in PAR1-mediated activation of YAP1<sup>437</sup>. While the YAP1/TAZ response to detachment and crowding can be inhibited by LATS activation, the response to matrix stiffness, requires not only an increase of filamentous actin but also the formation of stress fibers and seems to be independent of LATS activity<sup>437</sup>. In a recent study, Sun *et al* proposed a computational model integrating the mechano-sensing signaling network of YAP1 and TAZ, which integrated the relation of the latter and the Hippo pathway, by a synergistic effect between LATS2 and the F-actin regulator protein LIMK. The study also highlighted the influence of adhesion, stiffness, and RhoA over other regulators.

### iii. **Hippo kinases**

#### 1. **MST 1/2**

The gene coding for the *Drosophila* homologue of MST1/2, *hippo* (*hpo*) was first identified in 2003 in a genetic screen of overgrowth mutants in the fruit fly, as a gene promoting both cell cycle exit and cell death<sup>438</sup>. It was identified at the same time by other group in a screening of homozygous mutant cells in the eyes of otherwise heterozygous fruit flies, which also detected *sav* and *wts*<sup>439</sup>. In the human, MST1 and MST2 are Ste20-related serine/threonine kinases, which are at the core of the Hippo kinase tumor suppressor cascade. MST1 (487 amino acids) and MST2 (491 amino acids) share more than 95% of the catalytic domains are 75% identical<sup>440</sup>. The most characteristic feature of these kinases is the presence of a SARAH domain (Salvador-RASSF-Hippo), which is approximately 50 amino acids long, and mediate the homo- or heterodimerization and subsequent stabilization of MST1/2.

Regarding its enzymatic activity, auto-transphosphorylation *in vitro* has been described in purified MST1/2 in the presence of Mg<sup>2+</sup> and ATP, however, the relevance of this phenomenon *in vivo* remains unknown<sup>440</sup>. On the other hand, two main regulators of the MST1/2 activity in the cell have been described: RASSF polypeptides and SAV1 (WW45, Salvador).

The RASSF family of tumor suppressor proteins are adaptor proteins that interact with GTP-Ras by their RA-domain. There are ten isoforms, and isoforms RASSF1 to RASSF6 have been described to also possess a SARAH-domain, which allows them to form complexes with MST1/2 and SAV1<sup>441</sup>. This mechanism is particularly important in T cells, where the MST1/RASSF5 may facilitate the phosphorylation of MST1 by allowing the continuous interaction with the activated GTPase, thus impeding dephosphorylation of the kinase and also due to a concentration of MST1 in the membrane, facilitation transphosphorylation. The other regulator, SAV1 is a 383 amino acid, SARAH-domain containing protein that has the ability of binding both MST1/2 and its substrate LATS, thanks to the WW domain that links the PPXY in LATS<sup>440</sup>. Other stimuli can influence the activation of MST1/2. It is activated during apoptosis in the liver, by reactive oxygen species, and during the G2/M phase of the cell cycle, and on the contrary, inhibited by Akt-mediated non-canonical phosphorylation in response to EGF.

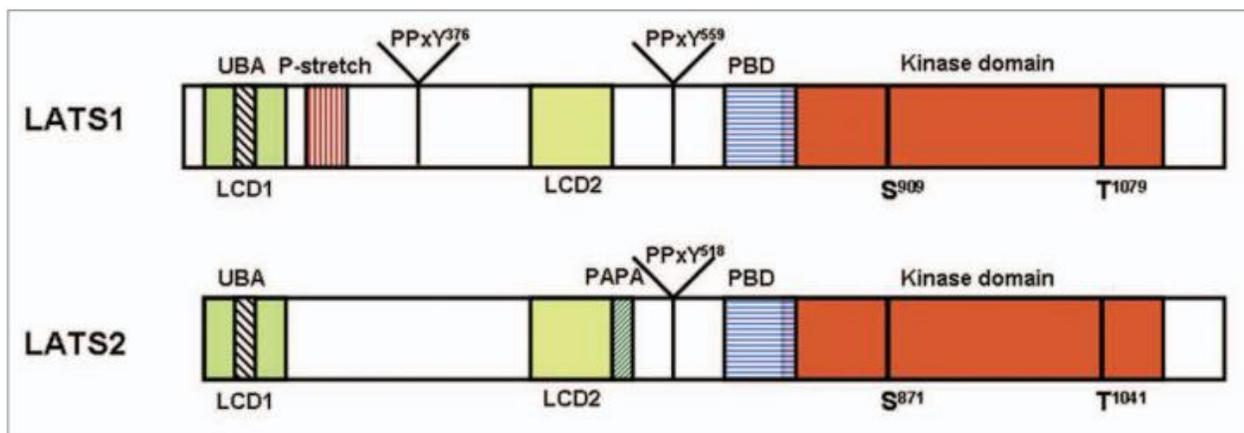
MST1/2 has various substrates. Downstream in the Hippo pathway, and the most studied are the LATS1/2 kinases and its regulators Mob1A/B. MST-mediated activation of LATS occurs in a sequential process that involves the collaboration of Mob1<sup>425</sup>. Autophosphorylation of MST1/2 creates a site for Mob1 recruitment, and relieves Mob1 from an autoinhibitory conformation, making it accessible to LATS. This allows the formation of a MST-Mob-LATS complex in which both Mob (Threonine 35) and LATS (Threonines 1079 in LATS1 and 1041 in LATS2) are phosphorylated by MST, rendering an active LATS and promoting the dissociation of the complex by phosphor-Mob1, which in addition favors the LATS autophosphorylation, also required for its activity. Besides the downstream elements of the Hippo pathway, there are other substrates of MST1/2, for example the apoptosis-related transcription factor FOXO1/3, histones H2B and H2AX, cell cycle related kinases Aurora A/B, among others.

## **2. LATS1/2**

The *Drosophila* homologue of LATS, warts (wts), was described in 1995 by Robin *et al* as a tumor suppressor gene whose loss resulted in overproliferation and hypertrophy of epithelial cells leading to abnormal deposit of extracellular matrix<sup>442</sup>. Based on the nucleotides sequence, the authors also reported a predicted serine/threonine kinase activity and homology to the human myotonic dystrophy protein kinase and the Cot-1 protein kinase from *Neurospora crassa*. The human homolog, identified initially as *h-warts* or *lats1* (*large tumor suppressor*), was located at chromosome 6q24-25.1 as a gene coding for a serine/threonine kinase as previously predicted, which localized to the centromeres in interphase cells and relocated dynamically during the different phases of mitosis and acted as a negative regulator of CDC2/cyclin A<sup>443,444</sup>. A second homologue, *lats2* was identified both in mouse and human, with high homology, especially in the kinase domain between the murine and human forms (75%) and also between human *lats1* and *lats2* (85%)<sup>445</sup>. Human *lats2* was found in chromosome 13q11-12 and coded for a 125-

116 kDa nuclear protein<sup>445</sup>, while a posterior study showed a cytoplasmic localization during interphase and in the mitotic apparatus during mitosis, and whose ectopic expression blocked the cell cycle in G1/S and caused a downregulation of CDK2/cyclin D<sup>446</sup>.

Regarding the protein structure, LATS1 and LATS2 share a high homology not only in the conserved kinase domain but also in the amino- and carboxy termini, as they both have a protein-bonding domain (PBD) upstream the catalytic domain, as well as the two LATS conserved domains LCD1 and LCD2<sup>447</sup>. The PBD domains binds to proteins like MOB1 and LIMK1, and while the exact function of the LCDs is unknown, they are indispensable for the tumor suppressor activity. The presence of PPxY motifs is also a common feature of both proteins, and allows the binding to YAP1 and TAZ. A schematic representation of the structure of LATS1/2 is showed in figure 12.



**Figure 12. Structure of LATS1 and LATS2<sup>447</sup>.** LATS1 and LATS2 share several conserved domains, including the kinase domain, but also differ in the PPxY motifs, a P-stretch (present only in LATS1), and a PAPA repeat (present only in LATS2).

The link with the Hippo signaling was established when Lats1/2 were found to be phosphorylation substrates by MST1/2<sup>448</sup>. Phosphorylation of the kinase domain of Lats1/2 in the activation loop (autophosphorylation, serine 909/872) and the hydrophobic domain (MST1/2, threonine 1079/1041) stimulated their kinase activity<sup>447,449</sup> (figure 11). In addition to regulation by Hippo signaling, there are several mechanisms of LATS1/2 regulation at the transcription and post-transcriptional levels, including phosphorylation by other kinases (Table 11), subcellular localization and conformational regulation<sup>447,450</sup>. Post-transcriptional regulation by micro RNAs have been described for LATS2, notably the miR-372-373 cluster. In esophageal cancer cell lines and esophageal cancer tissue samples, miR-373 levels were shown to be inversely correlated to the expression of LATS2, and a direct inhibition of LATS2 was demonstrated in vitro in the cell lines<sup>451</sup>. Additional evidence of this regulation has been shown in a gastric carcinogenesis

model of AGS cell infected with *H. pylori*. Belair et al<sup>452</sup> reported a high expression of miR-372 in this cell line, where together with miR-373, it silences LATS2 promoting cell proliferation. Infection with *H. pylori* inhibits the synthesis of both microRNAs, in a CagA-dependent manner, allowing LATS2 expression and a cell cycle arrest in G1/S, and this may impair the epithelium renewal, not only representing the overcome of a major antibacterial defense mechanisms, but also potentially favoring atrophy of the mucosal epithelium (an initial step in the gastric carcinogenesis cascade).

**Table 11. Overview of the regulation of mammalian LATS kinases by phosphorylation.** Modified from <sup>450</sup>

Kinase	Upstream kinase	Site(s)	Role of phosphorylation
LATS1	Unknown	Ser909	Essential for kinase activity
LATS1	MST1/2	Thr1079	Essential for kinase activity
LATS1	Cdk1/Cyclin B	Thr490/ Ser613	Possible role in mitosis
LATS1	NUAK1	Ser464	Controls LATS1 protein stability
LATS2	Aurora A	Ser83/ Ser380	Plays a role in mitosis
LATS2	CHK1/2	Ser408	Plays a role in DNA damage response
LATS2	PKA	Ser172/ Ser380/ Ser592/ Ser598	Regulate LATS2 activity towards YAP1

YAP1 and TAZ, targets of the canonical Hippo signaling, are the main substrates of LATS1/2. Upon phosphorylation of these two proteins, LATS1/2 favor their cytoplasmic retention and degradation, acting like a tumor suppressor by blocking the pro-oncogenic transcriptional activity of the YAP1/TAZ-TEAD complex, described later in this chapter. However, there are other proteins that can be phosphorylated by LATS2. Interestingly, the EMT-associated transcription factor Snail1 has been describe as a target of LATS2 phosphorylation in the breast epithelial cell line MCF10A, where LATS2-mediated phosphorylation in Snail1 Thr203 was found to retain this protein in the nucleus, enhancing the protein stability and function, and acting, contrary to its classic role, in tumor promotion<sup>453</sup>. Another substrate of LATS2 phosphorylation describe recently is p21, a cyclin-dependent kinase (CDK) inhibitor that regulates cell cycle and inhibits apoptosis<sup>454</sup>. In response to UV radiation in the human osteosarcoma cell line U2OS and in HeLa cells, Lats2 phosphorylates p21 in Ser146, causing a destabilization that induces apoptosis. Noteworthy, the activity of LATS2 induced by UV radiation is independent of Mst1/2 phosphorylation, and occurs in a different amino acid residue (Ser385) by the Chk-1 kinase. Ser835-phosphorylated LATS2 is not required to phosphorylate YAP1 in the Hippo canonical Ser127 position, and that stimulation by UV may

displace the classical targets of LATS2 in favor of other substrates such as p21, and represent an alternative induction of cell death via LATS2 in response to DNA damage.

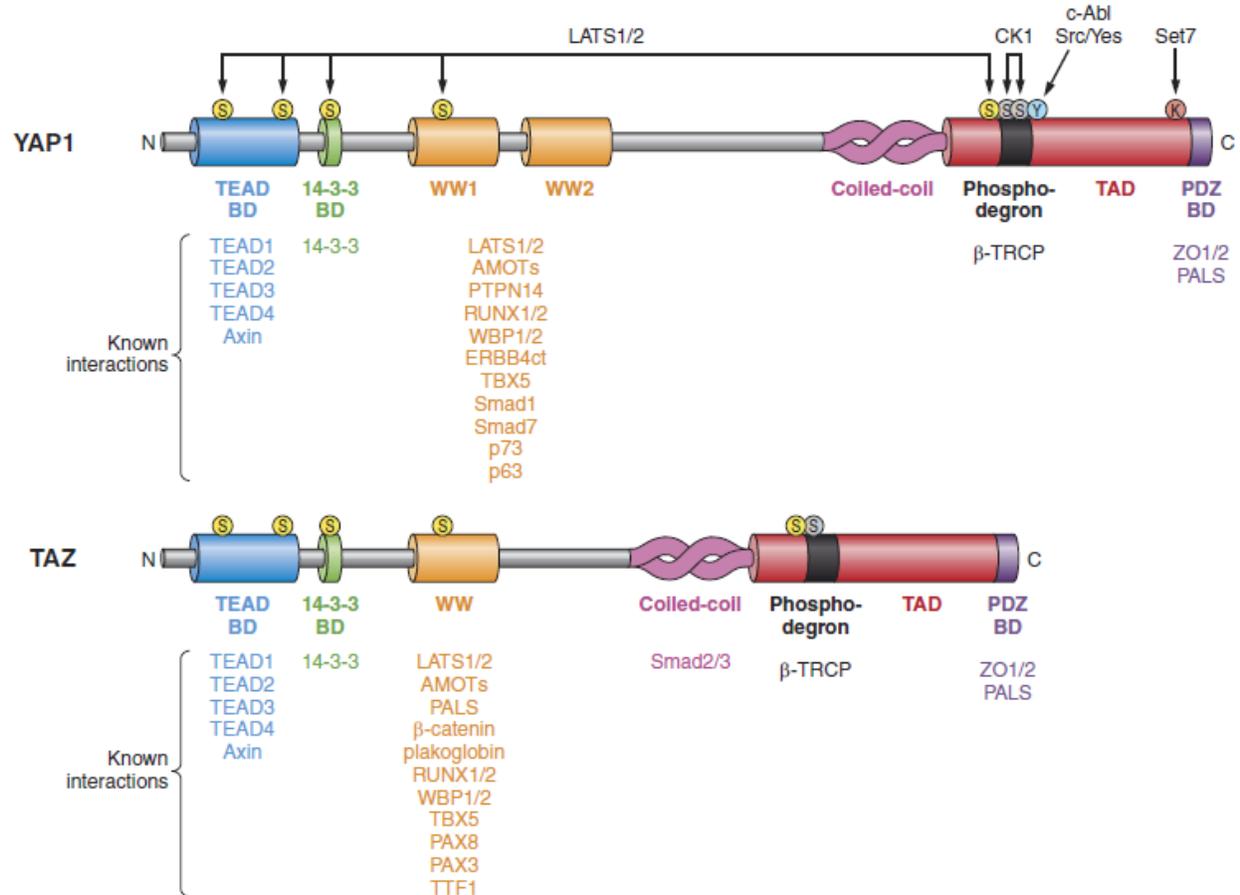
#### **iv. Downstream transcription co-activators YAP1 and TAZ**

The transcription co-activators Yes-associated protein 1 (YAP1, an orthologue of the *D. melanogaster* Yorkie) and its paralogue WW domain containing transcription regulator 1 (WWTR1 or TAZ) are the two major downstream effectors of the Hippo signaling pathway, and as such, they are involved in control of organ size, development, and tumorigenesis<sup>434</sup>. YAP1 and TAZ have similar functions and share structural features and mechanisms of regulation, however the redundancy between them is not absolute. Knock-out mice phenotypes illustrate some differences, as the YAP1-null mice are not viable due to defects in embryonic development, contrary to TAZ knock-out mice, which are viable but predisposed to disease.

##### **1. Structure**

The YAP1 gene was described in a screening of expression libraries aiming for proteins interacting with Src-homology 3 (SH3) by Sudol *et al*<sup>455</sup>. It encodes for protein of 65 kDa that has a modular structure including various domains: a WW (two conserved tryptophanes) domain that binds to PPxY (proline-proline-any amino acid-tyrosine) motifs, a PR (proline rich) domain, a TEAD-binding domain (TID), a SH3-binding motif (SH3-BM), and a transcriptional activation domain (TAD) containing a PDZ-binding motif (PDZ-BM). There are two isoforms of YAP1 generated by alternative splicing, YAP1-1 and YAP1-2, the latter have an additional WW domain (Figure 12).

Using a similar strategy of screening of cDNA libraries but for 14-3-3 protein binding partners, TAZ was identified by Kanai *et al* as a transcriptional co-activator sharing homology with YAP<sup>456</sup>. TAZ is a 45 kDa protein containing a conserved WW domain, a TEAD-binding domain, and a transcriptional activating domain with a PDZ-BM, common with YAP1, but it lacks the SH3-BM and the PR-domain (Figure 13). The TAZ gene is located in chromosome 3q24.



**Figure 13. Structure of YAP and TAZ<sup>457</sup>.** Schematic representation depicting the multiple domains of YAP1 and TAZ, the sites of interaction with other proteins and the sites of phosphorylation.

## 2. Phosphorylation and proteasomal degradation

The most studied regulation of YAP1 and TAZ occurs by LATS1/2-mediated phosphorylation in response to Hippo pathway signaling, as these proteins have five and four consensus sites (HxRxxS) for LATS1/2-serine phosphorylation, respectively<sup>457</sup>. YAP1-Ser127 and TAZ-Ser89 phosphorylations are very important in YAP1/TAZ regulation as they cause YAP1/TAZ cytoplasmic retention in  $\alpha$ -catenin/14-3-3 complexes in cell junctions, preventing their nuclear disponibility to act as transcriptional co-activators<sup>458</sup>. Other kinases may also phosphorylate the YAP1-Ser127, such as Akt (for which is was originally identified), JNK, and other unknown kinases in keratinocytes and liver. Thus LATS1/2-mediated phosphorylation could be seen as a predisposing event to nuclear exclusion and not necessarily a primary inactivation event.

A second LATS-phosphorylation site, YAP1-Ser381 and TAZ-Ser311, is important in the stability of YAP1/TAZ, because it primes a following round of phosphorylation by CK1 kinases, which in turn generates a phosphodegion recognized by ubiquitin-ligase adaptators that lead to poly-ubiquitination and

proteosomal degradation. YAP1 requires two CK1-mediated phosphorylations in order to be degraded and TAZ requires only one, which could explain the differences in stability observed between the two proteins. While the half-life of TAZ is short (<2h), YAP1 is more stable and its regulation is mostly by cytoplasmic retention<sup>457</sup>.

### **3. Physical regulation**

Cytoplasmic sequestration is a crucial regulator of YAP1/TAZ co-transcriptional activities. Part of this retention is dependent of LATS1/2-mediated phosphorylation, as previously explained, but there is also cytoplasmic retention independent from the phosphorylation status of YAP1/TAZ. Angiomotins and PTPN14 have PPxY domains capable of binding directly to WW domains of YAP1/TAZ, regardless their phosphorylation status, and retaining them in the cytoplasm<sup>458</sup>.

### **4. Nuclear functions and TEAD-transcription factor co-transcriptional activity**

As co-transcriptional regulators, YAP1 and TAZ exert their main function in the nucleus. When they are not phosphorylated in the LATS1/2-dependent phosphorylation site, YAP1/TAZ interact with and activate the TEA-domain (TEAD) transcription factors and promote the transcription of YAP1/TAZ-TEAD complex target genes. This interaction was demonstrated by the isolation of YAP1/TAZ as binding proteins of TEAD<sup>459</sup> and also by an increase of TEAD-luciferase reporter activity upon over expression of YAP1/TAZ<sup>460</sup>. In the fruit fly, the interaction has been described between the homologues of YAP1 and TEAD, Yorkie and Scalloped<sup>461</sup>.

TEAD-family transcription factors are evolutionary conserved transcription factors involved mainly in development, and were initially described for their capacity to bind to viral enhancers such as SV40 and HPV16 and activate the transcription of the controlled genes<sup>462</sup>. There are four TEAD transcription factor, TEAD1-4, and together, they are expressed in almost every tissue. The structure is conserved between the four members of the family, with a C-terminal TEA-domain, which binds DNA in the consensus sequence 5'-GGAATG-3', and an N-terminal transactivation domain that binds the co-activators<sup>462</sup>. Three groups of co-activators have been described for binding TEAD transcription factors: the Hippo pathway-controlled YAP1 and TAZ, the VGLL proteins, and the p160 family, and the complexes formed have different function at the transcriptional level.

TEAD-related transcription plays an important role in development, and TEAD transcription factors are necessary for myogenesis, cardiogenesis, development of the neural crest and other embryonic tissues<sup>462</sup>. Gene inactivation studies in mice embryos have shown, not only that TEAD transcription factors

are required for different developmental processes, but also that the roles for each TEAD are different, and that their activities may be redundant sometimes, but not necessarily identical in the same moment or in the same tissue. For example, mouse embryos null for TEAD1 do not survive due to lethal cardiac defects, proving a role of TEAD in heart development, but the silencing of TEAD4 results in a failure of endometrial implantation, but if it is silenced after implantation, the normal development is not affected, which suggest a role for TEAD4 particularly in the specification for the trophoctoderm<sup>460</sup>.

## **5. YAP1/TAZ/TEAD target genes**

The transcriptional targets of YAP1/TAZ-TEAD complexes include several genes such as CTGF, CYR61, ANKRD1, BIRC5, AXL, Ki67, surviving, c-myc, InhA, Col8a1, and others<sup>457,462</sup>. Remarkably, most of those are growth promoting factors, and the most important amongst them are the CCN family proteins CTGF (CCN2) and CYR61 (CCN1). Proteins of the CCN family are regulatory ECM-associated proteins, and in general, control cell migration, proliferation, cell lineage commitment and tissue specification, morphogenesis and angiogenesis, and are therefore required for embryonic development, tissue turnover, and injury repair<sup>463</sup>. Both CYR61 and CTGF, together with WISP2 (CCN5) are indispensable for embryonic viability, as deficiency of any of those compromised fetal development in mice, adding evidence to the importance of the YAP1/TAZ-TEAD role in this process.

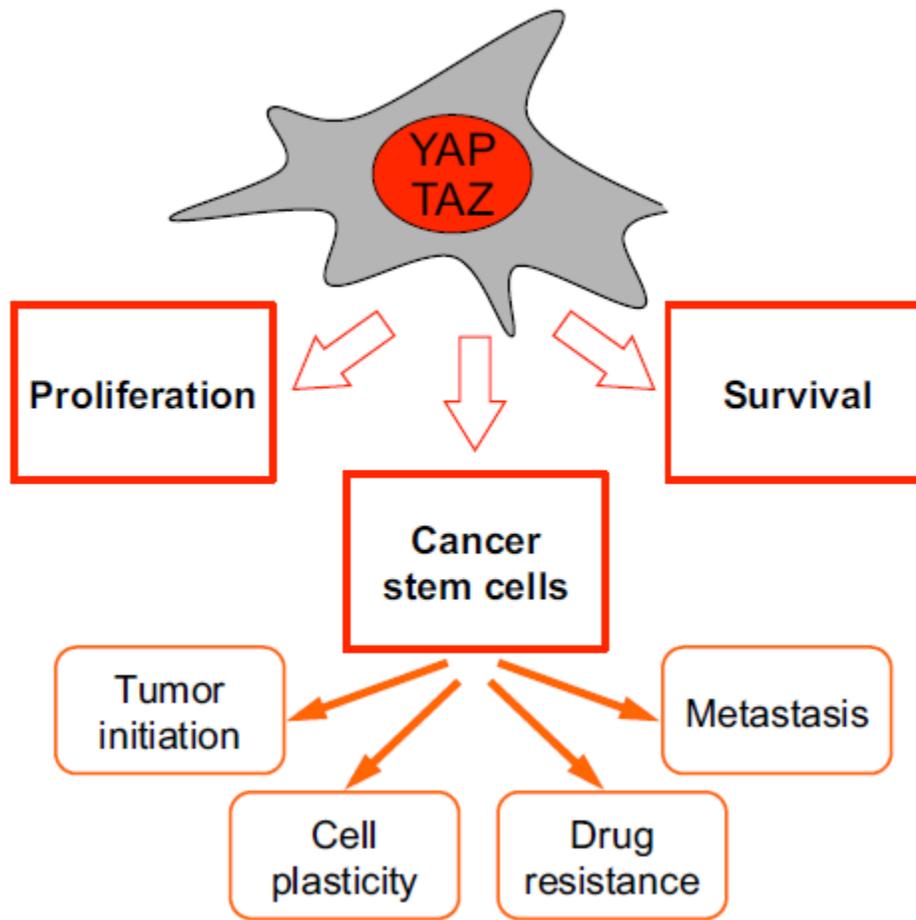
Both CTGF and CYR61 share common structural features, a signal peptide and an IGF1P-domain in N-terminal, followed by a von Willebrand C (vWC)-domain, a thrombospondin-type repeat 1 (TSP-1), and a Carboxy-terminal domain with a cysteine knot motif<sup>463</sup>. Through these domains, they are capable of binding to integrins ( $\alpha\beta3$ ,  $\alpha\beta5$ ), growth factors such as VEGF and TGF- $\beta$ , low-density lipoprotein receptor-related proteins, ECM components such as heparin sulfates, MMPs, and fibronectin, among others<sup>463,464</sup>. CTGF and CYR61 are co-expressed in the notochord, which suggests overlapping functions of both proteins in early stages of development. However, their relative importance varies in the different tissues. For example, in the mouse CTGF is more related to development of cartilage and epithelial structures of lung and pancreas, and CYR61 is widely expressed in the cardiovascular system.

In adult tissues, over expression of CTGF and CYR61 has been associated with pathological conditions. CYR61 increased expression is associated with inflammation and tissue repair. It has been found to be increased in obese men with metabolic syndrome, colitis, rheumatoid arthritis, and atherosclerosis; and polymorphisms of the gene have been linked to higher levels of plasma cholesterol and disorder of bone metabolism<sup>463</sup>. Increased expression of CTGF is linked to wound healing and fibroproliferative disorders, being correlated to excessive accumulation of ECM proteins in fibrotic tissues. While CTGF is highly expressed in some healthy tissues such as the spleen, the gastrointestinal tract, prostate, heart, amongst others, it is virtually absent in others, including brain, peripheral leucocytes, and

liver, except in pathological conditions<sup>463</sup>. A study showed that CTGF expression was elevated in hepatocellular carcinoma (HCC) samples and peritumoral non-cirrhotic and cirrhotic tissues<sup>465</sup>. Furthermore, the induction of CTGF expression in HCC cell lines and primary hepatocytes increased cell growth and dedifferentiation, expression of inflammation- and carcinogenesis-related genes, and resistance to doxorubicin.

#### **v. Hippo pathway and cancer**

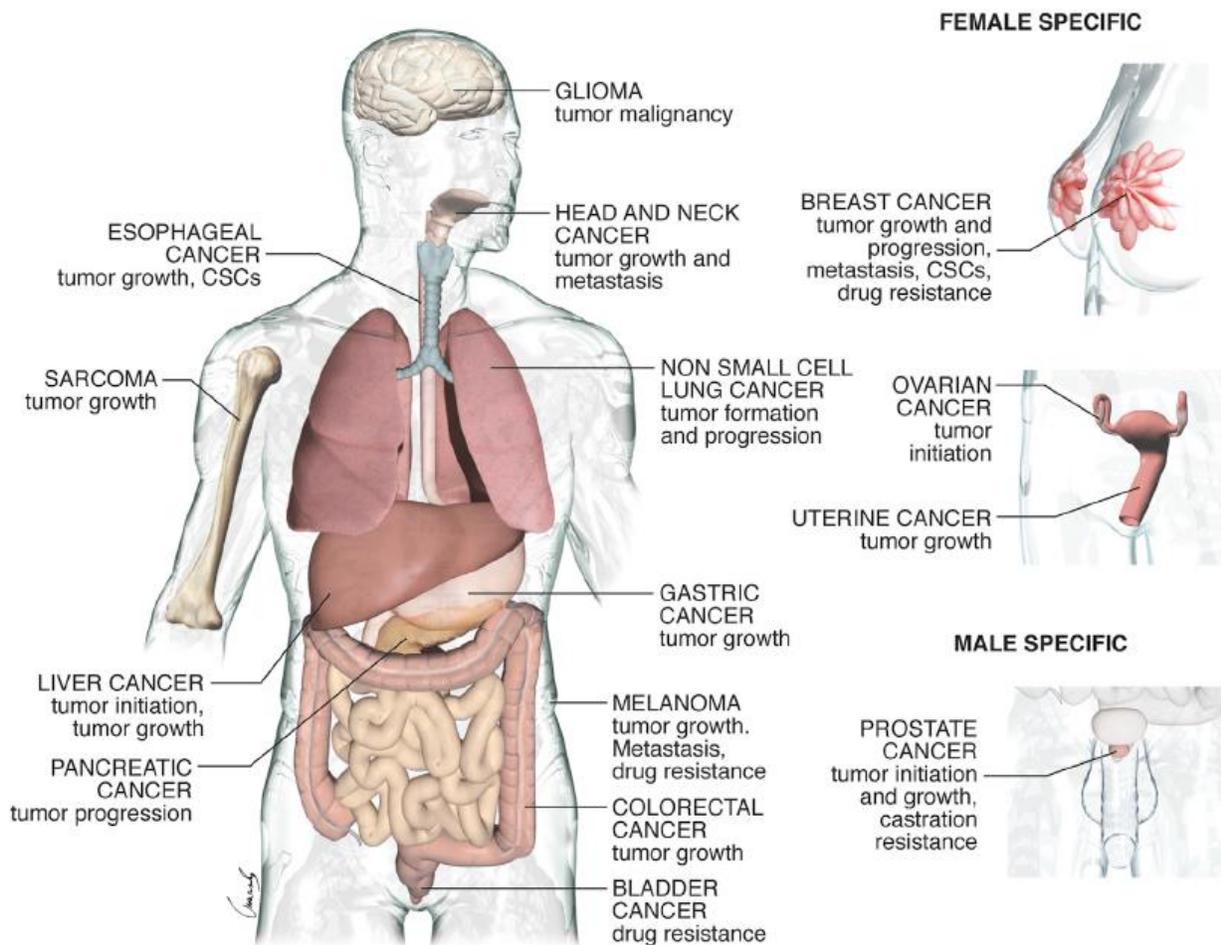
Several processes controlled by the Hippo pathway are important features of cancer cells (figure 14). For example, altered cell proliferation is a key aspect of tumorigenesis. It has been shown that hyperactivation of YAP1/TAZ transcriptional activity stimulates aberrant cell proliferation, not only by increasing the transcription of pro-growth factors, but also by counteracting tumor suppressor mechanisms such as RB-induced senescence<sup>466</sup>. LATS2 can activate p53 and its pro-apoptotic activity at least by two mechanisms: LATS2 can directly phosphorylate ASPP1 (apoptosis-stimulating protein of p53-1) to drive its translocation to the nucleus where it mediates p53 response, an effect that can be antagonized by YAP1<sup>467</sup>; and it can also bind to MDM2 and inhibiting its E3 ligase activity, therefore preventing the degradation of p53<sup>468</sup>. Furthermore, in response to H-Ras mutation, LATS2 is activated and translocated to the nucleus where it induces p53-mediated apoptosis, and the cells that survive usually display low levels of LATS2 protein, LATS2 gene promoter hypermethylation, and genomic instability, suggesting that silencing of LATS2 expression enables tumor development and progression<sup>469</sup>. This insensitivity to apoptotic cell death leads to another cancer cell property regulated by the Hippo pathway, cell survival. YAP1 over expression can promote cell survival by blocking TNF- and FAS-induced apoptosis in the mouse liver<sup>466</sup>. As described before, the target genes of the YAP1/TAZ/TEAD are involved mainly in growth promotion. Besides from their physiological roles, TEAD transcription factors have been involved in carcinogenesis, and their over expression has been described for various types of cancer<sup>470</sup>.



**Figure 14. Features of cancer cells controlled by the Hippo pathway<sup>471</sup>.**  
 YAP1/TAZ/TEAD transcriptional activity controls the expression of genes involved in proliferation, survival and cancer stem cell properties.

The Hippo pathway can also control stem cell properties, which are now considered features of the carcinogenic process, including replicative potential, dedifferentiation, and drug resistance. Increased YAP1/TEAD activity has been described in various types of stem cells and YAP1 is usually over expressed in the stem cell compartments of different tissues and drives the expansion of progenitor cells in the gastrointestinal tract<sup>466</sup>. Hippo pathway deregulation has been implied in cell transformation and tumor initiation, especially in the acquisition of mesenchymal properties at the origin of CSCs<sup>472</sup>. An essential role of TAZ in the metastatic and stem cells properties has been shown in breast cancer cell models, where it is required to sustain CSC-properties and the expression of a constitutively active TAZ (TAZS89A) is enough to confer self-renewal capacity<sup>472</sup>. Furthermore, TAZ knock-down inhibited the CSC-properties induced by Snail and Twist in breast cancer, likely acting downstream of these transcription factors, as they repress the expression of cell polarity and adhesion proteins, which in turn govern the localization of TAZ.

An increasing quantity of epidemiological and experimental data supports the role of the Hippo/YAP1/TAZ signaling pathway in different types of cancer, both of epithelial and non-epithelial origin, which are thoroughly reviewed by Zanconato *et al*<sup>471</sup> (figure 15). Gastric cancer is not the exception, and even if there are less studies than in breast or colorectal cancer, evidence is available. In a study by Xu *et al*, the expression of MST1/2 and LATS1 was decreased in tissue sections from gastric cancer compared to those of normal gastric mucosa<sup>473</sup>, and several studies have revealed an upregulation of YAP1 mRNA and protein in gastric adenocarcinomas, as well as a correlation of YAP1 expression and nuclear localization with a bad outcome<sup>471</sup>.



**Figure 15. YAP1/TAZ in human tumors<sup>471</sup>.** YAP1/TAZ have been linked epidemiologically and functionally with several types of human cancers.

By studying 84 cases of gastric patients, Jiao *et al*<sup>474</sup> found an association of YAP1 mRNA levels with tumor size, differentiation and pathologic staging, and with *H. pylori* status, and the immunohistochemistry staining of tissue arrays revealed higher YAP1 positivity rates in gastric cancer and dysplastic lesions compared to normal tissue. Consistently, the levels of YAP1/TEAD target genes CTGF and CYR61 were also increased. Interestingly, the authors also studied the expression of the tumor suppressor VGLL4, which was significantly decreased in gastric cancer and precancerous lesions and inversely correlated with the expression of CTGF and CYR61. The YAP1/VGLL4 mRNA ratio strongly correlated with clinical features of advanced gastric cancer and to *H. pylori* status, leading to the propositions that the YAP1/VGLL4 ratio could be an important factor to take in account for diagnosis and classification of gastric cancer. Furthermore, gastric cancer cell lines were found to have increased YAP1 and decreased VGLL4 levels, and over expression of VGLL4 induced apoptosis, reduced cell viability and decreased YAP1 transcriptional activity, evidenced by diminished TEAD reporter activity and expression of YAP1/TEAD target genes.

A study by Li *et al* described a role for TEAD1 as a negative regulator of the tumor suppressor VGLL4 via the stimulation of miR-222<sup>475</sup>. Hypomethylation in the TEAD4 promoter and a consequent overexpression have been described in gastric cancer tissues, and its knock down impaired cell growth both in vivo and in vitro, pointing out the contribution of TEAD4 to gastric cancer development<sup>476</sup>. Altogether, these studies point out the role of the Hippo signaling in gastric carcinogenesis.

## **1. Crosstalk with other oncogenic signaling pathways**

Both YAP1/TAZ and TEADs are also capable of binding to other proteins, and therefore participate in other signaling mechanisms aside from their canonical role of oncogenes suppressed by the Hippo pathway activation. These pathways include Wnt/ $\beta$ -catenin and TGF- $\beta$ /SMAD signaling, among others.

### **a. YAP1/TAZ - Wnt/ $\beta$ -catenin crosstalk**

YAP1 and TAZ are components of the  $\beta$ -catenin destruction complex and recruit  $\beta$ -transducing repeat-containing protein ( $\beta$ -TrCP) to facilitate  $\beta$ -catenin degradation, which places them as critical mediators to the  $\beta$ -catenin degradation when the Wnt signaling is inactive<sup>457</sup>. When YAP1 and TAZ are released from the complex due to Wnt ligand signaling,  $\beta$ -catenin accumulates as it becomes undetectable to the  $\beta$ -TrCP, and YAP1 and TAZ are free to go to the nucleus and stimulate their target genes transcription. So, functionally cytoplasmic YAP1 and TAZ able to terminate Wnt signaling, by sequestration of  $\beta$ -catenin, impairing its nuclear activity, and of Dvl, further inhibiting Wnt target gene transcription. In

addition, YAP1/TAZ can interact with and retain in the cytoplasm the SHP-2 phosphatase, whose nuclear localization promotes  $\beta$ -catenin dependent transcription<sup>458</sup>. In antagonizing Wnt/ $\beta$ -catenin, YAP1 is considered as a tumor suppressor in this context<sup>477</sup>. On the contrary, nuclear YAP1 and TAZ may serve as transcriptional mediators of Wnt signaling.

#### *b. YAP1/TAZ - TGF- $\beta$ /Smad crosstalk*

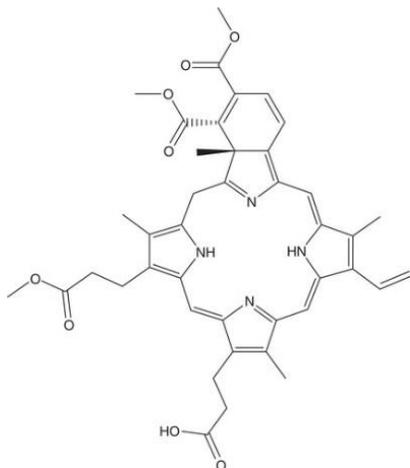
TGF- $\beta$  is characterized for having opposite roles depending on which state of tumorigenesis the signaling is activated. During tumor initiation, it has been reported to inhibit cell cycle progression, but in later stages, the TGF- $\beta$ /SMAD activation promotes cancer cell proliferation, acquisition of CSC-properties through EMT, and metastasis. This dichotomy could be in part defined by the YAP1/TAZ status of the cell. The presence of YAP1/TAZ in the nucleus enhances SMAD activity, and on the contrary, cytoplasmic accumulation of YAP1 and TAZ decreased it. According to this, in normal polarized cells where YAP1/TAZ are cytoplasmic, TGF- $\beta$ /SMAD signaling is hampered whilst in cells with nuclear YAP1/TAZ, the sensitivity to TGF- $\beta$ /SMAD is enhanced.

## **2. Hippo pathway targeting in cancer therapy**

Both in the cancer setting and in physiological conditions, YAP1 and TAZ are key to the TEAD-transcriptional activity and vice versa, as it has been shown that knock-down of TEADs or disruption of the YAP1-TEAD interaction decreased transcription of YAP1 target genes and oncogenic transformation. Dysregulation of YAP1/TAZ has been established as a signature in multiple types of cancer, notably in the liver, the ovaries and the lungs, amongst many others, and it has been showed that the pairing with TEADs is necessary for the promotion of tumorigenesis. Therefore, the ways to disrupt YAP1/TAZ-TEAD interaction have been studied as potential strategies for cancer treatment.

#### *a. Verteporfin*

Verteporfin (VP, C<sub>41</sub>H<sub>42</sub>N<sub>4</sub>O<sub>8</sub>, molecular weight: 718.8 Da, figure 16) is a benzoporphyrin derivative initially used as photosensitizer for photodynamic therapy (PDT) in the treatment of neovascular age-related macular degeneration, choroidal neovascularization due to pathologic myopia and chronic central serous chorioretinopathy<sup>478</sup>. It is commercialized by Novartis under the name of Vysudine®. In PDT, the VP molecules is stimulated with a light source with a wavelength of 688-692 nm, leading to the generation of oxygen-free radical that cause cytotoxic damage<sup>478</sup>.



**Figure 16. Chemical structure of verteporfin.** The benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin), chlorin-type molecule derived from porphyrin. A simplified outline of BPD-MAC, one of the two racemic regioisomers (BPD-MAC and BPD-MAD), is shown. Porphyrins have substituents in the peripheral positions of the pyrrole rings (1 – 8), on the four methine carbons (meso-positions), and/or coordinated metals.

Asides from the use in ophthalmology, VP-PDT have been tested as an anti-cancer therapy. Using an athymic rat model of breast cancer bone metastases, Akens *et al* showed the safety and efficacy of VP-PDT, as treatment induced tumor cell dissociation, infiltration with inflammatory cells and tumor cell necrosis and apoptosis<sup>479</sup>. In pancreatic cancer, following promising results from preclinical studies, a phase I/II clinical trial in 14 patients with locally advanced disease showed an induction of tumor necrosis after VP-PDT<sup>480</sup>.

Light-independent activity of VP have also been reported. VP was identified as an inhibitor of autophagosome accumulation in a screening of more than 3500 compound in MCF-7 cells<sup>481</sup>. VP treatment inhibited drug- and starvation-induced autophagic degradation and reduced cell viability in nutrient-depleted conditions, but it had no effect in a nutrient-rich environment.

Liu-Chittenden *et al* identified VP in a screening for small molecules that inhibit the YAP1/TEAD using a YAP1/TEAD luciferase reporter<sup>482</sup>. VP decreased luciferase reporter activity in more than 50% from concentrations as low as 2.5  $\mu$ M, as a result of a selective binding of YAP1 and the possible induction of conformational changes that result in an abrogated interaction with TEAD. The result was the same when the VP treatment was tested in the *Drosophila* homologues Yorkie and Scalloped. The authors next examined the anti-oncogenic properties of VP *in vivo* using two murine models (YAP1-induced hepatomegaly and liver-specific knock-out of NF2/Merlin) treated with a 100 mg/kg systemic dose of VP and DMSO as control, and they found that in both cases VP treatment suppressed liver overgrowth resulting from YAP1 overexpression or overactivation.

The role of VP in the inhibition of the YAP1/TEAD complex formation and the effects of this inhibition in CSC properties have been studied in cells of gastrointestinal origin. In a study by Song *et al* in esophageal cancer cells, where the CSC population was associated with a high YAP1 activity and the expression of Sox9, VP treatment reduced the CSC properties (sphere formation and ALDH activity) *in vitro* and the growth of tumors derived from xenografts in immunodeficient mice<sup>483</sup>. Furthermore, the same

group showed later that inhibition of YAP1 activity with VP sensitizes esophageal cancer cells to 5-FU et docetaxel *in vitro* and enhanced the inhibition of tumor growth by 5-FU *in vivo*<sup>484</sup>. Similar results have been reported in a murine model of azoxymethane/dextran sulfate sodium-induced colorectal carcinogenesis, where VP treated mice showed less numerous and minimally dysplastic tumors in treated groups versus the control group, decreased cell proliferation and expression of CTGF and CYR61, and increased apoptosis, without affecting the normal intestinal homeostasis or displaying any apparent toxicity in kidney, liver, or intestinal tissues<sup>485</sup>. These results were confirmed on patient-derived xenografts of colorectal cancer, in which VP inhibited tumor growth. Two other light-independent effects of VP were described in this study. First, a YAP1-independent inhibition of STAT3/IL-6 signaling leading to a decrease of tumor cell growth, which has also been reported in tubule-interstitial fibrosis of the kidney<sup>486</sup>. Second, the capacity of VP to induce oligomerization of high molecular weight proteins, which increases apoptotic cell death. More recently, the HMWP oligomerization mechanism has been addressed in detail and described as light-dependent<sup>487</sup>. Until now, the anti-oncogenic properties of VP have not been described in gastric cancer.

*b. VGLL4-mimicking “Super-TDU” inhibitor peptide*

Following the finding of VGLL4 as a tumor suppressor that disrupts the YAP1/TEAD activity, Jiao *et al*<sup>474</sup> deciphered the precise molecular mechanism of VGLL4-mediated disruption of the YAP1/TEAD complex, which lays on a competition between YAP1 and VGLL4 for the binding of TEADs, and while the YAP1 is an activator of transcription, VGLL4 is a repressor. Tandem Tondu (TDU) domains of VGLL4 were identified as crucial to the inhibitory activity and structural analysis allowed the identification of the precise residues, and the synthesis of an inhibitor peptide termed “Super-TDU” which targets the interaction between YAP1 and TEAD4. *In vitro*, the peptide inhibited cell viability and colony formation, and down-regulated the expression of CTGF and CYR61, and *in vivo*, the weight and size of tumors in a model of xenograft of gastric cancer cells in BALB/c mice, with acceptable levels of toxicity and specificity. The inhibitor peptide also showed a therapeutic effect in a model of *H. pylori* infected mice.



## II. Results/Articles



a. **Article 1: LATS2 and YAP1 are co-upregulated in response to *Helicobacter pylori* infection and control cancer stem cell properties in gastric epithelial cells**

*Submitted for publication*



**LATS2 and YAP1 are co-upregulated in response to *Helicobacter pylori* infection and control cancer stem cell properties in gastric epithelial cells**

Molina Castro S.E.<sup>1,2</sup>, Staedel C.<sup>3</sup>, Giraud J.<sup>1</sup>, Tiffon C.<sup>1</sup>, Boeuf H.<sup>4</sup>, Sifre E.<sup>1</sup>, Giese A.<sup>1</sup>, Belleannée G.<sup>5</sup>, Fernandez S.<sup>1</sup>, Lehours P.<sup>1,5</sup>, Bessède E.<sup>1,5</sup>, Mégraud F.<sup>1,5</sup>, Dubus P.<sup>1,5</sup>, Varon C.<sup>1</sup>.

1 INSERM, Univ. Bordeaux, UMR1053 Bordeaux Research in Translational Oncology, BaRITOn, F-33000 Bordeaux, France

2 University of Costa Rica, San José, Costa Rica

3 INSERM, Univ. Bordeaux, UMR1212, Bordeaux, France

4 INSERM, Univ. Bordeaux, UMR1026, Bordeaux, France

5 CHU de Bordeaux, F-33000 Bordeaux, France

**Running title: LATS2 and YAP control *H. pylori*-induced EMT & CSC phenotype**

**Key words:** CD44, Hippo pathway, TEAD, epithelial-mesenchymal transition, gastric cancer, biomarker.

**Correspondence.** Dr. Christine Varon, INSERM U1053 Bordeaux Research in Translational Oncology, University of Bordeaux, 146 rue Leo Saignat, Bordeaux F-33000, France. Email: [christine.varon@u-bordeaux.fr](mailto:christine.varon@u-bordeaux.fr). Tel: +33(0)557579575, Fax: +33(0)556514182.

## ABSTRACT

Gastric carcinoma (GC), which is mostly related to infection with CagA<sup>+</sup>-*Helicobacter pylori*, is the third leading cause of cancer-related mortality worldwide. *H. pylori* infection disrupts the gastric mucosa turnover and induces, *via* an epithelial-mesenchymal transition (EMT), the emergence of CD44<sup>high</sup> cells with properties of cancer stem cells (CSC) at the origin of GC. The Hippo pathway, involved in stem cell homeostasis, growth and cancer, was investigated in this context in human gastric epithelial cell lines (hGEC) and patients-derived tissues. *H. pylori* infection regulated Hippo signaling in a CagA-dependent manner in AGS and MKN74 hGECs, revealing concomitant up-regulations of Hippo kinases, downstream effectors and YAP/TEAD target genes. LATS2 and its target YAP1 were co-upregulated in response to *H. pylori* both *in vitro* and in the gastric mucosa of *H. pylori*-infected patients with gastritis and intestinal metaplasia. *In vitro*, YAP1 was triggered as early as 2 hours post-infection which subsequently stimulated the transcription of YAP1/TEAD target genes among them *ctgf* and *cyr61*, and then was tightly retro-controlled by LATS2 upregulation and phosphorylation of YAP1 on Ser127. Interestingly, we showed that LATS2 and YAP1 reciprocally maintained each other's expression both under basal and infected conditions. Using siLATS2 in hGECs, we showed that LATS2 restricted *H. pylori*-induced EMT and the emergence of invasive CD44<sup>+</sup> CSC-like cells forming tumorspheres *in vitro*. In GC tissues, LATS2 and YAP1 were mostly nuclear, heterogeneously expressed and colocalized in CD44<sup>+</sup> CSC-rich areas. Their co-expression also corroborated in the GC cell lines of the Cancer Cell Lines Encyclopedia. This study points out for the first time that *H. pylori*, *via* CagA, affects the Hippo pathway and unveils an unexpected co-upregulation of LATS2 and YAP1 upon infection, which in turn tightly control gastric CSCs properties and appear as new preneoplastic biomarkers of the gastric carcinogenesis cascade leading to GC.

## INTRODUCTION

Stomach cancer is the third cause of cancer-related deaths worldwide [1]. The most frequent are gastric adenocarcinomas (GC), which have a 5-years survival rate of <25% [2]. Current treatments include chemo- and radiotherapies, with surgery for the non-metastatic forms. The chronic infection with the gram-negative bacterium *Helicobacter pylori*, which is a type I carcinogen [3], is a strong risk factor for GC [4][5] and nearly 93% of non-cardia GC are *H. pylori*-positive [6]. *H. pylori* specifically colonizes the stomach of half the world population and provokes a chronic inflammation of the gastric mucosa likely to evolve into ulcers and in ~1% of the cases into GC. *H. pylori* strains positive for the *cag*-pathogenicity island, encoding a type 4 secretion system (T4SS) and the virulence oncoprotein CagA, are strongly associated with malignancy development [7]. Upon *H. pylori* adherence on human gastric epithelial cells (hGECs), the T4SS forms a *pilus*, which translocates the bacterial CagA and peptidoglycans into the epithelial cytoplasm triggering cell innate immunity and other signaling pathways that alienate the mucosa homeostasis [8].

Epithelial turnover, resulting from the balance between progenitor cell proliferation and differentiated cell death, is a major host defense mechanism against pathogens and is commonly altered during bacterial infections and chronic inflammatory diseases. In an animal model, it has been demonstrated that *H. pylori* via CagA delivery suppresses apoptosis at the superficial gastric pit cells, delaying gastric epithelium self-renewal [9]. In *H. pylori*-infected hGECs *in vitro*, we previously reported that *H. pylori* via CagA blocks cell cycle progression by up-regulating the cell cycle regulator LATS2 (Large Tumor Suppressor 2) and elicits an epithelial-to-mesenchymal transition (EMT) prominently governed by the ZEB1 transcription factor [10][11]. EMT is a developmental process characterized by loss of epithelial cell polarity and cell-cell interactions, reorganization of the cytoskeleton, and acquisition of the migratory properties of mesenchymal cells [12]. Influencing cell growth and survival, EMT in *H. pylori*-infected patients may contribute to a reduced renewal and an aberrant differentiation of the gastric mucosa, which leads to preneoplastic atrophic gastritis and metaplasia states [5]. EMT is also implicated in the emergence of cancer stem cells (CSC) [13]. CSCs correspond to a subpopulation of cells within the primary tumor; They

express markers of immaturity, are able to self-renew and divide asymmetrically, and are at the origin of the more or less differentiated cells that constitute the tumor. CSCs are responsible for tumor initiation, progression and metastasis, and have increased resistance to conventional radio/chemo-therapies [14]. In the context of gastric carcinogenesis, we previously demonstrated that challenging hGECs with *cagA*-positive *H. pylori* generates CD44<sup>high/+</sup> cells harboring a mesenchymal phenotype [15]. CD44, the cell surface hyaluronate receptor, has been identified as a CSC marker in many solid tumors including GC [16][17][18]. Interestingly, the *H. pylori*-induced CD44<sup>high/+</sup> hGECs share phenotypic properties with CSCs, such as the invasion, migration, tumorspheres formation *in vitro* and tumor formation *in vivo* compared to their CD44<sup>low/-</sup> counterparts [15]. CD44 and the mesenchymal markers ZEB1 and Snail1 are overexpressed in the gastric mucosa of *H. pylori*-infected patients and in mouse models of experimental *H. pylori* infection, within the isthmus region of the gastric glands where gastric epithelial stem cells reside [15][18][19].

*H. pylori*-driven LATS2 upregulation and emergence of CSC-like properties prompted us to analyze the Hippo pathway, an evolutionarily conserved signaling cascade involved in stem cell homeostasis and cancers [20][21], and which has never been studied in the context of *H. pylori* infection. In humans and mice, the pathway activates a kinase core involving MST1/2, their cofactor Salvador, and the tumor suppressors LATS1/2. In the activated Hippo pathway, which restricts tissue overgrowth, LATS1/2 phosphorylate the transcriptional co-activator YAP1 on serine 127, inactivating YAP1 by retention within the cytoplasm and degradation by the proteasome. On the contrary, when the Hippo pathway is inactivated, YAP1 translocates into the nucleus and acts as co-activator for the transcription enhancer factors TEADs to promote cell proliferation. The Hippo pathway is connected *via* its components and effectors to other pathways such as Wnt/ $\beta$ -catenin, Ras, junctional complexes, NF- $\kappa$ B, TGF $\beta$  and many others [22].

In order to get a deeper insights into the molecular mechanisms governing *H. pylori*-mediated gastric carcinogenesis, we aimed here to determine the role of the Hippo pathway, focused on LATS2 and YAP1,

on the EMT-like process and on the emergence of *H. pylori*-induced CSCs *in vitro* as well as in patients-derived gastric mucosa and GC.

## RESULTS

### 1. **LATS2 and nuclear YAP1 are co-upregulated in the gastric epithelial cells of *H. pylori*-infected patients**

LATS2, YAP1 and CD44 were evaluated by immunohistochemistry in gastric mucosae of either non-infected or *H. pylori*-infected patients (Fig.1a), and their expression was scored (Table 1, Fig.1b). Both LATS2 and YAP1 were found upregulated and strongly expressed in the nuclei of cells of the infected gastric mucosa, and are therefore associated with gastritis. Their scores were even higher in intestinal metaplasia. LATS2 and YAP1 over-expressions were found precisely within the isthmus in the fundus and in the crypts of the gastric glands in the antrum, where CD44 is also over-expressed in response to *H. pylori* infection, and which corresponds to the regenerative epithelial stem cells location [15].

### 2. **The Hippo pathway genes are affected by *H. pylori* in cultured hGECs.**

As primary hGECs cannot be easily isolated and expanded in tissue culture, the AGS and MKN74 established hGEC lines have been widely used to recapitulate *in vitro* the early events of *H. pylori* infection occurring within an actively replicating gastric mucosa. Both cell lines grow as monolayers of cohesive, cobblestone-shaped cells, and respond to *cagA*-positive *H. pylori* strains by an EMT and the emergence of CSC-like properties [11][15].

Global gene expression in response to *H. pylori* was performed using whole-genome microarrays. Genes involved in the Hippo pathway and whose expressions were significantly altered by infection are presented in Fig.S1 and summarized in Fig.1c. In both infected AGS and MKN74, the Hippo kinase core genes were globally upregulated about twice. LATS2 was significantly up-regulated in infected MKN74 but not in AGS (Fig.S1). We previously reported that LATS2 is post-transcriptionally down-regulated by miR-372/373,

which are down-regulated upon *H. pylori* infection leading to LATS2 upregulation and blockade of cell cycle progression [10]. In parallel, the transcriptional co-factors YAP1 or TAZ, which are both LATS2 substrates, were slightly up-regulated in AGS and MKN74, respectively. YAP1 interacts primarily with the TEAD transcription-enhancer factors, thereby promoting cell growth. VGLL4, a YAP1 negative competitor for TEAD binding [23], which prevents its oncogenic function, was also upregulated.

Other transcription factors such as members of the RunX family, the Smad family or p73 that are speculated to be interacting with YAP were variably affected according to the cell line, with the exception of RUNX1 which was upregulated in both. Several YAP1/TEAD target genes involved in cell proliferation and survival were significantly affected upon infection, including *cyr61* and *ctgf* encoding cysteine-rich extracellular matrix proteins that function as integrin ligands and activate cell proliferation and which are among the main target genes assessing YAP/TEAD transcriptional activity [20-22] (except *ctgf* in MKN74). It is noteworthy that, in infected MKN74, an outstanding increase was found in IGFBP3, an insulin growth-factor binding protein with either anti-proliferative or context-dependent EMT- and tumor-promoting activities [24].

The Hippo pathway upstream effectors as well as some components of the cell polarity complexes and intercellular junctions, which contribute to the activation of the Hippo cascade [25], were variably affected upon infection. *H. pylori* upregulated *cdh1* encoding E-cadherin accordingly to a previous report [11], as well as *crb3* encoding a component of the Crumbs complex maintaining the epithelial integrity [26], and *ajuba* encoding a scaffold protein of cell junctions and binding partner of LATS which negatively regulates YAP1 [27]. Thus, the analysis of the Hippo transcriptome in hGECs upon infection revealed concomitant up-regulations of the core kinase, downstream effector genes and YAP/TEAD canonical target genes.

### **3. Biphasic kinetics of Hippo activation upon *H. pylori* infection of hGECs *in vitro***

To assess how LATS2 and YAP1 are orchestrated during infection, we analyzed the time-course of their expressions in the hGECs challenged by *cagA*-positive *H. pylori*. As TEAD is considered the primary

transcriptional partner of YAP1, we used a TEAD-luciferase reporter assay to sense TEAD-mediated transcription (Fig.2a). In AGS, the TEAD transcriptional activity clearly exhibited biphasic kinetics characterized by an early and transient stimulation during the first 2 hours post infection (hpi) followed by a decreasing activity thereafter. This biphasic pattern was accompanied by parallel changes in the expression of the direct TEAD-target genes *ctgf* and *cyr61* (Fig.2b), indicating that *H. pylori* transiently inactivated the Hippo pathway triggering YAP/TEAD activity in AGS cells, and then progressively activated it rendering YAP/TEAD co-transcriptionally inactive. In MKN74, the TEAD transcriptional activity was highly variable before the first 5 hpi, but modestly decreased thereafter. Nevertheless, both *ctgf* and *cyr61* remained upregulated during the entire infection time-course, accordingly to the transcriptomic data but not to the YAP1/TEAD activity (Fig.S1 and Fig.2a).

*H. pylori* progressively triggered LATS2 and YAP expressions in hGECs at both the mRNA (Fig.2c) and protein levels (Fig.2d). The variations in proteins occurred earlier and were more marked than in mRNAs, suggesting initial post-transcriptional activating mechanisms. LATS2 and YAP1 proteins accumulated as soon as 2 hpi in both hGECs (Fig.2d), and YAP1 was mainly in its YAP1-P<sup>Ser127</sup> form. YAP1 accumulation was accompanied by enhanced, although delayed, *yap* transcription in AGS, but not in MKN74 (Fig.2c), in accordance with the transcriptomic data.

By immunofluorescence analyses, YAP1 was mainly observed in its nuclear active form (YAP-P<sup>Ser127</sup> negative) at 2 hpi and accumulated thereafter in the cytoplasm in its inactive form (YAP-P<sup>Ser127</sup>) (Fig.2e). MKN74 exhibited a greater heterogeneity of YAP1 labeling than AGS, thereby explaining why the TEAD activity was less strikingly affected than in AGS (Fig.2a). LATS2 progressively accumulated in both hGECs upon infection. Remarkably, LATS2 transiently accumulated in the cytoplasm of MKN74 (2 hpi) before becoming nuclear at 24 hpi, whereas LATS2 was nuclear in AGS at all time points.

Altogether these data show that LATS2 and YAP1 were regulated during *H. pylori* infection in a biphasic pattern. In the early period of infection, *H. pylori* transiently triggered YAP1 expression and co-transcriptional activity, most likely by post-transcriptional mechanisms, along with LATS2. This early

period of infection corresponds to an inactive Hippo pathway and is followed by a progressive activation, most likely sustained by LATS2 accumulation and the subsequent YAP Ser127-phosphorylation, preventing its nuclear translocation and TEAD-mediated transcriptional co-activation. It should be noted that *cagA*-deficient *H. pylori* isogenic mutants affected neither TEAD transcriptional activity nor LATS2 or YAP expressions (Fig.S2). This indicates that the activation of the Hippo pathway was CagA-dependent and thereby related to the oncogenic potential of the bacteria.

#### **4. LATS2 and YAP positively regulate each other's expression in response to *H. pylori***

To further assess to what extent LATS2 and YAP are specifically involved in hGEC responses to *H. pylori*, we knocked down their expression using small interfering RNAs (respectively siLATS2 and siYAP) (Fig.3). Under basal conditions, siLATS2 successfully knocked down LATS2 expression (Fig.3a,c) and activity as assessed by decreased YAP-P<sup>Ser127</sup> and enhanced TEAD activity, reflected by the stimulations of both the TEAD reporter and CYR61 and CTGF expression (except CTGF in AGS, Fig.3c). Conversely, transfecting a constitutively active LATS2 expression vector [10] inhibited TEAD transcriptional activity by 70% compared to the control pEGFP vector in AGS (Fig.3b).

siYAP knocked down YAP1 expression (Fig.3a,c) and inhibited TEAD activity in both hGECs, as expected (Fig.3b). siYAP severely down-regulated CTGF and Cyr61 in AGS, but barely affected them in MKN74 (Fig.3b-c), suggesting that these genes could be transcribed independently of YAP/TEAD. Conversely, transfection of vectors encoding either wild type YAP1 (pYAP) or the constitutively active, unphosphorylatable YAP<sup>S127A</sup> mutant (pSer127YAP) stimulated TEAD activity in AGS by 3 and 6 fold, respectively. In MKN74, only pSer127YAP but not pYAP triggered the TEAD reporter under basal conditions (Fig.3b), suggesting a tighter regulation of YAP1 in this cell line, likely by LATS2, which is expressed at a higher level in MKN74 than in AGS under basal conditions (Fig.2b,e).

Unexpectedly siLATS2 not only decreased YAP-P<sup>Ser127</sup> but also total YAP protein in both hGECs (Fig.3a) and YAP1 mRNA in MKN74 (Fig.3c). In turn, siYAP reciprocally downregulated LATS2 under basal condition (Fig.3a,c). Accordingly, we also found a strict co-expression of YAP1 and LATS2 in other hGECs (Fig.S3). Under infected conditions, siLATS2 maintained the TEAD activity at a high level compared to siControl infected cells, in which it was repressed compared to non-infected hGECs. To the contrary, siYAP further decreased it (Fig.3d). These observations are corroborated by parallel changes in CTGF and CYR61 expression (except CYR61 in AGS, Fig.3e). Upon infection, siLATS2 and siYAP not only efficiently prevented the respective LATS2 and YAP1 *H. pylori*-mediated up-regulations, but siLATS2 also impaired YAP up-regulation and reciprocally (Fig.3e).

Taken together, these data show that YAP1 is the major coactivator of TEAD-mediated transcription, and that it is prominently controlled by LATS2-mediated YAP1 inhibitory phosphorylation. Remarkably, LATS2 and YAP1 reciprocally positively regulate each other's expression reciprocally both under basal conditions and in response to *H. pylori* infection.

##### **5. LATS2 restricts the EMT and CSC-like properties induced by *H. pylori* in hGECs**

AGS morphology was not noticeably affected by either siLATS2 or siYAP under non-infected conditions (Fig.S4a). Nevertheless, LATS2 repression accelerated the acquisition of the EMT-characteristic "hummingbird" phenotype early as 2 hpi in siLATS2 cells, in contrast to 5 hpi in siControl or siYAP AGS cells (Fig.S4a). The remarkable feature occurring specifically in siLATS2-transfected MKN74 cells was the round cell clusters loosely attached to the epithelial layer upon infection (Fig.S4b) that appeared at a much higher number than in siControl MKN74 or siYAP and also correspond to a mesenchymal phenotype [11].

The induction of the mesenchymal phenotype observed in siLATS2 compared to siControl hGECs was associated with a ZEB1 up-regulation under both basal and infected conditions, along with Snail1 EMT inducer, in siLATS2 MKN74 (Fig.4a). The nuclear accumulation of ZEB1 in siLATS2 hGECs was confirmed by

immunofluorescence (Fig.4b). ZEB1 is reciprocally linked to the miR-200 microRNA family members in a negative feedback loop [28]. The miR-200s are produced from two microRNA clusters, *miR-200b-200a-429* and *miR-200c-141*, the promoters of which harbor ZEB1 binding sites and are repressed by ZEB1. Interestingly, siLATS2 decreased the expression of miR-200b and -200c in AGS and prevented their *H. pylori*-mediated upregulation [11] in MKN74 (Fig.4a). Thus, the decreased miR-200 levels in infected siLATS2 hGECs may contribute to an acceleration of their EMT upon infection. The exacerbated EMT phenotype in siLATS2-treated cells was further confirmed by increased invasive properties under both basal and infected conditions, compared to the siControl (Fig.4c).

We previously reported that *H. pylori*-induced EMT promotes the emergence of cells with CSC-like properties, such as high CD44 expression and the ability to grow as spheroids under non-adherent culture conditions [15]. Accordingly, CD44 was upregulated at 24 hpi in siControl hGECs (Fig.5a). The percentage of CD44+ cells which is <1% in MKN74 cells under basal conditions (contrarily to >60% in AGS cells [15]), increased by 4 times upon LATS2 knockdown and up to 23% at 24 hpi (Fig.5b) (along with increased mean cell fluorescence intensities, data not shown). The hGECs' ability to grow as tumorspheres was significantly increased in siLATS2 AGS cells both under basal and infected conditions, but not in siLATS2 MKN74 cells, in which this CSC property was hampered despite the CD44 enhancement (Fig.5c). The inability of siLATS2 MKN74 to form tumorspheres may be correlated to their strong upregulation of the cyclin-dependent kinase inhibitor p21/CDKN1A along with the downregulations of S-phase markers E2F1 and PCNA compared to siControls, which did not occur in AGS cells (Fig.S5).

Collectively these results support a restricting role of LATS2 in the acquisition of EMT/CSC-like phenotype and properties upon *H. pylori* infection.

## 6. LATS2 and YAP1 are heterogeneously expressed, nuclear and co-upregulated in *H. pylori*-induced gastric adenocarcinoma in cancer patients.

We previously reported that GC are heterogeneous, composed of variably differentiated cells within the primary tumor, including a subpopulation of less than 30% CD44<sup>+</sup> cells comprising the CSCs [17]. Here we show that LATS2 and YAP1 immunolabeling is heterogeneous among the GCs and among the cells within the tumors (Fig. 6a) as far as both expression level and subcellular localization are concerned. LATS2 expression was for the majority nuclear and positively correlated to nuclear YAP1 expression on GC tissue sections (Fig.6b), thus corroborating the LATS2 and YAP1 co-expressions in the different hGECs *in vitro* (Fig.S3). The transcriptomic data of the 38 GC cell lines of the Cancer Cell Line Encyclopedia (CCLE) [29] also revealed a positive correlation between LATS2 and YAP1 expression (Fig.6c). Interestingly the CYR61 YAP1/TEAD target gene expression is also strongly correlated to LATS2 expression (R=0.758, p<0.0001, Table S1), suggesting that LATS2 could also be considered as a YAP1/TEAD target gene. Moreover, the cells overexpressing nuclear YAP1 and LATS2 were found mainly at the periphery of the tumor foci, where also the CD44<sup>+</sup> CSCs were also detected. However, CD44 and nuclear LATS2/YAP1 expressions were not correlated neither at the protein level in GC tissue sections nor at the transcriptomic level in the CCLE GC cell lines (Fig.6c): this further argument is in favor of LATS2 repressing CD44 as depicted in Fig.5b.

## DISCUSSION

LATS and YAP1 constitute the downstream core kinase/effector tandem within the Hippo pathway, an evolutionary conserved signaling cascade regulating tissue growth and stem cell homeostasis, and which has never been studied in the framework of *H. pylori* infection so far. We previously reported that on one hand carcinogenic strains of *H. pylori* up-regulate the tumor suppressor LATS2 [10], and on the other hand express CSC-like features [15] in hGECs. However, the role of LATS2 and its oncogenic targets YAP1/TEAD remained to be elucidated in this context.

We show here that LATS2 and YAP1 are co-upregulated, nuclear and therefore activated in hGECs of *H. pylori* chronically infected patients as early as the gastritis stage and prior to their neoplastic transformation. This observation was unexpected in regard to most data reporting an inverse correlation of LATS1/2 and YAP1 expression, with LATS1/2 downregulated while YAP1 was overexpressed and functional in the nuclei in many tumor cells [30] including those of GC [31][32]. LATS2 has been reported as a dynamic regulator of proliferation and functional maturation of stem cells *via* YAP1 restriction. YAP1 has been extensively portrayed as a “stemness factor” localized within the stem cell niche of adult organs [33-36] and also constitutes a Wnt/ $\beta$ -catenin regulator [37][38]. YAP1 has been found to be essential to regulate stem cell growth when these cells are stirred up to ensure tissue regeneration [35], a condition fulfilled here during the *H. pylori* assault of the gastric mucosa. LATS2 and YAP1 co-localization with the CSC and EMT marker CD44 in the gastric glands may reveal an extension of the stem cell reservoir naturally present in the gastric mucosa. Furthermore, in the context of the *H. pylori*-triggered chronic inflammation, regenerative hyperplasia and metaplasia, these stem cells acquire mesenchymal and pre-neoplastic features, most likely predisposing them to transform and generate self-renewing CSCs.

Second, using hGEC lines challenged by *H. pylori in vitro*, we found that the bacterium affects LATS2 and YAP1 in a coordinated biphasic pattern, characterized by an early and transient YAP1/TEAD transcription stimulation and followed by LATS2 upregulation leading to YAP1 phosphorylation and nuclear exclusion, in a CagA-dependent manner. At this point, these data on infected hGECs do not support our observations in infected patients' gastric mucosa showing concomitant LATS2 and nuclear YAP1 up-regulations. Although this wave of YAP1/TEAD activation is rapidly retro-controlled following LATS2 induction in response to *H. pylori in vitro*. Repeated and chronic aggression of the gastric epithelium by *H. pylori in vivo* could lead to constant waves of YAP1/TEAD activation and LATS2 induction, resulting, as observed, in a co-overexpression of both and the nuclear localization of YAP1 reflecting its co-transcriptional activity. The siLATS2- and siYAP1-mediated loss-of-function experiments revealed that LATS2 and YAP1 depend on each other for their expression in hGECs, and knocking down either of the two weakens the expression of the other, suggesting that LATS2 and YAP1 reciprocally maintain each other's expression under both non-

infected and infected conditions. Moroishi *et al.* reported that YAP1/TEAD directly induces *lats2* transcription upon binding to its promoter [39]. LATS2 in turn inactivates YAP1 *via* Ser<sup>127</sup>-phosphorylation: this negative feedback loop is likely to be responsible for the transient YAP1 activation in *H. pylori*-challenged hGECs *in vitro*. The YAP/TEAD-dependent transcription of LATS2 observed in the hGECs of this study is also supported by the strong LATS2/YAP1 and LATS2/CTGF-CYR61 correlations that we unveiled in the CCLE data base. This leads to the conclusion that LATS2 can be considered as a YAP1 target gene aimed, as a negative feedback loop, to tightly control but not completely silence YAP1/TEAD oncogenic transcriptional activity, maintaining by the way its own expression.

LATS2 is also involved in the cell cycle control in response to microtubule stress during which LATS2 binds and inhibits Mdm2, stabilizing p53 and thereby maintaining mitotic fidelity and genomic stability [40]. During this process, LATS2 moves from the cytoplasm to the nucleus, which is also observed in MKN74 as early as 2 hpi (Fig.2e). According to its function in the mitotic apparatus, loss of LATS2 results in mitotic defects, which could become very critical for the more differentiated hGECs like MKN74 compared to AGS for survival under extreme growth conditions such as tumorspheres. This is in relation with the upregulation of p21 and the downregulation of E2F1 and PCNA observed in siLATS2 MKN74 cells, and consequently to the decrease in tumorsphere formation (Fig.2e).

Third, LATS2 knockdown exacerbating the EMT and CSC markers ZEB1, Snail1 and CD44 indicates that LATS2 restricts the EMT and migrating-CSC phenotype in hGECs, accordingly to its tumor suppressor function. Indeed, Hippo signaling is associated with stem cell maintenance and normal epithelial architecture, characterized by specialized cell-cell junctions [35][36]. Its dysfunction leads to increased nuclear and under-phosphorylated YAP1 that promoted oncogenic transformations due to TEAD activation, revealed here by CTGF and Cyr61 upregulation. Interestingly, these YAP1/TEAD targets involved in cancer progression are also ZEB1 targets [41]: ZEB1 mechanistically and functionally binds YAP1, promoting the expression of a common ZEB1/YAP1 target oncogene set. This could be the reason why CTGF or CYR61 transcription was not totally abolished upon siYAP treatment in MKN74 like in AGS: the remnant CTGF or Cyr61 levels after YAP1 downregulation may be attributable to ZEB1. Contrarily to AGS,

MKN74 may harbor a functional YAP1/ZEB1 transcriptional relationship. ZEB1 is a NF- $\kappa$ B target gene during *H. pylori*-induced EMT in hGECs [11]. The rise in ZEB1 and Snail1 expression induced by siLATS2 (but not by siYAP, data not shown) could be explained by an indirect action of LATS2 on ZEB1 *via* NF- $\kappa$ B, which was found negatively regulated by LATS2 in non-small-cell lung carcinoma [42]. In addition, silencing LATS2 in non-transformed mammary epithelial cells increases NF- $\kappa$ B association with p53, thereby promoting cell migration [43]. CD44 is a Wnt/ $\beta$ -catenin target gene, a pathway which is activated by *H. pylori* *via* CagA [44][45][46]. Its upregulation upon LATS2 knockdown could be explained by the ability of LATS2 to interfere with  $\beta$ -catenin/BCL-9 interaction within the nucleus thereby preventing Wnt/ $\beta$ -catenin signaling [47].

Finally, one open question is how are LATS2 and YAP1 alerted by *H. pylori* infection? In addition to the Hippo kinases MST1/2 and p53 mentioned above, LATS2 is subject to regulation by numerous proteins, including MAP4Ks, Aurora A and AJUBA, NF2/Merlin and Crb3 [30][27][54][26]. Mechanical signals in relationship with the microenvironment represent also an additional pillar for YAP1 function [48][20][35]. The destabilization of hGECs cell/cell junctions and cell shape is deeply affected by CagA, as evidenced by the “hummingbird” phenotype of infected AGS and the transcriptomic data (Fig.1c, Fig.S1, Fig.2e; [11][15]), thanks to its ability to binds to numbers of host proteins involved in cell junctions [45][49][50]. It is important to note that CagA targets and triggers the SHP-2 phosphatase, which can also directly interact with YAP1 and potentiate its cotranscriptional function [51][52]. This tripartite connection CagA/SHP2/YAP may be critical in the early phases of human gastric carcinogenesis [53]. Nuclear localization of YAP1 is the sum of multiple, possibly parallel, regulatory layers including G protein-coupled Receptor and Src kinases [30], which are also CagA-interacting proteins [8][49].

Epithelia exposed to environmental assaults, including pathogen infection, require constant stem cell-driven renewal and signaling tuning to maintain tissue homeostasis. *H. pylori* infection constitutes the greatest risk factor for GC mainly due to its oncoprotein CagA which interacts with numerous host cell proteins, subsequently triggering several signaling pathways including, in this case, the LATS2/YAP1/TEAD axis of the Hippo pathway. The co-overexpression of LATS2/YAP1 appears at all stages of the gastric

carcinogenesis cascade, in *H. pylori*-associated gastritis and metaplasia as well as in GC, therefore constituting novel additional markers of *H. pylori*-triggered gastric stem cell expansion leading to CSCs and GC.

## **MATERIALS AND METHODS**

**Human gastric tissues.** Gastric tissue samples (tumors and non-cancerous mucosa) from consenting patients (both sexes, 68-85 years old) undergoing gastrectomy for distant non-cardia adenocarcinoma were included in the study, in agreement with the tumor bank of the Bordeaux University Hospital (France), as previously reported [17][55][15].

**Bacterial culture.** *H. pylori* strain 7.13 and isogenic *cagA*-deleted mutant were cultured as previously described [11][15]. The coculture experiments were performed at a multiplicity of infection of 50 following existing protocols [11][15].

**Gastric epithelial cell culture.** The AGS (ATCC CRL-1739) and MKN74 (HSRR Bank JCRB0255) cell lines were cultured in DMEM/F12-Glutamax or RPMI1640-Glutamax media, respectively, supplemented with 10% heat-inactivated fetal calf serum (all from Invitrogen), at 37°C in a 5% CO<sub>2</sub> humidified atmosphere [11][15]. The experiments were performed with cells seeded at low density (2.5x10<sup>4</sup> cells/well in 24 well plates).

**Transfection and reporter assays.** Cells were grown in 24 well plates and transfection of plasmids and siRNA were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) as recommended. Two rounds of siRNA transfection were performed. Previously validated non-silencing siRNA (siCtrl, Dharmakon, Lafayette, CO, USA)[10][19] with no known homology to mammalian genes was used as a negative control and siRNA directed against human LATS2 (Qiagen)[10] and YAP1 (Qiagen) were used at 20 nM. The 8xGTII-Luciferase, pcDNA-Flag-Yap1, and pCMV-flagS127A-YAP vectors, used at 100 ng/well were gifts from Stefano Piccolo [56], Yosef Shaul [57] and Kunliang Guan [58], respectively. TAL-luciferase (100 ng/well) reporter was from

Becton Dickinson, and pRL-SV40 (10 ng/well) and pEGFP-C3 (100 ng/well) were from Promega. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Assay (Promega). Firefly luciferase activities were normalized for transfection efficiency by *Renilla* luciferase.

**Immunohistochemistry scoring.** 3  $\mu\text{m}$  tissue sections were prepared from formalin-fixed paraffin-embedded human tissues and submitted to standard IHC protocols (see supplementary materials and methods). Relative quantification of the percentage of gastric epithelial cells expressing CD44, LATS2, and YAP1 and with nuclear LATS2 and YAP1 was determined in a double blind lecture using a scale from 1 to 4 (1, <5%; 2, 5-25%; 3, 25-50%; 4, >50%). The coefficients R and R2, and the p values were calculated for each pair.

Ethical statements and procedures concerning RNA extraction, transcriptome and RT-qPCR, western blotting, immunocytofluorescence, immunohistochemistry, flow cytometry, invasion assay, tumorsphere assay, and statistical analysis are detailed in supplementary materials.

## **Acknowledgments**

Silvia Elena Molina Castro is a PhD fellowship recipient of the University of Costa Rica (San José, Costa Rica) and the ministry of sciences and technologies (MICIT, Costa Rica). This project and Julie Giraud and Camille Tiffon were financially supported by the French National Institute for Cancer INCa (grant 2014-152). This project was supported by the SIRIC BRIO (Bordeaux Research in Translational Oncology - Site de Recherche Intégrée sur le Cancer, grant INCa-DGOS-Inserm 6046). We thank R. Peek (Vanderbilt University, Nashville, TN, USA) for the *H. pylori* strains 7.13 and isogenic *cagA*-deleted mutant, Professor Nojima (Osaka, Japan) for the pCMVmyc-LATS2 plasmid, Yannick Lippi (GeTRix Platform, Toulouse, France) for statistical analyses of the transcriptome, and Jacques Robert (INSERM U1218, University of Bordeaux, France) for CCLE database analyses. We thank Julie Pannequin (IGF, University of Montpellier, France) and Michel Moenner (CNRS UMR5095, University of Bordeaux, France) for helpful discussions.

## **Disclosures**

All authors declare no financial, professional, or personal conflict of interest.

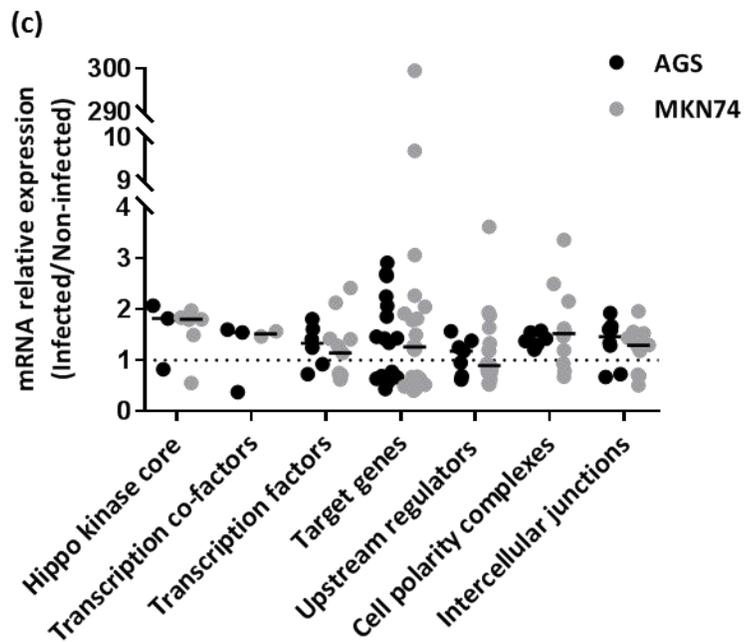
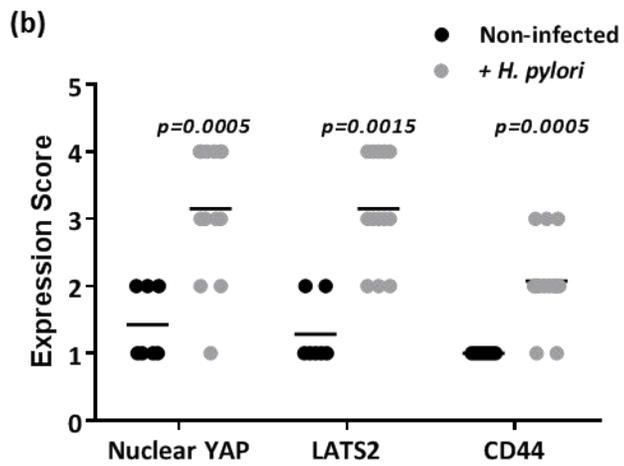
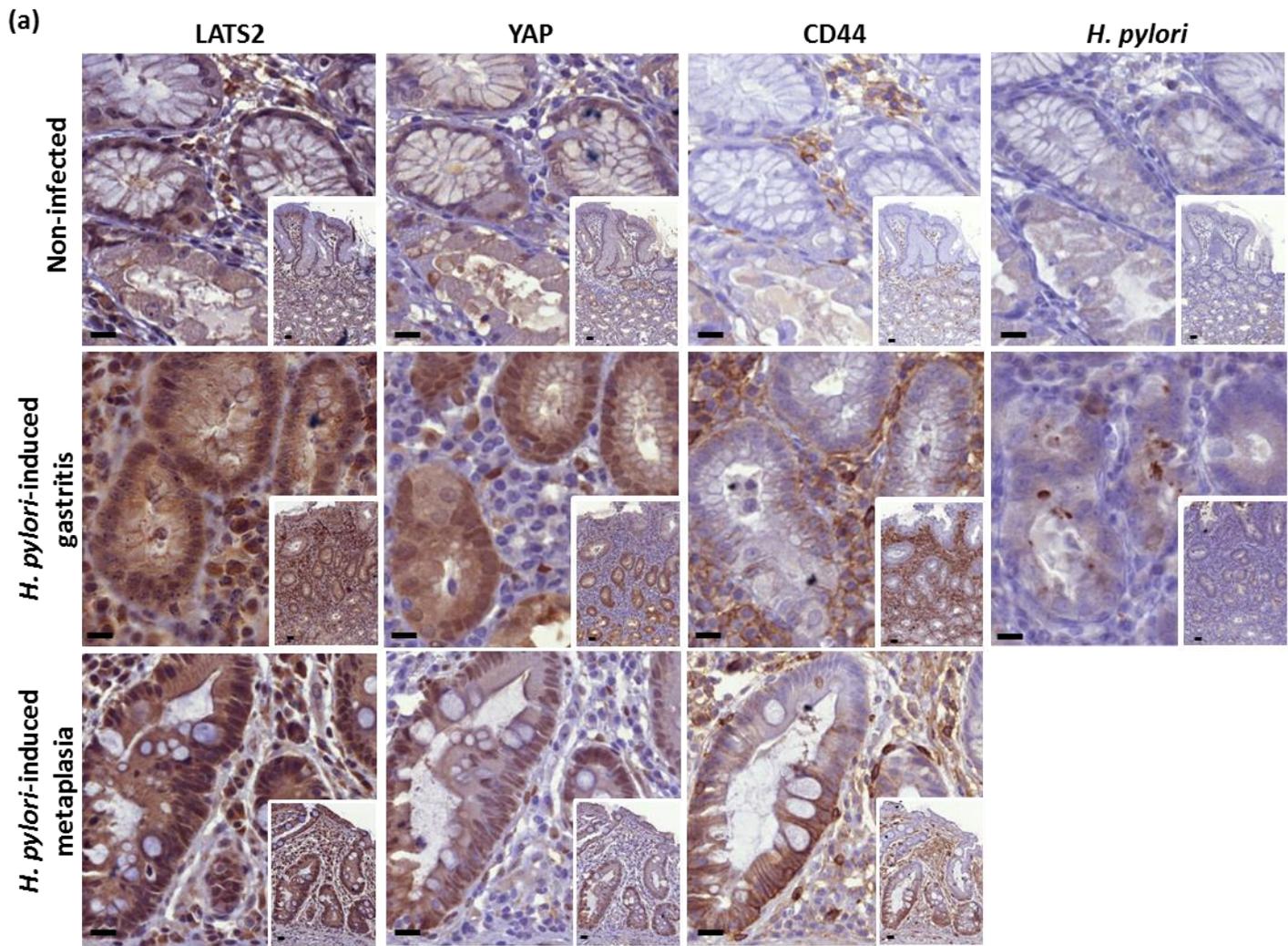
## References

- [1] Bray F, Jemal A, Grey N, Ferlay J, Forman D. Global cancer transitions according to the Human Development Index (2008-2030): a population-based study. *Lancet Oncol*. 2012 Aug;13(8):790-801.
- [2] Gloria L, Bossard N, Bouvier AM, Mayer-da-Silva A, Faivre J, Miranda A. Trends in net survival from stomach cancer in six European Latin countries: results from the SUDCAN population-based study. *Eur J Cancer Prev*. 2017 Jan;26 Trends in cancer net survival in six European Latin Countries: the SUDCAN study:S32-S9.
- [3] IARC'Working'Group. Schistosomes, liver flukes and *Helicobacter pylori*:Views and expert opinion of IARC Working Group on the evaluation of carcinogenic risks to humans. 1994;61:177-240. Lyon, IARC Monographs. International Agency for research on Cancer.
- [4] Rugge M. Gastric Cancer Risk in Patients with *Helicobacter pylori* Infection and Following Its Eradication. *Gastroenterology clinics of North America*. 2015 Sep;44(3):609-24.
- [5] Correa P, Houghton J. Carcinogenesis of *Helicobacter pylori*. *Gastroenterology*. 2007 Aug;133(2):659-72.
- [6] Gonzalez CA, Megraud F, Buissonniere A, Lujan Barroso L, Agudo A, Duell EJ, et al. *Helicobacter pylori* infection assessed by ELISA and by immunoblot and noncardia gastric cancer risk in a prospective study: the Eurgast-EPIC project. *Ann Oncol*. 2012 May;23(5):1320-4.
- [7] Megraud F, Bessede E, Varon C. *Helicobacter pylori* infection and gastric carcinoma. *Clin Microbiol Infect*. 2015 Nov;21(11):984-90.
- [8] Backert S, Selbach M. Role of type IV secretion in *Helicobacter pylori* pathogenesis. *Cellular microbiology*. 2008 Aug;10(8):1573-81.
- [9] Mimuro H, Suzuki T, Nagai S, Rieder G, Suzuki M, Nagai T, et al. *Helicobacter pylori* dampens gut epithelial self-renewal by inhibiting apoptosis, a bacterial strategy to enhance colonization of the stomach. *Cell host & microbe*. 2007 Oct 11;2(4):250-63.
- [10] Belair C, Baud J, Chabas S, Sharma CM, Vogel J, Staedel C, et al. *Helicobacter pylori* interferes with an embryonic stem cell micro RNA cluster to block cell cycle progression. *Silence*. 2010 Oct 25;2(1):7.
- [11] Baud J, Varon C, Chabas S, Chambonnier L, Darfeuille F, Staedel C. *Helicobacter pylori* initiates a mesenchymal transition through ZEB1 in gastric epithelial cells. *PLoS one*. 2013;8(4):e60315.
- [12] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009 Nov 25;139(5):871-90.
- [13] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008 May 16;133(4):704-15.
- [14] Clevers H. The cancer stem cell: premises, promises and challenges. *Nature medicine*. 2011 Mar;17(3):313-9.
- [15] Bessede E, Staedel C, Acuna Amador LA, Nguyen PH, Chambonnier L, Hatakeyama M, et al. *Helicobacter pylori* generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene*. 2014 Aug 7;33(32):4123-31.
- [16] Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, et al. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem cells (Dayton, Ohio)*. 2009 May;27(5):1006-20.
- [17] Nguyen PH, Giraud J, Chambonnier L, Dubus P, Wittkop L, Belleanne G, et al. Characterization of Biomarkers of Tumorigenic and Chemoresistant Cancer Stem Cells in Human Gastric Carcinoma. *Clin Cancer Res*. 2017 Mar 15;23(6):1586-97.
- [18] Bessede E, Dubus P, Megraud F, Varon C. *Helicobacter pylori* infection and stem cells at the origin of gastric cancer. *Oncogene*. 2015 May 14;34(20):2547-55.

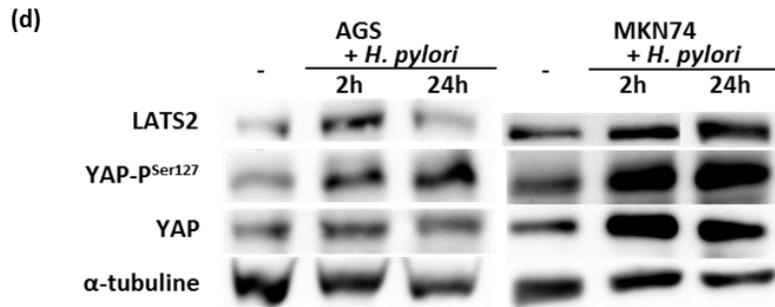
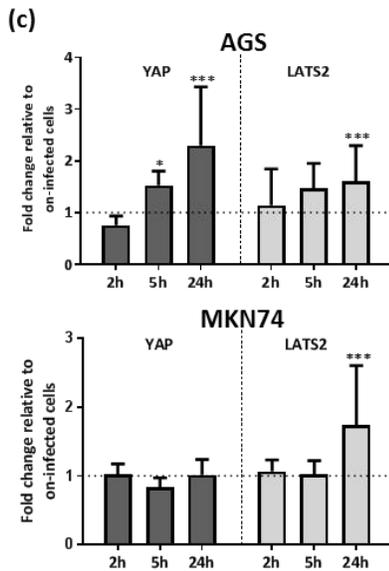
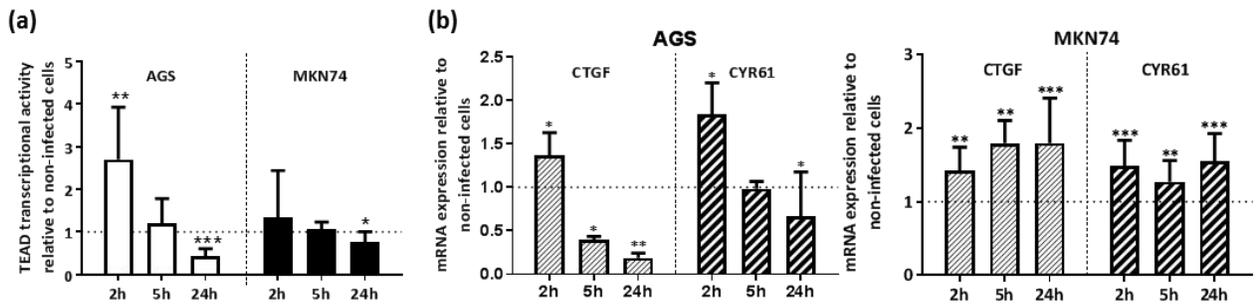
- [19] Bessede E, Molina S, Amador LA, Dubus P, Staedel C, Chambonnier L, et al. Deletion of IQGAP1 promotes *Helicobacter pylori*-induced gastric dysplasia in mice and acquisition of cancer stem cell properties in vitro. *Oncotarget*. 2016 Dec 06;7(49):80688-99.
- [20] Piccolo S, Dupont S, Cordenonsi M. The biology of YAP/TAZ: hippo signaling and beyond. *Physiological reviews*. 2014 Oct;94(4):1287-312.
- [21] Zanonato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. *Cancer Cell*. 2016 Jun 13;29(6):783-803.
- [22] Hansen CG, Moroishi T, Guan KL. YAP and TAZ: a nexus for Hippo signaling and beyond. *Trends in cell biology*. 2015 Sep;25(9):499-513.
- [23] Jiao S, Li C, Hao Q, Miao H, Zhang L, Li L, et al. VGLL4 targets a TCF4-TEAD4 complex to coregulate Wnt and Hippo signalling in colorectal cancer. *Nature communications*. 2017 Jan 04;8:14058.
- [24] Natsuzaka M, Ohashi S, Wong GS, Ahmadi A, Kalman RA, Budo D, et al. Insulin-like growth factor-binding protein-3 promotes transforming growth factor- $\beta$ 1-mediated epithelial-to-mesenchymal transition and motility in transformed human esophageal cells. *Carcinogenesis*. 2010 Aug;31(8):1344-53.
- [25] Martin-Belmonte F, Perez-Moreno M. Epithelial cell polarity, stem cells and cancer. *Nature reviews*. 2011 Dec 15;12(1):23-38.
- [26] Szymaniak AD, Mahoney JE, Cardoso WV, Varelas X. Crumbs3-Mediated Polarity Directs Airway Epithelial Cell Fate through the Hippo Pathway Effector Yap. *Developmental cell*. 2015 Aug 10;34(3):283-96.
- [27] Tanaka I, Osada H, Fujii M, Fukatsu A, Hida T, Horio Y, et al. LIM-domain protein AJUBA suppresses malignant mesothelioma cell proliferation via Hippo signaling cascade. *Oncogene*. 2015 Jan 02;34(1):73-83.
- [28] Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO reports*. 2008 Jun;9(6):582-9.
- [29] Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012 Mar 28;483(7391):603-7.
- [30] Yu FX, Zhao B, Guan KL. Hippo Pathway in Organ Size Control, Tissue Homeostasis, and Cancer. *Cell*. 2015 Nov 05;163(4):811-28.
- [31] Zhang J, Yang YC, Zhu JS, Zhou Z, Chen WX. Clinicopathologic characteristics of YES-associated protein 1 overexpression and its relationship to tumor biomarkers in gastric cancer. *International journal of immunopathology and pharmacology*. 2012 Oct-Dec;25(4):977-87.
- [32] Xu ZP, Zhu JS, Zhang Q, Wang XY. A breakdown of the Hippo pathway in gastric cancer. *Hepato-gastroenterology*. 2011 Sep-Oct;58(110-111):1611-7.
- [33] Yi J, Lu L, Yanger K, Wang W, Sohn BH, Stanger BZ, et al. Large tumor suppressor homologs 1 and 2 regulate mouse liver progenitor cell proliferation and maturation through antagonism of the coactivators YAP and TAZ. *Hepatology (Baltimore, Md)*. 2016 Nov;64(5):1757-72.
- [34] Park GS, Oh H, Kim M, Kim T, Johnson RL, Irvine KD, et al. An evolutionarily conserved negative feedback mechanism in the Hippo pathway reflects functional difference between LATS1 and LATS2. *Oncotarget*. 2016 Apr 26;7(17):24063-75.
- [35] Barry ER, Camargo FD. The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. *Current opinion in cell biology*. 2013 Apr;25(2):247-53.
- [36] Ramos A, Camargo FD. The Hippo signaling pathway and stem cell biology. *Trends in cell biology*. 2012 Jul;22(7):339-46.

- [37] Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, et al. YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response. *Cell*. 2014 Jul 3;158(1):157-70.
- [38] Vermeulen L. Keeping stem cells in check: a hippo balancing act. *Cell stem cell*. 2013 Jan 03;12(1):3-5.
- [39] Moroishi T, Park HW, Qin B, Chen Q, Meng Z, Plouffe SW, et al. A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. *Genes & development*. 2015 Jun 15;29(12):1271-84.
- [40] Aylon Y, Yabuta N, Besserglick H, Buganim Y, Rotter V, Nojima H, et al. Silencing of the Lats2 tumor suppressor overrides a p53-dependent oncogenic stress checkpoint and enables mutant H-Ras-driven cell transformation. *Oncogene*. 2009 Dec 17;28(50):4469-79.
- [41] Lehmann W, Mossmann D, Kleemann J, Mock K, Meisinger C, Brummer T, et al. ZEB1 turns into a transcriptional activator by interacting with YAP1 in aggressive cancer types. *Nature communications*. 2016 Feb 15;7:10498.
- [42] Yao F, Zhou W, Zhong C, Fang W. LATS2 inhibits the activity of NF-kappa B signaling by disrupting the interaction between TAK1 and IKKbeta. *Tumour Biol*. 2015 Sep;36(10):7873-9.
- [43] Furth N, Bossel Ben-Moshe N, Pozniak Y, Porat Z, Geiger T, Domany E, et al. Down-regulation of LATS kinases alters p53 to promote cell migration. *Genes & development*. 2015 Nov 15;29(22):2325-30.
- [44] Franco AT, Israel DA, Washington MK, Krishna U, Fox JG, Rogers AB, et al. Activation of beta-catenin by carcinogenic *Helicobacter pylori*. *Proceedings of the National Academy of Sciences of the United States of America*. 2005 Jul 26;102(30):10646-51.
- [45] Murata-Kamiya N, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, et al. *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene*. 2007 Jul 12;26(32):4617-26.
- [46] Suzuki M, Mimuro H, Kiga K, Fukumatsu M, Ishijima N, Morikawa H, et al. *Helicobacter pylori* CagA phosphorylation-independent function in epithelial proliferation and inflammation. *Cell host & microbe*. 2009 Jan 22;5(1):23-34.
- [47] Li J, Chen X, Ding X, Cheng Y, Zhao B, Lai ZC, et al. LATS2 suppresses oncogenic Wnt signaling by disrupting beta-catenin/BCL9 interaction. *Cell reports*. 2013 Dec 26;5(6):1650-63.
- [48] Enderle L, McNeill H. Hippo gains weight: added insights and complexity to pathway control. *Science signaling*. 2013 Oct 08;6(296):re7.
- [49] Hatakeyama M. *Helicobacter pylori* and gastric carcinogenesis. *Journal of gastroenterology*. 2009;44(4):239-48.
- [50] Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science (New York, NY)*. 2003 May 30;300(5624):1430-4.
- [51] Coulombe G, Rivard N. New and Unexpected Biological Functions for the Src-Homology 2 Domain-Containing Phosphatase SHP-2 in the Gastrointestinal Tract. *Cellular and molecular gastroenterology and hepatology*. 2011 Jan;2(1):11-21.
- [52] Tsutsumi R, Masoudi M, Takahashi A, Fujii Y, Hayashi T, Kikuchi I, et al. YAP and TAZ, Hippo signaling targets, act as a rheostat for nuclear SHP2 function. *Developmental cell*. 2013 Sep 30;26(6):658-65.
- [53] Yamazaki S, Yamakawa A, Ito Y, Ohtani M, Higashi H, Hatakeyama M, et al. The CagA protein of *Helicobacter pylori* is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. *The Journal of infectious diseases*. 2003 Jan 15;187(2):334-7.
- [54] Li W, Cooper J, Zhou L, Yang C, Erdjument-Bromage H, Zagzag D, et al. Merlin/NF2 loss-driven tumorigenesis linked to CRL4(DCAF1)-mediated inhibition of the hippo pathway kinases Lats1 and 2 in the nucleus. *Cancer Cell*. 2014 Jul 14;26(1):48-60.

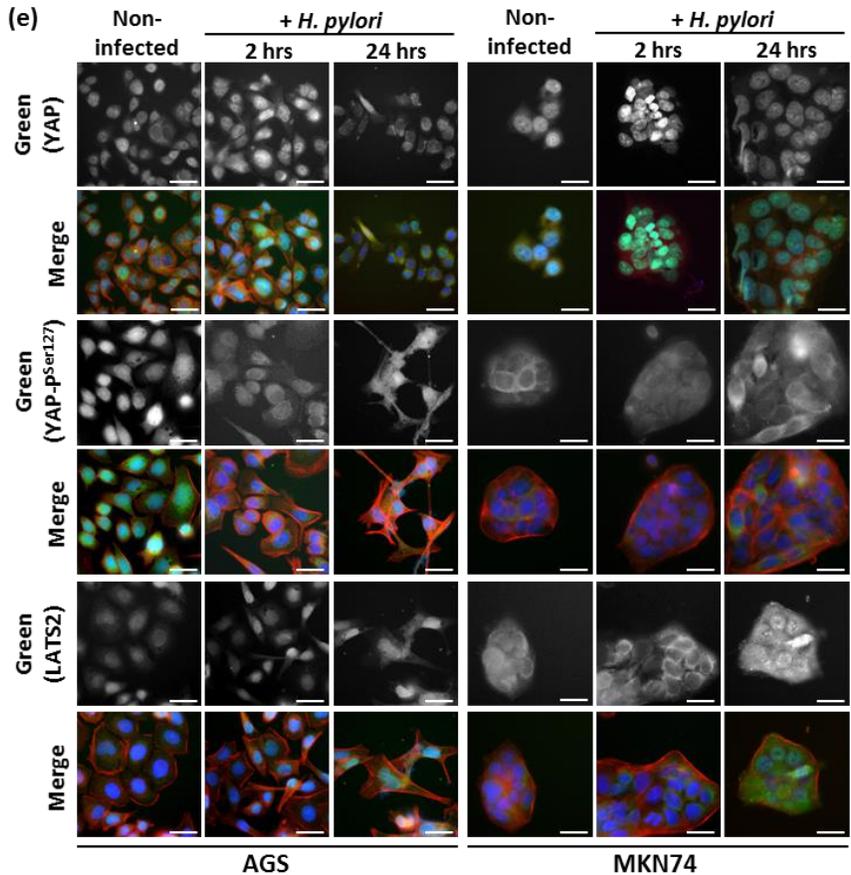
- [55] Nguyen PH, Giraud J, Staedel C, Chambonnier L, Dubus P, Chevret E, et al. All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. *Oncogene*. 2016 Oct 27;35(43):5619-28.
- [56] Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. *Nature*. 2011 Jun 08;474(7350):179-83.
- [57] Levy D, Adamovich Y, Reuven N, Shaul Y. The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73. *Cell death and differentiation*. 2007 Apr;14(4):743-51.
- [58] Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development*. 2007 Nov 01;21(21):2747-61.



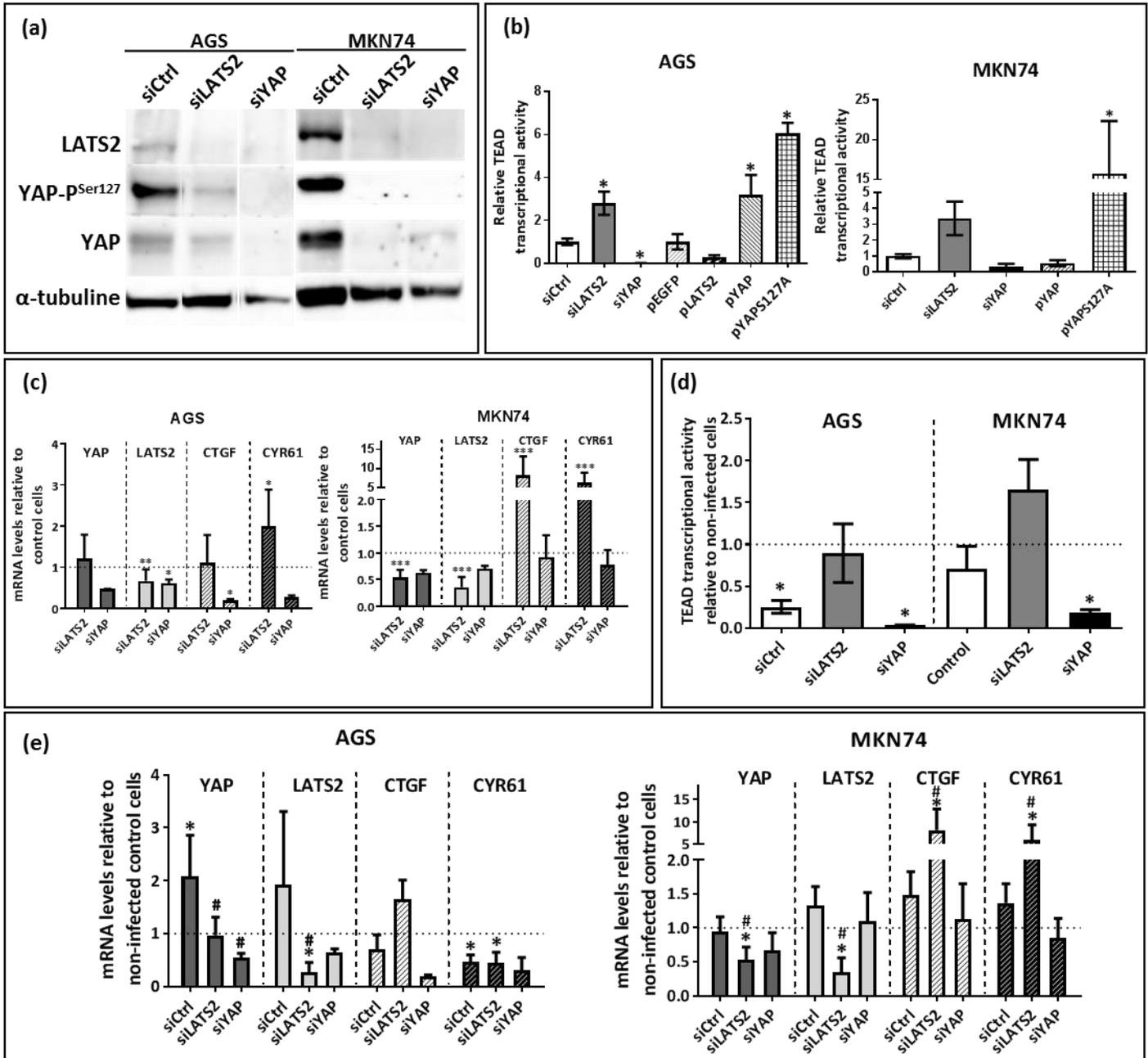
**Figure 1. LATS2, YAP1 and CD44 expressions in *H. pylori*-infected and non-infected human gastric mucosa, and relative expression of the Hippo pathway genes in *H. pylori* infected hGECs.** (a) Representative images of the immunohistochemistry staining of LATS2, YAP1, CD44, and *H. pylori* in the gastric mucosa of non-infected individuals (upper panels, n=7), and patients with *H. pylori*-associated gastritis (middle panels, n=7) and metaplasia (lower panels, n=4). Bars, 10  $\mu$ m. (b) Scores of the relative percentages of LATS2, nuclear YAP1 or CD44 positive cells determined from *H. pylori* negative (n=7) and *H. pylori* positive (n=11) patients from the experiments shown in (a). The difference between both groups was evaluated using a Fisher's exact test. (c) Relative expression of the differentially expressed Hippo-related genes in response to *H. pylori* infection in AGS and MKN74 cells determined according to microarray-based transcriptome analyses. Only genes with statistically significant changes in expression are shown (n=4, p<0.05 in t-test with Benjamini-Hochberg correction). Bars are medians.



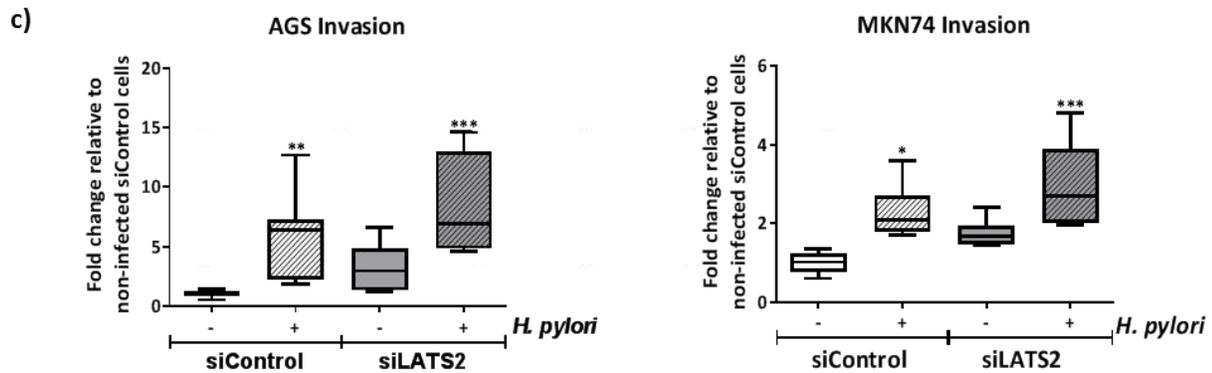
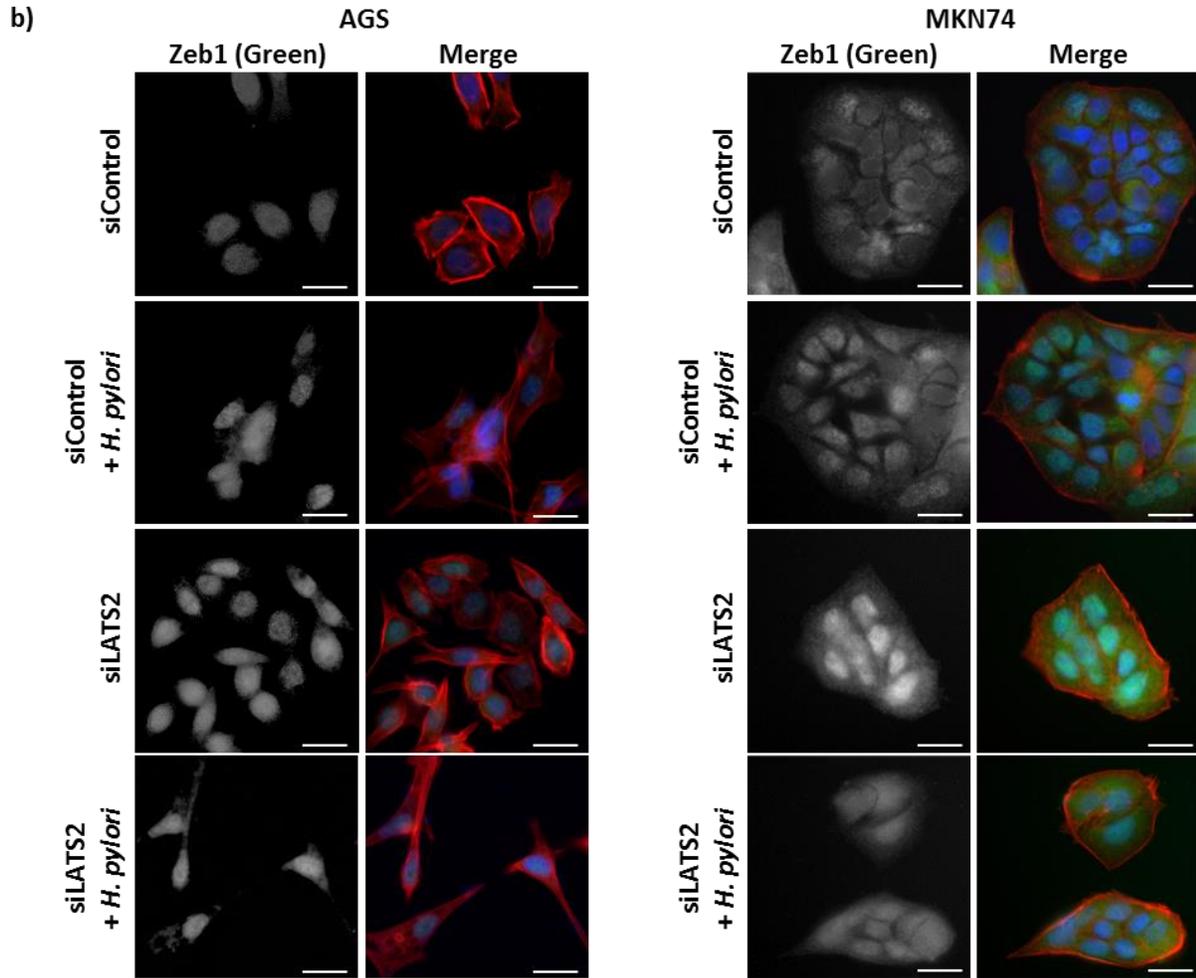
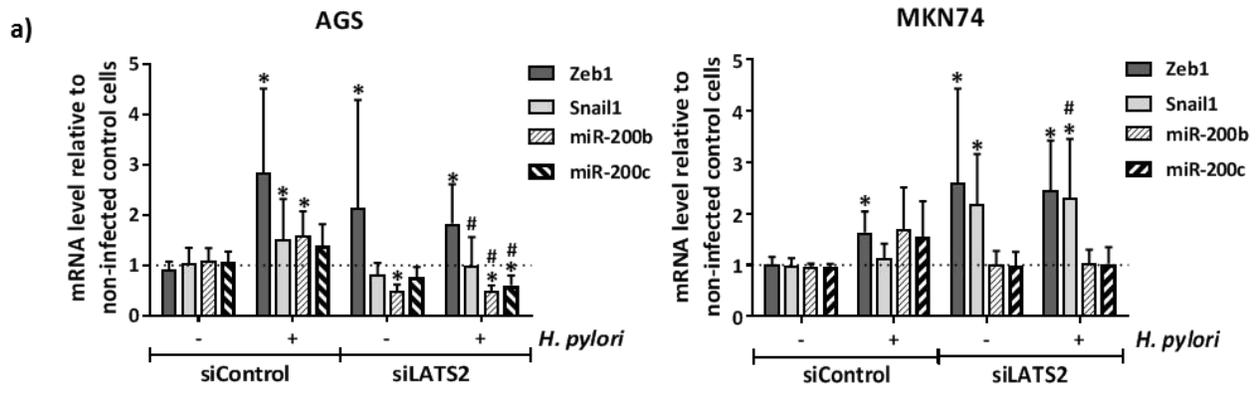
	AGS		MKN74	
	2h NI $\pm$ SD ( <i>p</i> )	24h NI $\pm$ SD ( <i>p</i> )	2h NI $\pm$ SD ( <i>p</i> )	24h NI $\pm$ SD ( <i>p</i> )
LATS2	2.29 $\pm$ 1.09 (0.004)	1.52 $\pm$ 0.36 (0.012)	1.40 $\pm$ 0.43 (0.018)	1.35 $\pm$ 0.22 (0.010)
YAP-p <sup>Ser127</sup>	1.95 $\pm$ 0.08 (0.115)	2.93 $\pm$ 0.11 (0.003)	1.48 $\pm$ 0.27 (0.094)	3.68 $\pm$ 1.90 (0.009)
Total YAP	2.18 $\pm$ 0.78 (0.030)	1.62 $\pm$ 0.81 (0.504)	2.00 $\pm$ 0.77 (0.031)	1.84 $\pm$ 1.14 (0.031)



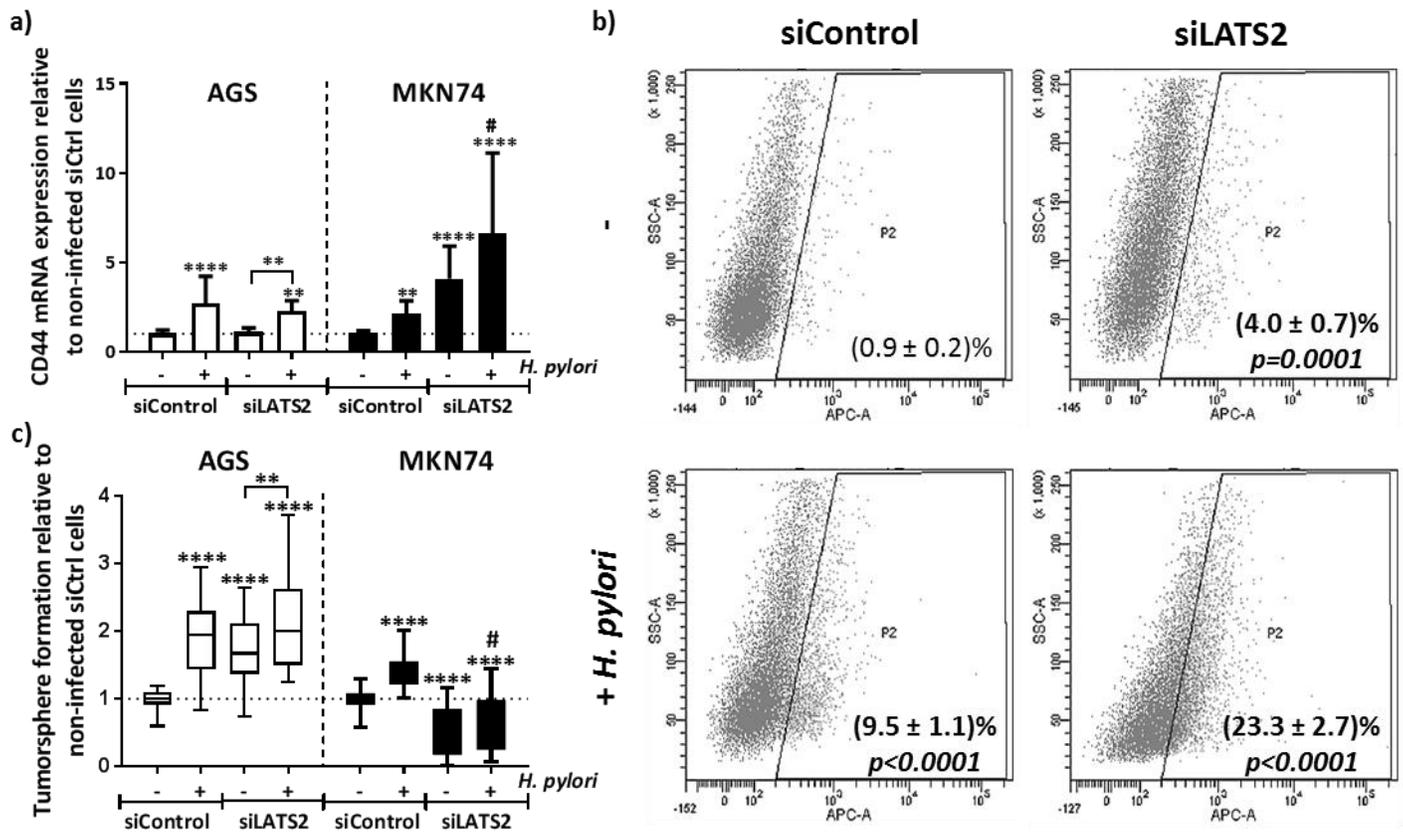
**Figure 2. Biphasic kinetics of *H. pylori*-induced changes in LATS2 and YAP1 expressions and subcellular localizations in hGECs *in vitro*.** (a) The time-course of LATS2 and YAP1 expressions was analyzed in the hGECs infected with *H. pylori* 7.13. TEAD-Luciferase reporter activity measured at 2, 5, and 24 hpi in AGS (white bars) and MKN74 (black bars) cells. (b, c) Relative mRNA levels for YAP1/TEAD target genes CTGF (white/black bars) and CYR61 (black/whites bars), YAP1 (dark grey bars) and LATS2 (light grey bars) assessed by RT-qPCR. Fold changes relative to non-infected cells are presented. (d) LATS2, total YAP and YAP-P<sup>Ser127</sup> protein levels were quantified by Western Blot in non-infected cells, and 2 and 24 hpi. Mean band intensities  $\pm$  SD relative to  $\alpha$ -tubulin are shown in the table below the blot image (n=4). (e) Representative images of the expression and subcellular localization of LATS2, total YAP1 and YAP1-P<sup>Ser127</sup> (in green) evaluated by immunofluorescence. Actin was marked with phalloidin-Alexafluor-546 (red) and nuclei were marked with DAPI (blue). Bars, 10  $\mu$ m. (a-c) Bars represent the mean  $\pm$  SD of 3<n<5 independent experiments. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs non-infected in Kruskal-Wallis test.



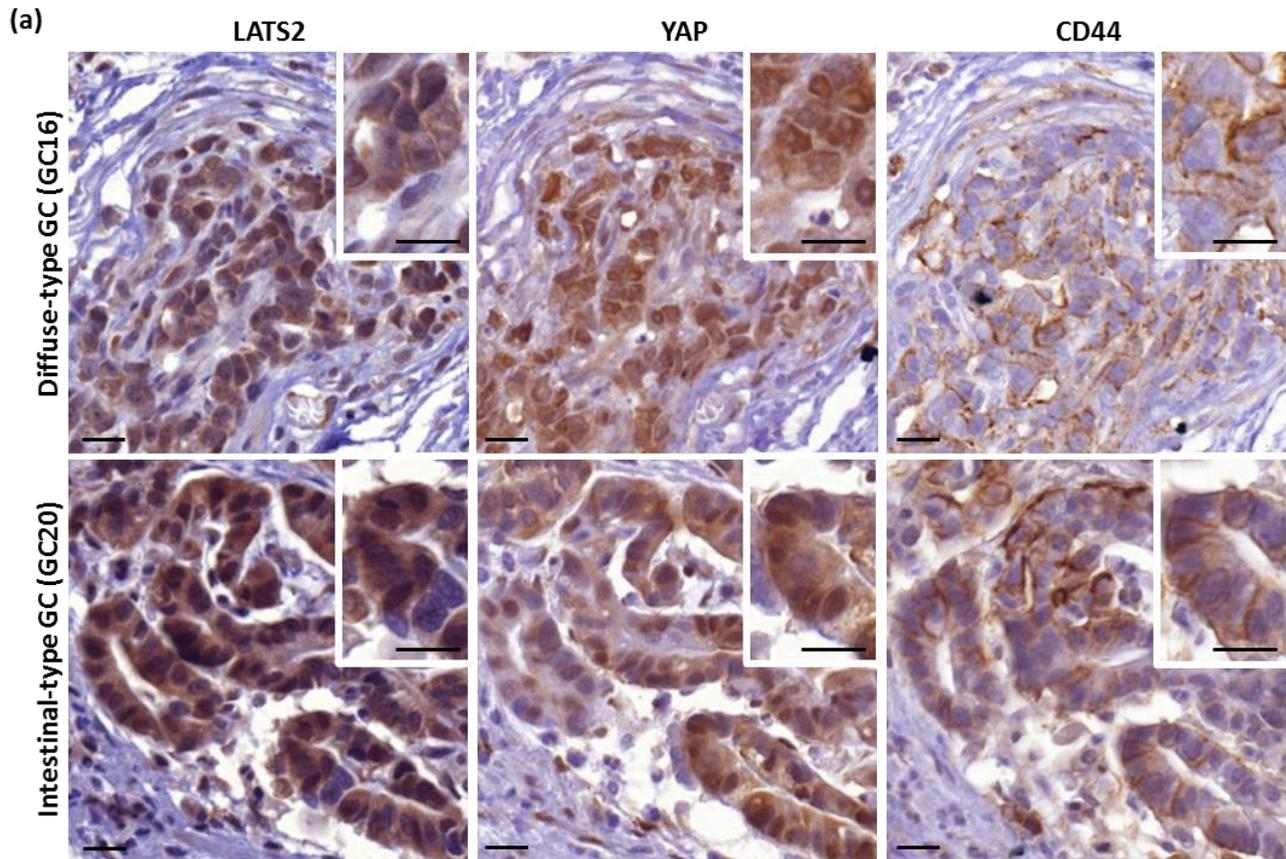
**Figure 3. LATS2 and YAP1 regulate each other both in basal conditions and in response to *H. pylori* infection.** (a) Western blot analysis of LATS2, YAP1, and YAP1-P<sup>Ser127</sup> protein levels in non-infected conditions, 48h after the last transfection with siRNA negative control (siControl) and against LATS2 (siLATS2) and YAP1 (siYAP). (b) TEAD-Luciferase reporter activity in non-infected AGS and MKN74 cells 48h after transfection with siCtrl, siLATS2 and siYAP. Cells transfected with pEGFP, pLATS2, pYAP and pYAPS127A were included as controls. (c) Relative mRNA levels for YAP1/TEAD target genes CTGF (white/black bars) and CYR61 (black/white bars), YAP1 (dark grey bars) and LATS2 (light grey bars) in non-infected conditions were evaluated at the same time point. (d, e) TEAD-Luciferase reporter activity (d) and YAP1, LATS2, CTGF or CYR61 mRNA levels (e) relative to non-infected siControls, upon infection with *H. pylori* 7.13. (b-e) Bars represent the mean  $\pm$  SD of 3<n<6 experiments. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs non-infected siControls in Kruskal-Wallis test, #p<0.05 vs *H. pylori*-infected siControls in Kruskal-Wallis test.



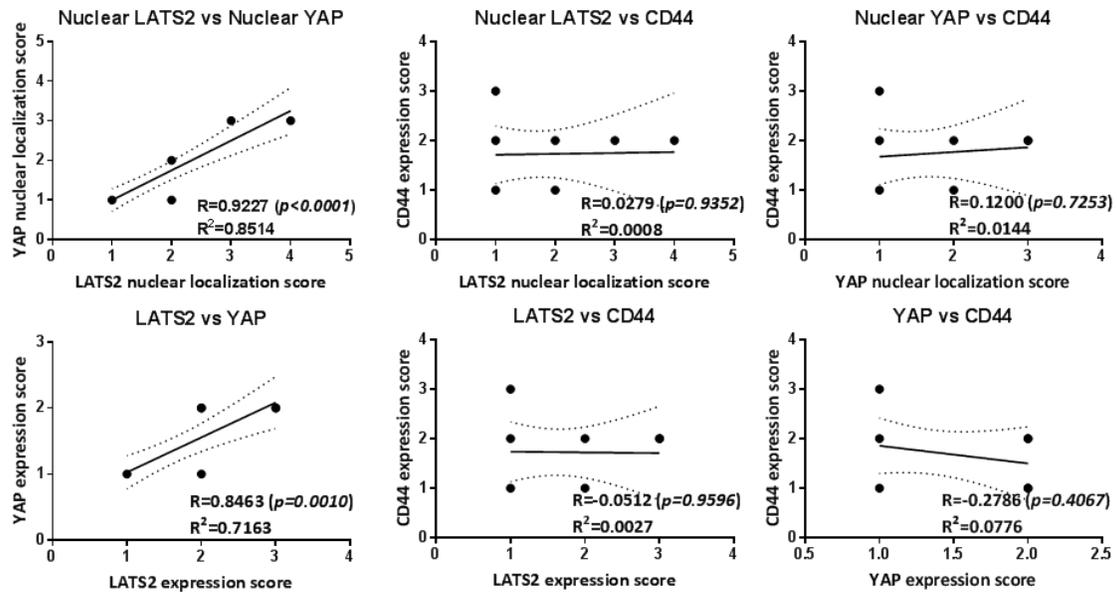
**Figure 4. LATS2 restricts *H. pylori*-induced EMT in hGECs *in vitro*.** (a) Relative mRNA levels for EMT-markers Zeb1 (dark grey bars), Snail1 (light grey bars), and mature miR-200b (white/black bars) and miR-200c (black/white bars) measured 24 hpi with *H. pylori* 7.13 in siControl-, and siLATS2-transfected AGS and MKN74 cells. (b) Expression and subcellular localization of ZEB1 (green) assessed by immunofluorescence in AGS and MKN74. F-Actin was marked with Alexa-546-labelled phalloidin (red) and nuclei with DAPI (blue). Bars, 10  $\mu$ m. (c) Invasion capacity through collagen I-coated Transwell inserts determined after 18h for *H. pylori*-infected and non-infected siControl-, and siLATS2-transfected AGS and MKN74 cells. (a, c) Bars represent the mean  $\pm$  SD of 3<n<6 independent experiments. \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 vs non-infected siControl in Kruskal-Wallis test, # p<0.05 vs *H. pylori*-infected siControl in Kruskal-Wallis test.



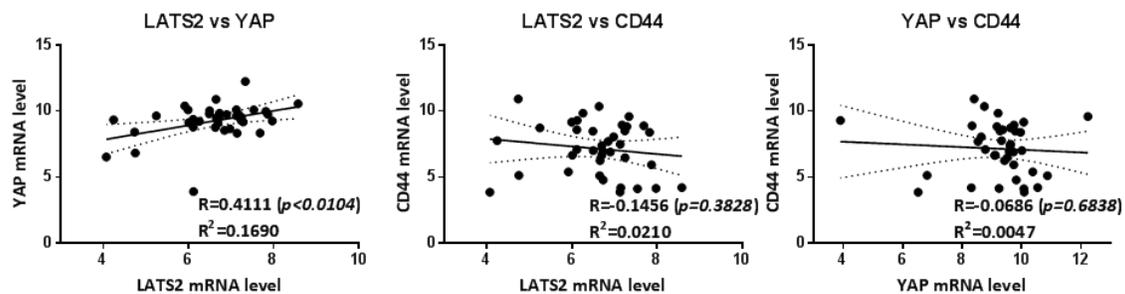
**Figure 5. LATS2 represses CD44 and CSC-like properties in hGECs *in vitro*.** (a) Relative mRNA levels of CD44 at 24 hpi with *H. pylori* 7.13 in siControl-, and siLATS2-transfected AGS (white bars) and MKN74 (black bars) cells. (b) Percentage of CD44-positive cells assessed by flow cytometry analyses at 24 hpi with *H. pylori* 7.13 in siControl-, and siLATS2-transfected MKN74 cells. (c) Number of tumorspheres determined after 5 days culture in serum free medium and non-adherent conditions of *H. pylori* 7.13-infected and uninfected siControl-, and siLATS2-transfected AGS (white boxes) and MKN74 (black boxes) cells. (a-c) Bars represent the mean ± SD of 3<n<6 independent experiments. \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 vs non-infected siControls in Kruskal-Wallis test, # p<0.05 vs *H. pylori*-infected siControl in Kruskal-Wallis test.



(b) Gastric tumor tissue sections from patients



(c) Gastric cancer cell lines from CCLE



**Figure 6. LATS2, YAP1 and CD44 expressions of in diffuse- and intestinal-type patient's GC tissue samples and GC cell lines of the CCLE.** (a) Representatives images of the LATS2, YAP1, and CD44 immunohistochemistry stainings of diffuse (upper panel, n=4) and intestinal (lower panel, n=7) tissue sections from GC patients. Scale bars, 10  $\mu$ m. (b) Scores of LATS2, YAP1, and CD44 relative expression determined from immunohistochemistry analyses in (a) (n=11). The scores for each case were plotted and R, R<sup>2</sup>, and *p* values were calculated for each pair. (c) The expression level of LATS2, YAP1, and CD44 transcripts obtained from the 38 GC cell lines included in the CCLE were plotted and R, R<sup>2</sup>, and *p* values were calculated for each pair.

## **Supplementary materials and methods.**

**Ethic statements on human tissue samples and animal experiments.** Studies on paraffin-embedded tumors from GC patients were performed in agreement with the Direction for Clinical Research and the Tumor and Cell Bank of the University Hospital Center of Bordeaux (Haut-Leveque Hospital). Tumor xenograft experiments with non obese diabetous/severe combined immunodeficiency /interleukin-2Ry null (NSG) immunodeficient mice were performed in level 2 animal quarters of the University of Bordeaux, in agreement with the local Ethic Committee on Animal Experiments CEEA50 of Bordeaux (agreement number 50120151-A). Activities of preparation and conservation of tissues of human origin were declared at the French Ministry of Research (DC-2008-412).

**RNA extraction, transcriptome and RT-qPCR.** Cell RNAs were extracted using Trizol reagent (Invitrogen) and quantified by their absorbance at 260 nm. For the transcriptome, RNAs were extracted using RNeasy (Qiagen), and the RINs were determined on the TapeStation (Agilent). The transcriptome was performed at the GeTRix Platform (Toulouse, France) with the G3 HGE 8x60 microarrays (Agilent). For RT-qPCR, retrotranscription was performed with 1 µg total RNA using the Quantitect Reverse Transcription kit (Qiagen). Quantitative-PCR was performed using the SYBR-qPCR-Premix Ex-Taq (TAKARA), 0.3 µM specific primers (Table S2) and 1:200 of the RT reaction volume. Relative expressions were calculated using the comparative Ct method with HRPT1 and TBP as normalizers. Mature miR-200 levels were quantified as previously described [11].

**Immunohistochemistry.** Tissue sections 3 µm thick were prepared from formalin-fixed paraffin-embedded human tissues and submitted to standard IHC protocols with CD44 (mouse anti-CD44 from BD, 1:100, 2 h), LATS2 (rabbit anti-LATS2 from Bethyl; 1:100, 2 h), YAP (rabbit anti-YAP from Cell Signaling; 1:100, 1 h) or and *H. pylori* (rabbit anti-*H. pylori* from DAKO; 1/100, 2 h) antibodies, and then with Horse Radish Peroxidase-labelled anti-rabbit EnVision System (DAKO; 30 min). Immunolabeling was revealed upon 10 min incubation in liquid diaminobenzidine-chromogen substrate (DAKO). Slides were counterstained with hematoxylin, dehydrated and mounted with Eukitt-mounting medium (Labonord).

**Western blotting.** Cells were lysed on ice in ProteoJET™ reagent (Fermentas) supplemented with protease/phosphatase inhibitor cocktail (Sigma). The extracted proteins were submitted to SDS-PAGE and western blotting for immunolabeling. Immunolabelling was performed using rabbit anti-YAP or anti-YAP-PSer127 (each at 1:2000; Cell Signaling), anti-LATS2 (1:2000; Bethyl), mouse anti- $\alpha$ -tubulin (1:8000; Sigma-Aldrich), Horseradish Peroxydase-coupled anti-rabbit and anti-mouse secondary antibodies (DAKO) and chemoluminescent detection (ECL<sup>+</sup>, Amersham). A representative blot out of four is shown. The band intensities relative to  $\alpha$ -tubulin were quantified using ImageJ software and the results are shown in the table below the gel image (mean  $\pm$  SD of 4 blots).

**Immunocytofluorescence.** 25,000 cells cultured on glass coverslips were fixed with 4% paraformaldehyde solution for 10 minutes and processed for immunofluorescent staining as described [19]. Expression and subcellular localization of LATS2, total YAP and YAP-PSer127 (labelled in green) assessed by immunofluorescence using the same antibodies used in western blot, at a 1:300 dilution and anti-rabbit Alexa-488-labelled secondary antibody (1:250, Molecular Probes). F-Actin (in red) was marked with Alexa-546-labelled phalloidin (1:250, Molecular Probes) and nuclei (in blue) with DAPI. Images were taken using an Eclipse 50i epi-fluorescence microscope (Nikon) with Nis Element acquisition software and x40 oil immersion objective.

**Invasion assay.** Cells were recovered by trypsinization and  $5 \cdot 10^3$  cells per condition were placed in the upper side of a 8-mm pore size Transwell (Corning, NY, USA) insert, previously coated with rat-tail type 1 collagen (Becton Dickinson, Le Pont de Claix, France) as described elsewhere, in 24-well culture plates with medium containing 5% fetal bovine serum. After 18h of incubation at 37C, the Transwell inserts were fixed in cold methanol and processed for hematoxylin staining, as described previously [15]. Cells having migrated through the lower side of the inserts were counted on five different randomly chosen fields per insert under light microscopy using a 20x objective.

**Flow cytometry.** Cells were recovered using trypsin-EDTA and  $10^5$  cells per condition were stained with anti-CD44-APC antibody (1:25 each, BD Pharmingen, San Jose, CA, USA) in PBS, 0.5% bovine serum

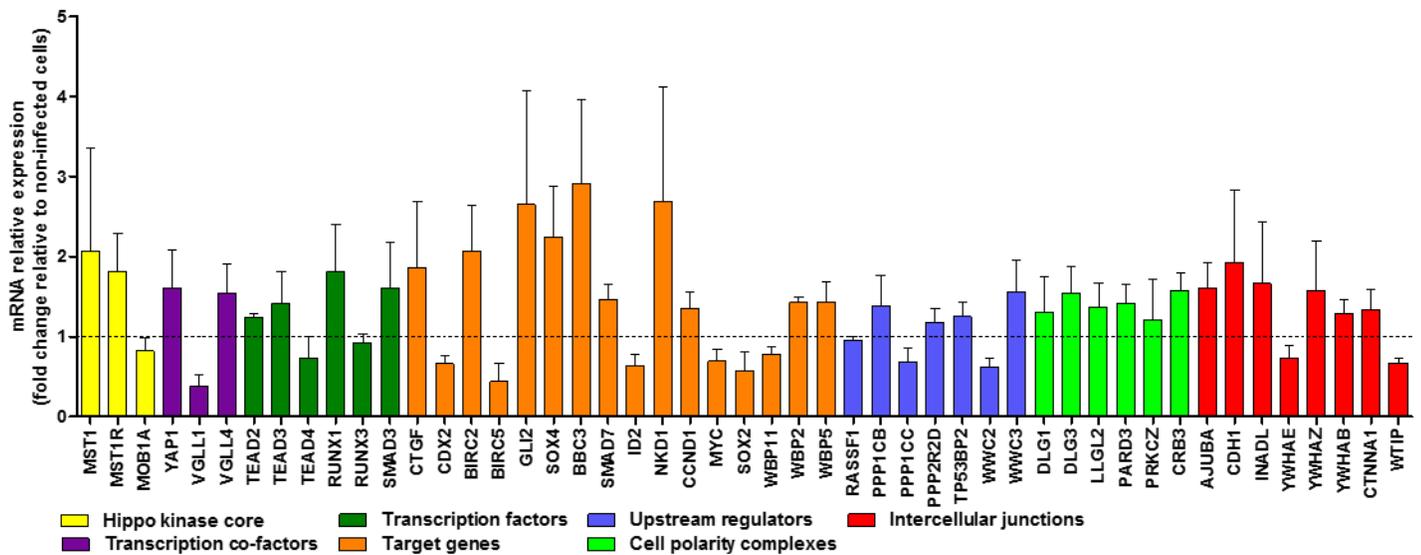
albumin, 2 mM EDTA solution. Flow cytometry was performed using a FACS Canto II instrument and DIVA software (both from Becton Dickinson, Le Pont de Claix, France). Results were based on the analysis of 10000 cells.

**Tumorsphere assay.** Cells were recovered and 500 cells were plated on non-adherent 96-well culture plates (coated with a 10% polyHEMA (Sigma, Saint-Quentin Fallavier, France) solution in absolute ethanol and dried overnight at 56°C). After plating, cells were incubated for 5 days at 37°C in a serum-free medium consisting of DMEM-F12 Glutamax supplemented with 20ng/ml of EGF, 10ng/ml of basic-FGF, 1:100 N2-supplement 100x, 0.3% glucose, 5mg/ml of gentamicin, 50IU/ml of penicillin and 2.5mg/ml of amphotericin B (all from Invitrogen and Sigma) [17]. The number of spheroids per well was counted under light microscopy using a x10 objective.

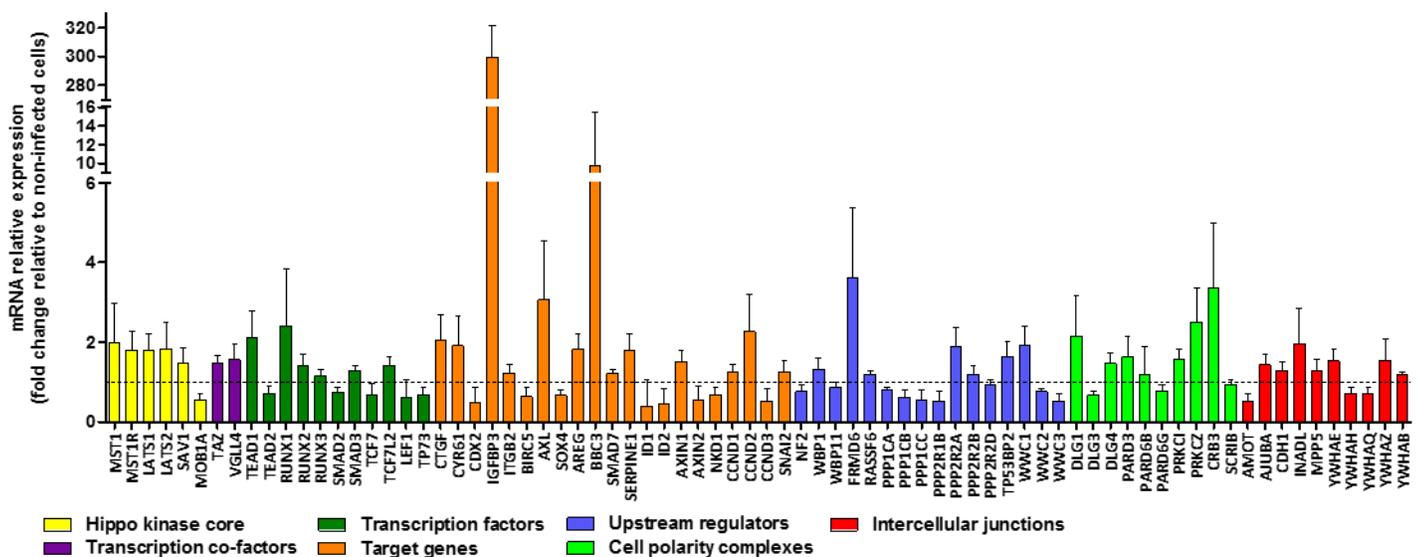
**Statistical analysis.** Quantification values represent the means of three or more independent experiments, each performed by triplicate or more  $\pm$  standard deviation, except for the tumorsphere formation experiments where five independent experiments with n=10 each were carried-on. Mann-Whitney test was used to compare between two groups and Kruskal-Wallis test with Dunn's post-test was used for multiple comparisons. Statistics were performed on GraphPadPrism 7.02 (USA).

## Supplementary figures

(a) AGS

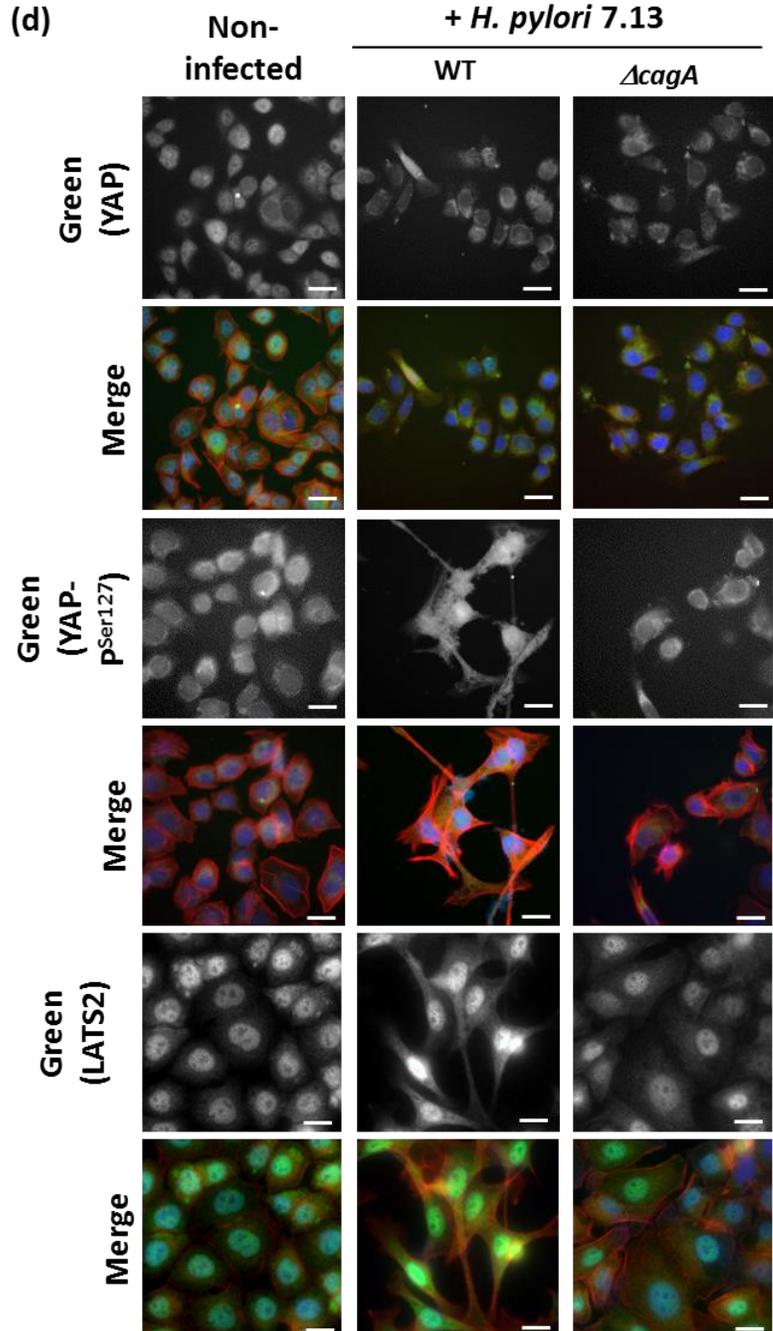
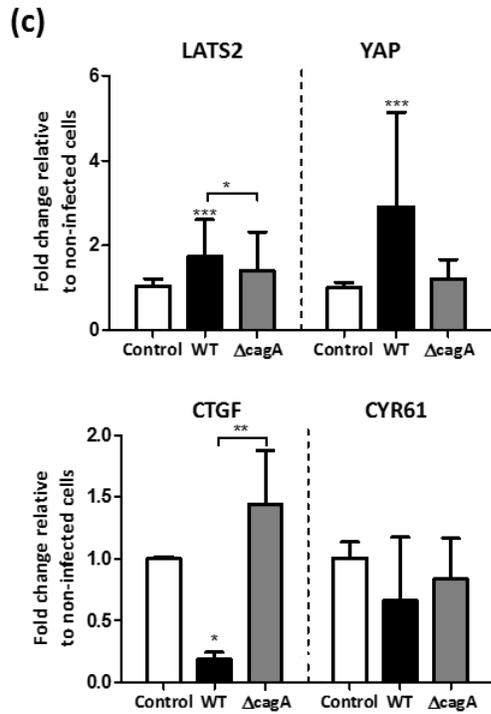
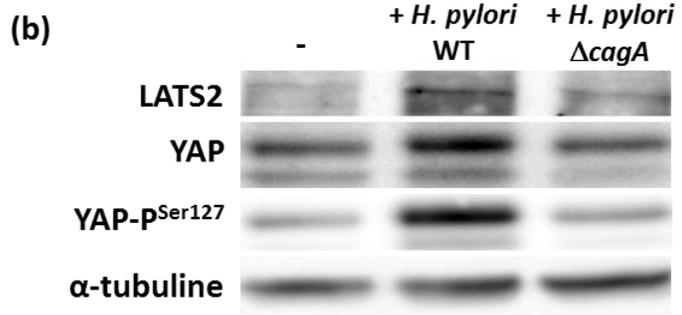
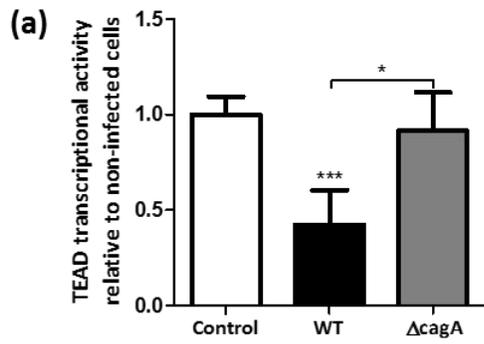


(b) MKN74

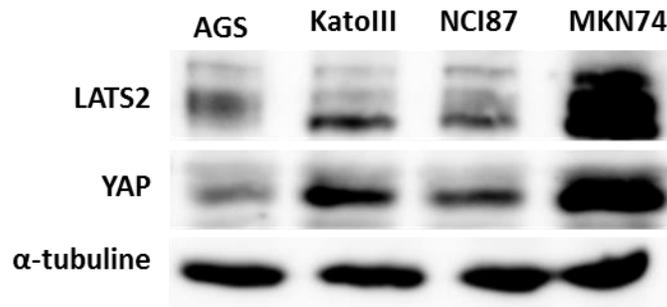


**Figure S1. Expression of the Hippo pathway genes upon *H. pylori* infection of cultured human gastric epithelial cells.** Microarray-based identification of differentially expressed Hippo pathway associated genes in response to *H. pylori* infection in AGS (a) and MKN74 (b) cells. Hippo kinase core genes (yellow bars), transcriptional co-factors YAP or TAZ (purple bars), YAP/TAZ/TEAD target genes CTGF and CYR61 (orange bars) were upregulated. Hippo pathway upstream effectors (blue bars), components of the cell polarity complexes (light green bars) and intercellular junctions (red bars), TEAD transcription-enhancer

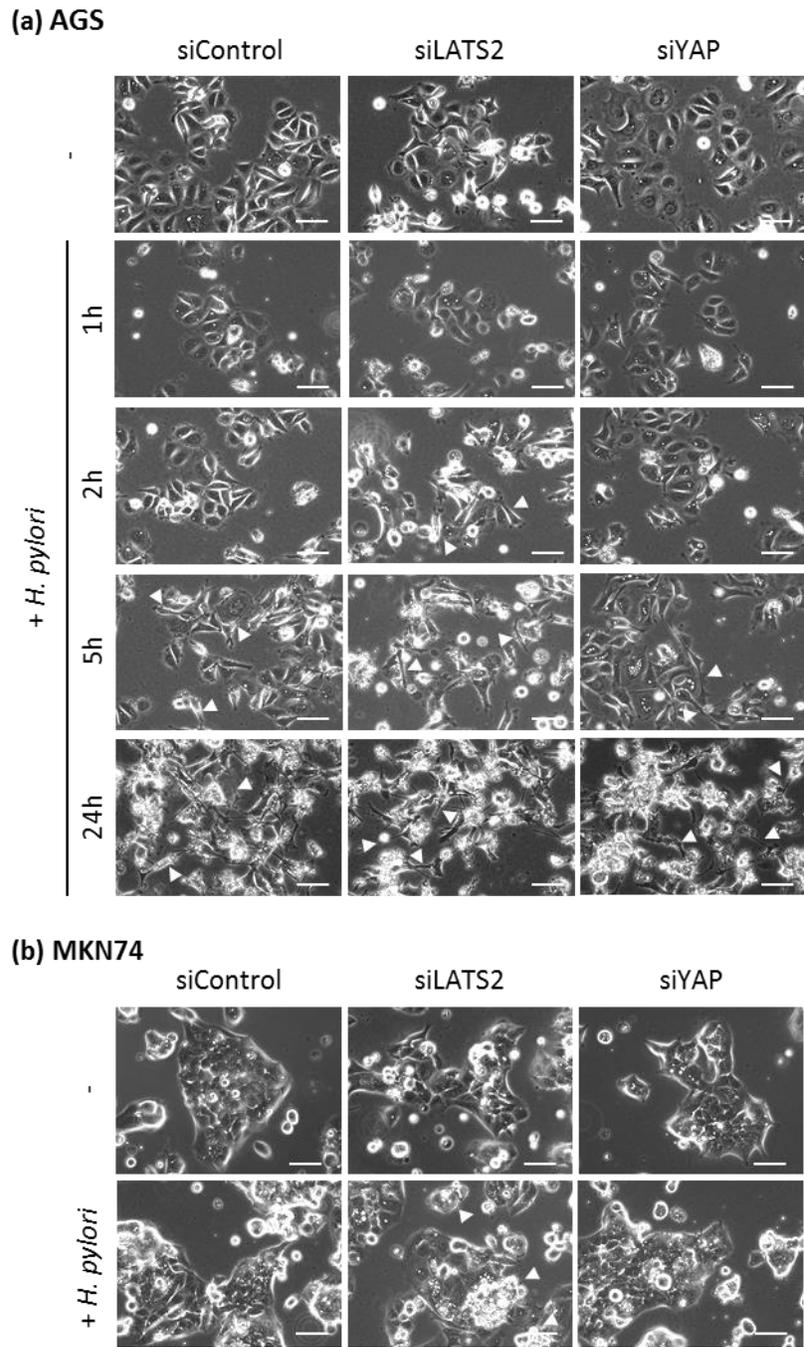
factors and other transcription factors (dark green bars), and other TEAD-target genes (orange bars) were variably affected. Only genes with statistically significant changes in expression are shown ( $p < 0.05$  in t-test with Benjamini-Hochberg correction).



**Figure S2. Helicobacter pylori regulates LATS2 and YAP in a CagA-dependent manner.** The infection of AGS cells with *cagA*-deficient *H. pylori* 7.13 isogenic mutant (grey bars) did not affect neither TEAD-Luciferase reporter activity 24h after infection (a) nor mRNA levels of YAP, LATS2, CTGF, and CYR61 (c), as they remained similar to non-infected cells (white bars) in contrast to cells infected with the wild-type strain (black bars). Protein level of LATS2, YAP and Ser-127 phosphorylation of YAP were measured by Western Blot with the same results (b). Expression and subcellular localization of LATS2, total YAP and YAP-PSer127 (marked in green) were evaluated by immunofluorescence (d). Actin was marked with phalloidin-Alexafluor-546 and nuclei were marked with DAPI. Scale bars, 10  $\mu$ m. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs non-infected cells in Kruskal-Wallis test.

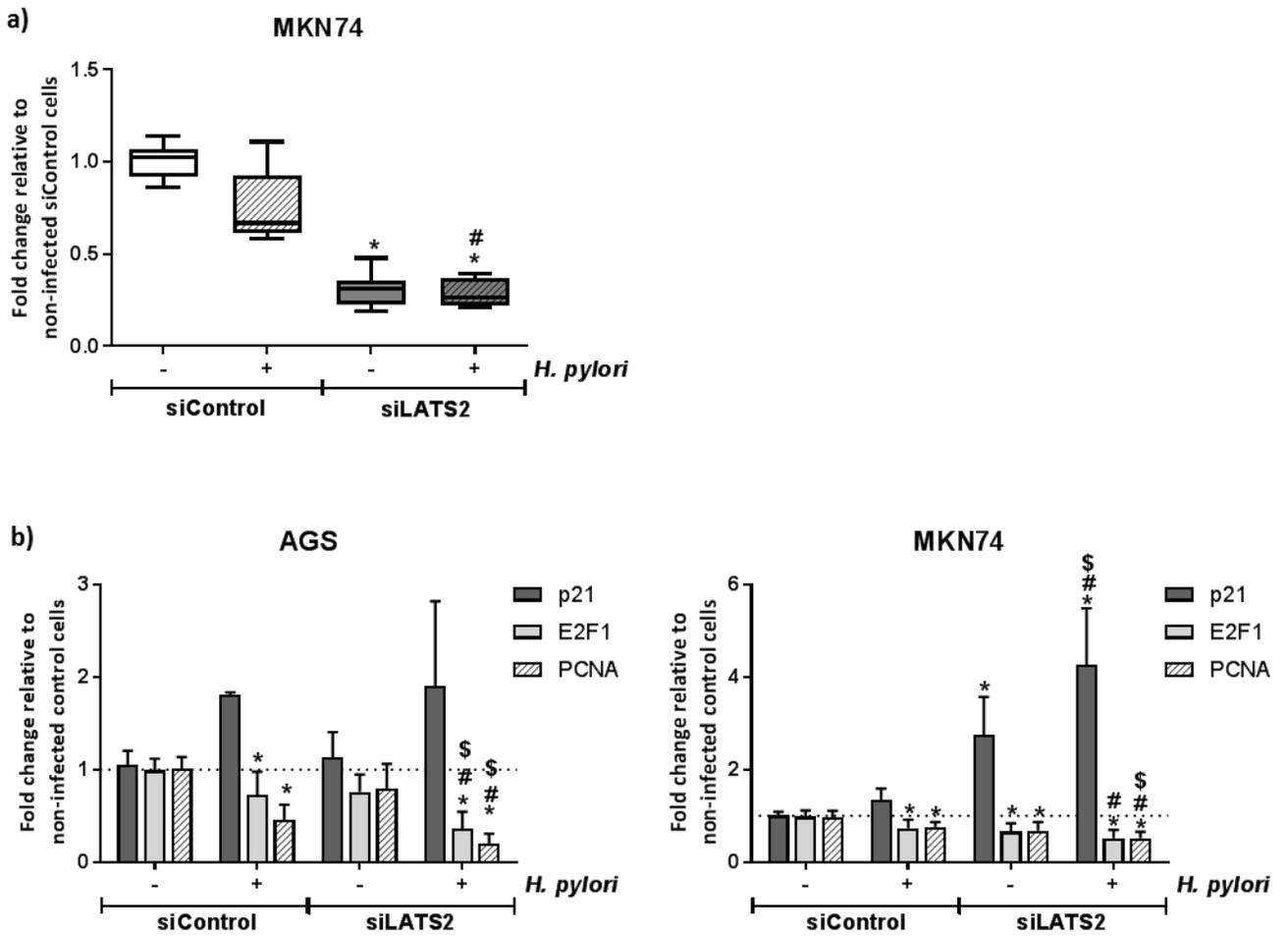


**Figure S3. Basal expression of LATS2 and YAP in four gastric epithelial cell lines.** Basal expression of LATS2 and YAP were evaluated by Western Blot in AGS, MKN74, NCI-87, and Kato III cell lines.



**Figure S4. Changes in cell phenotype upon *H. pylori*-infection of LATS2 and YAP knocked-down cells.**

Cellular phenotype changes upon *H. pylori* infection and LATS2 and YAP knock-down in were observed by phase contrast microscopy in (a) AGS and (b) MKN74 cells. Scale bars, 15  $\mu$ m. The acquisition of the mesenchymal “hummingbird” phenotype appeared from 2 hpi in siLATS2 cells in contrast to 5 hpi in siControl or siYAP AGS cells (a). In siLATS2-transfected MKN74 cells loosely attached round cell clusters appeared more frequently than in siControl or siYAP (b).



**Figure S5. Cell cycle-related gene expression upon *H. pylori*-infection of LATS2 knocked-down cells.** p21, E2F1, and PCNA mRNA levels were measured 24 hours after infection with *H. pylori* strain 7.13 in siControl-, and siLATS2-transfected AGS and MKN74 cells. \* $p < 0.05$ , vs non-infected siControl, # $p < 0.05$  vs *H. pylori*-infected siControl, \$ $p < 0.05$  vs non-infected siLATS2 in Kruskal-Wallis test.

**Supplementary Table S1.** Relative mRNA expression level of CD44 and Hippo/YAP related genes in the GC cell lines of the CCLE.

CCLE_CELL_LINE/mRNA level	CD44	CYR61	CTGF	LATS2	YAP1
23132/87	6,472997742	7,588963806	8,850265206	7,24624938	9,52513914
AGS	6,248519129	8,384566835	10,0945962	6,656237292	9,43200617
AZ-521	4,12080453	10,61293161	10,90798677	7,537679393	10,0798598
ECC10	5,127291819	4,294893044	7,415243006	4,753828751	6,82320264
ECC12	3,854176301	3,855889943	3,60320796	4,069451983	6,52323048
FU97	4,195111076	5,963697015	7,876144194	7,143295395	8,31416686
GCIY	7,693603267	10,04368659	8,553021165	6,847030856	8,53482354
GSS	3,872930175	5,976974878	5,827685793	7,128756857	10,0927972
GSU	8,716387899	4,954975098	6,227298676	5,247612629	9,63013371
HGC-27	6,660678918	6,875392969	4,685165162	6,006754235	9,1177632
Hs 746T	8,464912926	9,904875322	8,509118516	6,491887479	9,79991426
HuG1-N	9,834433737	6,186353331	6,555294932	6,256512013	9,21436073
IM95	7,075793505	6,145537984	8,43466728	6,112026219	8,7846035
KATO III	7,380399415	6,835190708	5,048130833	6,698631147	9,6133809
KE-39	5,102020418	7,977870163	3,36127356	6,648416484	10,8767428
LMSU	9,578416283	12,07342973	10,15922044	7,338217047	12,2339047
MKN1	8,949666419	9,724320551	9,25308584	7,174739945	9,74879977
MKN-45	9,2843709	7,448013894	5,345408214	6,122005606	3,90931299
MKN7	8,38636868	9,86978856	7,17421106	7,820866887	9,9672307
MKN74	4,20283475	10,71905398	7,075803084	8,577951269	10,549445
NCC-StC-K140	5,936691291	9,972517616	6,582006703	7,866842764	9,74387835
NCI-N87	6,640708404	7,15173809	6,455040415	6,695548385	9,09566842
NUGC-2	8,046017508	9,020160784	4,84344148	6,97606443	8,64516026
NUGC-3	8,49537633	11,06668343	8,204019344	7,241332202	9,27694425
NUGC-4	10,3432648	5,503154632	6,508748889	6,632272869	8,75121804
OCUM-1	5,390823493	5,583585253	5,170897261	5,913769618	10,3809619
RERF-GC-1B	4,782878747	8,601129513	6,97521937	6,729983474	9,82602535
SH-10-TC	8,818585195	10,94628079	7,459664095	7,288205157	9,17449632
SNU-1	4,14026619	10,62980281	9,645844772	7,970309796	9,24624799
SNU-16	10,9105867	5,513833581	7,0815203	4,738249773	8,40627028
SNU-216	7,476330286	10,8186569	8,163451313	7,130833429	9,63523776
SNU-5	6,944952906	6,39952233	7,184046146	6,72266539	9,58617946
SNU-520	8,581010297	5,983810744	4,280683131	6,113898253	9,37007607
SNU-601	6,898548932	4,292468395	8,121064233	6,898386894	9,7332712
SNU-620	7,750967742	4,491168811	6,43431847	4,244373347	9,33088594
SNU-668	8,878579591	10,00247739	4,472163645	7,683462564	8,33502848
SNU-719	9,152094383	5,673263055	7,775816255	5,99195843	10,0756885
TGBC11TKB	7,005714768	6,14846316	5,863155846	6,49790852	10,0137786

Correlation coefficient R	CD44	CYR61	CTGF	LATS2	YAP1	
CD44		1	0,047587663	0,006211469	-0,145694202	-0,06827919
CYR61			1	0,437166448	0,758708588	0,31952455
CTGF				1	0,353922975	0,27387874
LATS2					1	0,41107788
YAP1						1

Regression coefficient R <sup>2</sup>	CD44	CYR61	CTGF	LATS2	YAP1	
CD44		-	0,002264586	0,000038582	0,021226801	0,00466205
CYR61			-	0,191114503	0,575638721	0,10209594
CTGF				-	0,125261472	0,07500957
LATS2					-	0,16898502
YAP1						-

P value	CD44	CYR61	CTGF	LATS2	YAP1	
CD44		-	0,7766	0,9705	0,3828	0,6839
CYR61			-	0,0061	< 0,001	0,0505
CTGF				-	0,0293	0,0961
LATS2					-	0,0104
YAP1						-

**Supplementary Table S2.** Primers used in qPCR experiments.

Gene	Sequence (5'→3')	Reference
CD44	F : GGGCGTAACTCTGGAAGCAC R : TTAGCCCAGGTCACCTGAAAAT	[1]
LATS2	F : CAGGATGCGACCAGGAGATG R : CCGCACAATCTGCTCATTC	[2]
YAP1	F : CACAGCATGTTTCGAGCTCAT R : GATGCTGAGCTGTGGGTGTA	[3]
CTGF	F : GCCACAAGCTGTCCAGTCTAATCG R : TGCATTCTCCAGCCATCAAGAGAC	[4]
CYR61	F : ATGAATTGATTGCAGTTGGAAA R : TAAAGGGTTGTATAGGATGCGA	[5]
ZEB1	F : TCCCAACTTATGCCAGGCAC R : CAGGAACCACATTTGTCATAGTCAC	[1]
SNAI1	F : ACAATGTCTGAAAAGGGACTGTGA R : CAGACCAGAGCACCCCAT	[1]
E2F1	F : CAAGAAGTCCAAGAACCACATCC R : CTGCTGCTCGCTCTCCTG	[6]
P21	F : CCTCATCCCGTGTTCCTTT R : GTACCACCCAGCGGACAAGT	[7]
PCNA	F : AGGGCTCCATCCTCAAGAAGG R : TGGTGCTTCAAATACTAGCGC	[8]
HPRT1	F : TGGTCAGGCAGTATAATCCA R : GGTCTTTTCACCAGCAAGCT	[9]
TBP	F : GGGCATTATTTGTGCACTGAGA R : GCCCAGATAGCAGCACGGT	[9]

## Supplementary references

- [1] Bessede E, Staedel C, Acuna Amador LA, Nguyen PH, Chambonnier L, Hatakeyama M, et al. *Helicobacter pylori* generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene*. 2014 Aug 7;33(32):4123-31.
- [2] Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell*. 2006 Mar 24;124(6):1169-81.
- [3] Zhang W, Gao Y, Li P, Shi Z, Guo T, Li F, et al. VGLL4 functions as a new tumor suppressor in lung cancer by negatively regulating the YAP-TEAD transcriptional complex. *Cell research*. 2014 Mar;24(3):331-43.
- [4] Tao J, Calvisi DF, Ranganathan S, Cigliano A, Zhou L, Singh S, et al. Activation of beta-catenin and Yap1 in human hepatoblastoma and induction of hepatocarcinogenesis in mice. *Gastroenterology*. 2014 Sep;147(3):690-701.
- [5] Xie JJ, Xu LY, Wu JY, Shen ZY, Zhao Q, Du ZP, et al. Involvement of CYR61 and CTGF in the fascin-mediated proliferation and invasiveness of esophageal squamous cell carcinomas cells. *The American journal of pathology*. 2010 Feb;176(2):939-51.
- [6] Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. *Cancer research*. 2007 Sep 01;67(17):8293-300.
- [7] Moos PJ, Edes K, Mullally JE, Fitzpatrick FA. Curcumin impairs tumor suppressor p53 function in colon cancer cells. *Carcinogenesis*. 2004 Sep;25(9):1611-7.
- [8] Lin Z, Yang H, Tan C, Li J, Liu Z, Quan Q, et al. USP10 antagonizes c-Myc transcriptional activation through SIRT6 stabilization to suppress tumor formation. *Cell reports*. 2013 Dec 26;5(6):1639-49.
- [9] Varon C, Mocan I, Mihi B, Pere-Vedrenne C, Aboubacar A, Morate C, et al. *Helicobacter pullorum* cytolethal distending toxin targets vinculin and cortactin and triggers formation of lamellipodia in intestinal epithelial cells. *The Journal of infectious diseases*. 2014 Feb 15;209(4):588-99.

**b. Article 2: Deciphering and targeting YAP1/TAZ/TEAD activity in human gastric adenocarcinomas**

*Manuscript in preparation*



## Deciphering and targeting YAP/TAZ activity in human gastric adenocarcinomas

Silvia MOLINA-CASTRO<sup>1,2\*</sup>, Julie GIRAUD<sup>1\*</sup>, Julien IZOTTE<sup>1</sup>, Cathy STAEDEL<sup>3</sup>, Solène FERNANDEZ<sup>1</sup>, Pierre DUBUS<sup>1,4</sup>, Philippe LEHOURS<sup>1,4</sup>, Francis MEGRAUD<sup>1,4</sup>, Christine VARON<sup>1</sup>

<sup>1</sup> INSERM U1053 Bordeaux Research in Translational Oncology, University of Bordeaux, France

<sup>2</sup> INISA/School of Medicine, University of Costa Rica, San José, Costa Rica

<sup>3</sup> INSERM U1212 Régulations Naturelles et Artificielles des ARN, University of Bordeaux, France

<sup>4</sup> CHU de Bordeaux, France

\*These authors contributed equally to this work

### Abstract

Gastric cancer (GC) is the fifth most common cancer worldwide. Currently, cancer is considered a stem cell disease, which in the case of GC are identified with the marker CD44. The Hippo pathway is a conserved pathway that controls organ size by regulating the oncoproteins YAP and TAZ, and which has been linked to gastric cancer and cancer stem cells (CSC). YAP and TAZ function together with TEAD family transcription factors to promote cell survival and proliferation. Verteporfin is a small molecule with recently identified capacity of disrupting the YAP/TAZ/TEAD interaction. The aim of this work is to study the YAP/TAZ activity in gastric cancer cell lines and patient-derived primary tumor xenografts and evaluate the capacity of verteporfin to disrupt the YAP/TAZ/TEAD transcriptional activity, target CSC and inhibit tumor growth. Nuclear expression of YAP/TAZ is found in 10-30% of GC cells in primary tumors and frequently in the invasive CSC compartment. Verteporfin treatment decreased YAP/TEAD transcriptional activity in a dose dependent manner in GC cell lines and in patient-derived xenografts and decreased cellular proliferation, as well as the CSC pool assessed by the inhibition of tumorsphere formation and the diminished expression of CD44. *In vivo*, verteporfin decreased tumor growth in a murine model and inhibited the ability of residual cell to initiate tumorspheres. In conclusion, targeting YAP/TAZ/TEAD could be a promising strategy in the treatment of GC.

## i. Introduction

The Salvador-Warts-Hippo pathway is a highly evolutionarily conserved pathway, first described as a size-controlling pathway in *D. melanogaster*<sup>1</sup>. It is composed by a tumor suppressor module of two kinases: MST1/2 and LATS1/2, their regulatory proteins SAV1 and MOB1/2<sup>2</sup>, and their targets of phosphorylation, the oncoproteins YAP and TAZ. Upon phosphorylation by LATS1/2, YAP and TAZ are retained in the cytoplasm or undergo proteasomal degradation, hence limiting their nuclear activity. Active nuclear YAP and TAZ function together with TEAD family transcription factors to promote cell survival and proliferation<sup>2</sup>.

Stomach cancer is the fifth most common cancer overall and the third leading cause of cancer death in both sexes worldwide<sup>3</sup>. The diagnosis of the disease is often at advanced stages, the therapeutic options are limited, and the 5-year survival is poor<sup>4,5</sup>. Gastric cancer is a multifactorial disease, frequently associated to the chronic infection with *H. pylori*<sup>4</sup>. Our group has shown that *H. pylori* induces an activation of the Hippo signaling pathway and the Hippo pathway plays a role in the emergence of a CD44<sup>+</sup> cell population with CSC-properties induced upon infection. An increasing body of evidence links the Hippo pathway to gastric cancer. The expression of MST1/2 and LATS1 was decreased in tissue sections from gastric cancer compared to those of normal gastric mucosa<sup>6</sup>, and several studies have revealed an upregulation of YAP mRNA and protein in gastric adenocarcinomas, as well as a correlation of YAP expression and nuclear localization with a bad outcome<sup>7</sup>. Jiao *et al*<sup>8</sup> found an association of YAP mRNA levels with tumor size, differentiation, pathologic staging, and with *H. pylori* status, as well as higher YAP protein positivity rates in gastric cancer and dysplastic lesions compared to normal tissue. Promoter-hypomethylation and over expression of TEAD4 have been described in gastric cancer tissues, and its knock down impaired cell growth both *in vivo* and *in vitro*<sup>9</sup>. Altogether, these studies point out the role of the Hippo signaling in gastric carcinogenesis.

It has been shown that knock-down of TEADs or disruption of the YAP-TEAD interaction decreased transcription of YAP target genes and oncogenic transformation<sup>8</sup>. Therefore, the ways to disrupt YAP/TAZ-TEAD interaction have been studied as potential strategies for cancer treatment. Verteporfin (VP, C<sub>41</sub>H<sub>42</sub>N<sub>4</sub>O<sub>8</sub>, molecular weight: 718.8 Da), is a benzoporphyrin derivative initially used as photosensitizer for photodynamic therapy (PDT) in ophthalmology<sup>10</sup> and tested in anti-cancer therapy<sup>11,12</sup>. Light-independent activity of VP have also been reported. Liu-Chittenden *et al* identified verteporfin as an inhibitor of YAP/TEAD, with anti-oncogenic properties *in vivo* in liver cancer murine models<sup>13</sup>. VP inhibitory effects on CSC properties and enhancement of chemotherapy effectivity have been studied in esophageal cancer cells *in vitro* and *in vivo*<sup>14,15</sup>, and in a model colorectal carcinogenesis without affecting the normal

intestinal homeostasis or displaying any apparent toxicity<sup>16</sup>. Until now, the anti-oncogenic properties of VP have not been described in gastric cancer.

The aim of this work is to study the YAP/TAZ activity in gastric cancer cell lines and patient-derived primary tumor xenografts and evaluate the capacity of verteporfin to disrupt the YAP/TAZ/TEAD transcriptional activity, target CSC and inhibit tumor growth.

## ii. Results

### 1. Hippo pathway proteins are heterogeneously expressed in gastric tumors

Immunohistochemistry staining was performed in tissue samples of two poorly differentiated and four moderately differentiated gastric adenocarcinomas (Figure 1). YAP expression was mainly nuclear and the percentage of positive cells varied from 10 to 30% amongst the tumors. The YAP positive cells were often located in the invasive front of the tumors, where CSC have been described to reside.

Expression of YAP, TAZ and LATS2 mRNAs (Suppl figure 1a) and proteins (Suppl figure 1b) were evaluated in gastric cancer cell lines, healthy stomach tissue, and gastric cancer samples. YAP was detected in all samples excepting MKN45 cells, in variable levels. TAZ expression was also heterogenous but much lower than that of YAP, MKN45 cells presented the highest levels and it was absent from MKN74 and the healthy tissues. Regarding LATS2, the expression was heterogenous in the gastric cancer samples, decreased in the cell lines and higher in the healthy tissues, remarkably in the antrum.

### 2. Verteporfin inhibited cell proliferation, and YAP/TAZ/TEAD transcriptional activity in gastric cancer cell lines *in vitro*

MKN45 and MKN74 cell lines were treated with increasing concentrations of verteporfin and DMSO as control vehicle. In both cell lines, a significant decrease in cell growth was observed from 1  $\mu$ M in a dose dependent manner (Figure 2a), without an evident alteration of cell morphology (Figure 2b). The inhibition of cell proliferation occurred along a cell cycle arrest, with a significant accumulation of cells in G0/G1 phase, and decrease of cells in phase S at the 5  $\mu$ M concentration (Figure 2c-d). YAP/TAZ/TEAD transcriptional activity was studied with a TEAD-luciferase reporter and the mRNA levels of the YAP/TAZ/TEAD target genes CTGF, CYR61, IGFBP3, JAGGED1, and LATS2 (Figure 2e-f). TEAD-luciferase activity was significantly decreased from the 0.1  $\mu$ M dose of verteporfin in MKN45 cells, which also showed a decreased expression of all the studied YAP/TAZ/TEAD target genes in a dose-dependent manner from 0.1  $\mu$ M. In MKN74, a dose-dependent, decreasing trend of TEAD-luciferase activity from 0.1  $\mu$ M, but significant only at the 5  $\mu$ M concentration of verteporfin. The case was the same for the expression of

LATS2 in these cells, while the other target genes showed a significantly decreased expression from the 1  $\mu$ M concentration. MKN45 cells do not express YAP but they express TAZ and MKN74 cells express mainly YAP. The expressions of TAZ in MKN45 and YAP in MKN74 were decreased in the condition treated with the 5  $\mu$ M dose (Figure 2f).

### **3. Verteporfin treatment targeted CSC-properties in vitro**

In order to evaluate the CSC-targeting properties of verteporfin *in vitro*, we cultured gastric cancer cell lines and PDXs in non-adherent, nutrient defined conditions to promote tumorsphere formation and enrich the culture in CSCs and treated them with increasing doses of verteporfin. For the preventive treatment (Figure 3a), i.e. cells seeded in presence of verteporfin, a significant inhibitory effect in the formation of tumorspheres was observed in MKN45, GC04 and GC10 from the lowest concentration in comparison to the DMSO-treated control cells. The treatment was effective from 1  $\mu$ M in the MKN74 and GC07, and no effect was observed for the GC06. The action of verteporfin was observed both in the total tumorsphere count (Figure 3a) and size (illustrated in Figure 3b). We also tested the curative effect of verteporfin, i.e. the treatment was added to well established tumorspheres (Figure 3c). Significant reduction on the tumorsphere counts were observed from concentration 0.1  $\mu$ M in MKN74, GC04, and GC07, while a 1  $\mu$ M concentration was required to observe the inhibitory effects in MKN45, GC10, GC35, and remarkably, GC06.

CD44 CSC-marker expression upon verteporfin treatment was studied by RT qPCR in MKN45, MKN74, GC04, GC06, and GC07 (Figure 4a). CD44 expression was significantly decreased in a dose dependent manner in MKN74 and GC04 from the lowest concentration, and for MKN45, GC06, and GC07, a concentration of 1  $\mu$ M was required to observe the desired effect. The immunocytochemistry evaluation of CD44 in spheres from MKN45 confirmed the decrease on CD44 expression upon treatment with verteporfin 1  $\mu$ M (Figure 4b).

### **4. Verteporfin treatment inhibits tumor growth in a murine xenograft model**

To evaluate the antitumor activity of verteporfin, xenograft experiments were performed, where MKN45, MKN74, and GC10 cells were subcutaneously injected into the dorsal flank of immunodeficient NSG mice and tumor growth was determined by measuring tumor growth every other day. Once the tumor had reached a volume of 100 mm<sup>3</sup>, daily peritumoral treatment with a dose of 2 mg/kilogram of verteporfin and DMSO as a carrier vehicle for the control group was performed. For the three cell types, tumor growth slowed down in the group treated with verteporfin (Figure 5a). The strongest and fastest effect was observed in MKN45, where significant differences appeared as early as 6 days after the

beginning of treatment. For MKN74 and GC10, the treatment took 25-30 days to cause an observable decrease in the tumor growth rate.

When the tumors in the control group reached a volume of 500 mm<sup>3</sup>, treatment was stopped, mice were sacrificed and residual tumors were extracted (Figure 5b), weighed and processed for cellular dissociation and histology. A significant decrease in tumor weight was observed between controls and verteporfin-treated tumors in MKN45 and GC10, but not for MKN74 (Figure 5c). Noteworthy, an important heterogeneity was observed amongst the different tumors of the same group, in some cases pointing out a problem of distribution of the drug. Cells dissociated from the remaining treated tumors significantly initiated less tumorspheres *in vitro* in the verteporfin treated group compared with the control in MKN45 and MKN74, confirming that verteporfin treatment led to a decrease in the number of CSC *in vivo* (Figure 5d). That was not the case for GC10 where the number of tumorspheres was already low in the control condition.

The histological analysis of the residual tumors in MKN45 cells showed that after the verteporfin treatment, the tumor mass was decreased to smaller foci of cells in fibrotic tissue, compared to a more uniform mass observed in the control group. Regarding the expression of CSC markers, a slight decrease in the intensity of the CD44 staining was observed upon treatment, as well as in the percentage of cells strongly expressing ALDH staining. Proliferating cells expressing DNA replication marker Ki67 were also strongly reduced. These results support the *in vitro* results showing that verteporfin treatment targets CSCs, reducing proliferation and survival of tumor cells, reflected in an arrest of tumor growth.

### iii. Discussion

The Hippo signaling pathway controls several processes related to tumorigenesis, which define the features of cancer cells such as cell proliferation and survival. Deregulation of the pathway occurs at multiple levels. LATS2 can activate p53 and its pro-apoptotic activity can be antagonized by YAP<sup>17,18</sup> and silencing of LATS2 expression enables tumor development and progression<sup>19</sup>. YAP/TAZ transcriptional activity stimulates aberrant cell proliferation also by counteracting tumor suppressor mechanisms such as senescence<sup>20</sup>. YAP overexpression can promote cell survival by blocking TNF- and FAS-induced apoptosis in the mouse liver<sup>20</sup> and a TEAD overexpression has been involved in carcinogenesis and described in various types of cancer<sup>21</sup>. CTGF and CYR61, the most important YAP/TAZ/TEAD target genes, are regulatory ECM-associated proteins involved in growth promotion, cell migration, proliferation, cell lineage commitment and tissue specification, and participate in embryonic development, tissue turnover, and injury repair<sup>22</sup>. Their overexpression has been associated with inflammatory conditions, such as metabolic syndrome, colitis, rheumatoid arthritis, wound healing and fibroproliferative disorders, being correlated to excessive accumulation of ECM proteins in fibrotic tissues, and remarkably, with cancer<sup>22</sup>. In

hepatocellular carcinoma, CTGF was over expressed and responsible of increased cell growth and dedifferentiation, expression of inflammation- and carcinogenesis-related genes, and resistance to doxorubicin<sup>23</sup>.

The Hippo pathway can also control stem cell properties, including replicative potential, dedifferentiation, and drug resistance, and its deregulation has been implied in cell transformation and tumor initiation, especially in the acquisition of mesenchymal properties at the origin of CSCs<sup>24</sup>. Increased YAP/TEAD expression and activity has been described in various types of stem cells and stem cell compartments of different tissues, and drives the expansion of progenitor cells in the gastrointestinal tract<sup>20</sup>. TAZ is essential to metastatic and stem cells properties in breast cancer cell models, where it is required to sustain CSC-properties and confers self-renewal capacity<sup>24</sup>. Epidemiological and experimental data supports the role of the Hippo/YAP/TAZ signaling pathway in different types of cancer, both of epithelial and non-epithelial origin, which are thoroughly reviewed by Zanconato *et al*<sup>7</sup>. In addition to the studies by Xu *et al*<sup>6</sup> and Jiao *et al*<sup>8</sup>, our group recently showed that YAP1 is upregulated and activated in the gastric epithelium upon chronic infection with *H. pylori*, a major risk factor for gastric cancer, and appears at all stages of the gastric carcinogenesis cascade, co-localized with the CSC and EMT marker CD44 in the gastric glands (unpublished results). Altogether, these studies point out the role of the Hippo signaling in gastric carcinogenesis.

Given the importance of the role of YAP/TAZ/TEAD signaling in gastric carcinogenesis and CSC properties, this study evaluated the anti-tumor properties of verteporfin, showing that treatment with verteporfin decreased cell growth, YAP/TEAD transcriptional activity, and tumorsphere formation *in vitro* and tumor growth and CSC-properties and markers *in vivo*. These results show the CSC-inhibitory activity of verteporfin both *in vitro* and *in vivo*. We corroborated the results obtained by various groups in models of liver, colorectal, and esophageal carcinogenesis<sup>13-16</sup>, and to our knowledge, this is the first description of the anti-tumor activity of verteporfin in gastric cancer. Other mechanisms of tumor growth inhibition have been described for verteporfin<sup>26,27</sup>, which could also have a role in the effect found in our model. However, even if they can contribute to the effect, our results confirm a role for the YAP/TEAD disrupting action of verteporfin, reflected in the decrease of YAP/TEAD target genes expression.

Verteporfin has a selectivity for cancerous cells with high YAP/TEAD activity and it is an FDA-approved molecule for the routine treatment of other pathologies, making it suitable for a potential clinical application. Despite the observed significant action of verteporfin treatment in tumor reduction, the insolubility of the molecule limits its bioavailability and the possibility of reaching the adequate concentration in the tumor when administered in a systemic way, unless using very high quantities of the product which could carry systemic toxicity and increase the financial burden of the treatment. As an alternative, we used a peritumoral injection, which proved to fail in the homogeneous distribution of

verteporfin around the tumor and in some cases, caused the tumor to escape the treatment, invading towards the opposite side. However, the positive results obtained despite the flaws of the model, open the possibility of exploring new alternatives for verteporfin formulation that make it more suitable for implementing the treatment in patients. Targeting the YAP/TAZ/TEAD transcriptional activity was shown to be efficient in preventing tumor growth in a PDX model, therefore highlighting the potential of its inhibition to be implemented in gastric cancer therapy.

#### iv. **Materials and Methods**

**Human gastric tissues.** Gastric tissue samples (tumors and non-cancerous mucosa) from consenting patients (both sexes, 68-85 years old) undergoing gastrectomy for distant non-cardia adenocarcinoma were included in the study, in agreement with the tumor bank of the Bordeaux University Hospital (France), as previously reported<sup>28,29</sup>.

**Ethic statements on human samples and animal experiments.** Studies on fresh tumors from patient-derived gastric adenocarcinoma were performed in agreement with the Direction for Clinical Research and the Tumor and Cell Bank of the University Hospital Center of Bordeaux (Haut-Leveque Hospital) and of the Bergonié Institute of Bordeaux. Animal experiments with non-obese diabetous/severe combined immunodeficiency /interleukin-2R $\gamma$  null (NSG) immunodeficient mice were performed in level 2 animal facilities of the University of Bordeaux, with the agreement of the local Ethic Committee on Animal Experiments CEEA50 of Bordeaux (agreement number 50120151-A) and the French Committee of Genetic Engineering (approval number 4608). Activities of conservation and preparation of elements of human origin are declared at the French Ministry of Research (DC-2008-412).

**Histology and immunohistochemistry staining.** Tissue sections 3  $\mu$ m thickness were prepared from formalin-fixed paraffin-embedded tissues (PET) and submitted to standard Hematoxylin/Eosin/Safran (HES) staining (all from Sigma) and immunohistochemistry protocols<sup>28,29</sup>. Primary mouse monoclonal anti-human CD44 antibodies 1:100 (G44-26, BD) were incubated for 30 min at room temperature, followed by 30 min incubation at room temperature with anti-mouse Labelled Polymer-HRP DAKO Envision Systems (DAKO). Immunolabeling was revealed by 10 min incubation in liquid substrate-diaminobenzidine-chromogen (DAKO). Slides were counterstained with hematoxylin, dehydrated and mounted with Eukitt-mounting medium (Labonord, Templemars, France). Images of the staining were recorded on tissue sections using light microscopy and a x20 objective.

**Gastric epithelial cell lines culture and patient-derived tumor xenografts.** MKN45 and MKN74 (HSRR Bank JCRB0255 and JCRB0255) cell lines authenticated by STR profiling and mycoplasma free (verified by PCR), were cultured in RPMI1640-Glutamax media supplemented with 10% heat-inactivated fetal calf serum (all from Invitrogen), at 37°C in a 5% CO<sub>2</sub> humidified atmosphere<sup>30</sup>. GC04, GC06, GC07, GC10, and

GC35 are patient-derived GC, which were successfully established by serial transplantation in NSG mice as previously described<sup>30</sup>. 24 hour cultures of gastric cancer cell lines were treated with verteporfin (SML0534, Sigma) diluted in DMSO (D2650, Sigma) or DMSO as control for 48 hours and then harvested for cell proliferation count, cell cycle and luciferase reports assays, and RNA extraction.

**Cell cycle analysis by flow cytometry.** Treated cells were dissociated by trypsination and 100 000 cells per condition were stained with propidium iodide (P4864, Sigma) as describe elsewhere. Flow cytometry was performed using a BD FACSCanto II instrument and DIVA software (BD).

**Transfection and luciferase-reporter assay.** Cells were grown in 24 well plates and transfection of plasmids were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) as recommended. The 8xGTII-Luciferase reporter (100 ng/well) was a gift from Stefano Piccolo<sup>31</sup>, the TAL-luciferase reporter (100 ng/well) was from BectonDickinson, and pRL-SV40 (10 ng/well) was from Promega. Firefly and Renilla luciferase activities were measured using the Dual Luciferase Assay (Promega). Firefly luciferase activities were normalized for transfection efficiency by Renilla luciferase.

**RNA extraction and RT-qPCR.** Cell RNAs were extracted using Trizol reagent (Invitrogen) and quantified by their absorbance at 260 nm. For RT-qPCR, retrotranscription was performed with 1 µg total RNA using the Quantitect Reverse Transcription kit (Qiagen). Quantitative-PCR was performed using the SYBR-qPCR-Premix Ex-Taq (TAKARA), 0.3 µM specific primers (Table S1) and 1:200 of the RT reaction volume. Relative expressions were calculated using the comparative Ct method with HRPT1 and TBP as normalizers.

**Western blotting.** Cells were lyzed on ice in ProteoJET™ reagent (Fermentas) supplemented with protease/phosphatase inhibitor cocktail (Sigma). The extracted proteins were submitted to SDS-PAGE and western blotting for immunolabeling. Immunolabelling was performed using rabbit anti-YAP or mouse anti-TAZ (each at 1:2000; Cell Signaling), rabbit anti-LATS2 (1:2000; Bethyl), mouse anti-α-tubulin (1:8000; Sigma-Aldrich), Horseradish Peroxydase-coupled anti-rabbit and anti-mouse secondary antibodies (DAKO) and chemoluminescent detection (ECL+,Amersham).

**Tumorsphere culture.** 200 cells were seeded in non-adherent 96-well culture plates (previously coated with a 10% poly-2-hydroxyethyl methacrylate (polyHEMA, Sigma) solution in 95% (v/v) ethanol and dried overnight at 56 °C in serum-free GlutaMAX-DMEM/F12 medium supplemented with 20 ng/ml of epidermal growth factor, 20 ng/ml of basic- fibroblast growth factor, 0.3% glucose, 5 µg/ml of insulin, 50 IU/ml of penicillin and 50 µg/ml of streptomycin (from Invitrogen and Sigma) and cultured at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. For preventive treatment, verteporfin or DMSO were added to this medium in the desired concentrations before seeding the cells and spheres were quantified 7 days after seeding. For the curative treatment, tumorspheres were grown and then treatment was added, and tumorspheres were counted 48 hours after treatment. Tumorsphere counts were performed using a Nikon TE2000 inverted light microscope using a × 10 objective (Nikon, Champigny sur Marne, France).

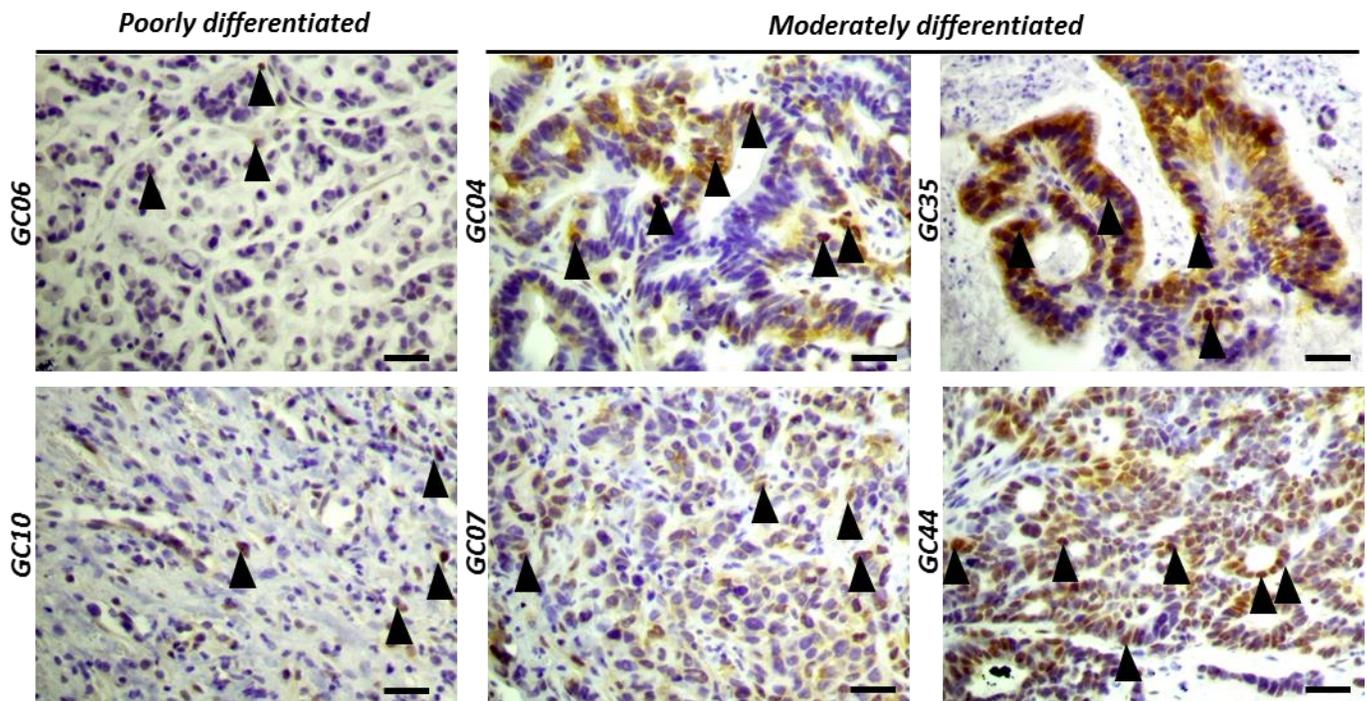
**In vivo xenograft experiments.** In all, 100 000 cells from MKN45 and MKN74 cell lines, and GC10 were suspended in 100  $\mu$ l of 7 mg/ml ice-cold Matrigel (BD Biosciences) and subcutaneously injected into the dorsal flank of randomly distributed 7-week-old NSG female mice. Tumors were measured blindly twice a week using a caliper and tumor volumes were calculated<sup>30</sup>. When tumor volume reached 100 mm<sup>3</sup>, 50  $\mu$ l of DMSO (control group) or verteporfin solution per mouse was subcutaneously injected at the tumor periphery daily for 15-25 days, after which mice were sacrificed (when tumors reached 500 mm<sup>3</sup>). Tumors were collected, fixed in a 3.7% buffered-formaldehyde solution and embedded in paraffin following standard procedures as described<sup>30</sup>.

**Statistical analysis.** Quantification values represent the means of three or more independent experiments, each performed by triplicate or more  $\pm$  standard deviation, except for the tumorsphere formation experiments where three to five independent experiments with n=10 each were carried-on. Mann-Whitney test was used to compare between two groups and Kruskal-Wallis test with Dunn's post-test was used for multiple comparisons. Statistics were performed on GraphPadPrism 7.02 (USA).

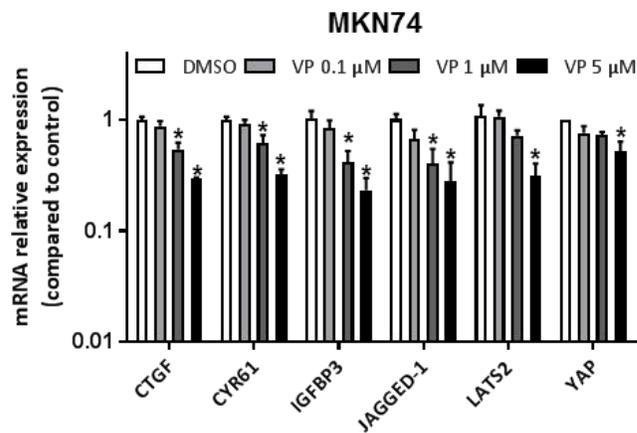
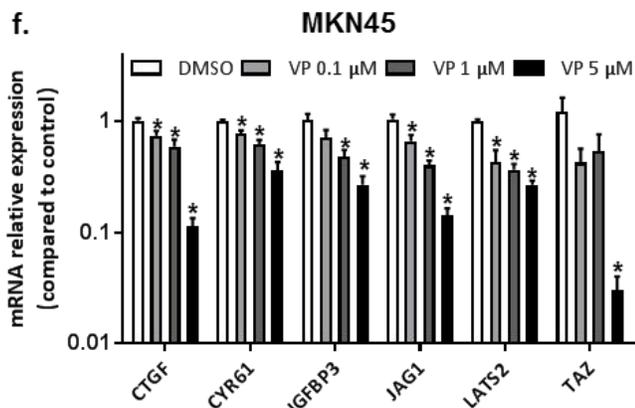
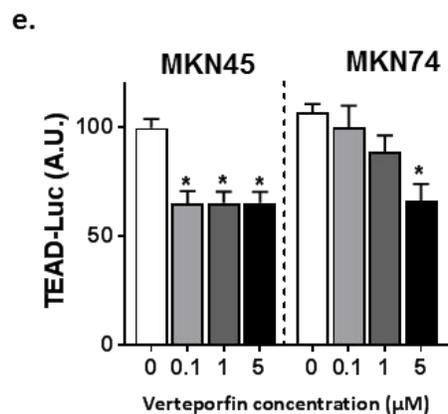
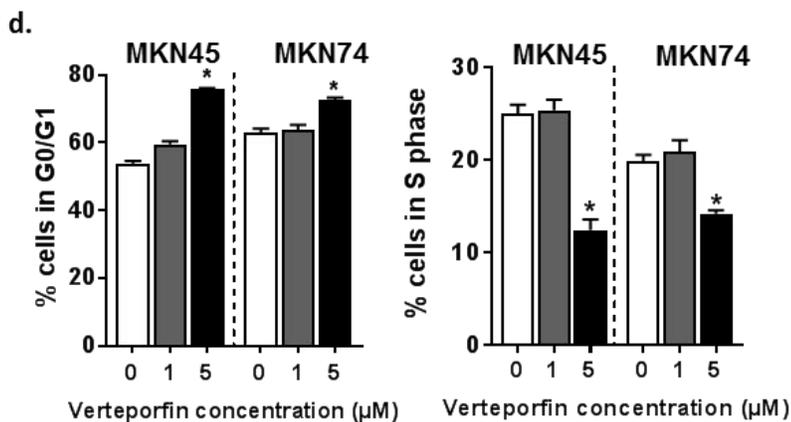
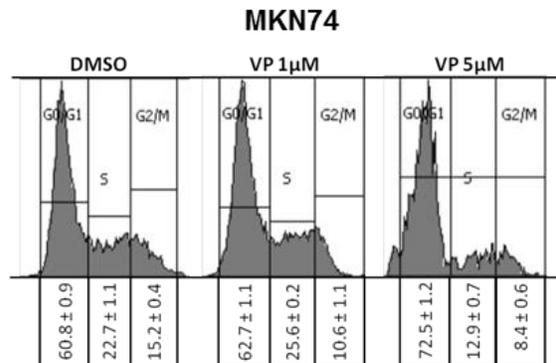
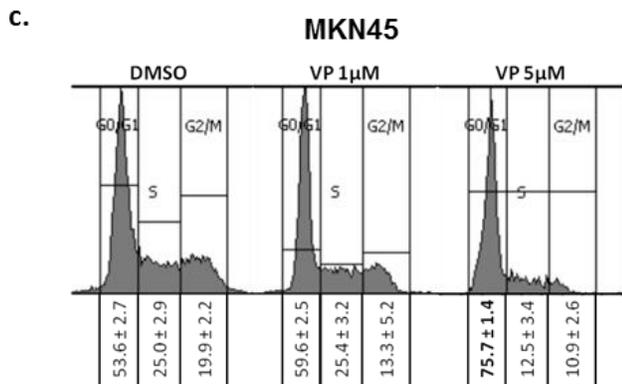
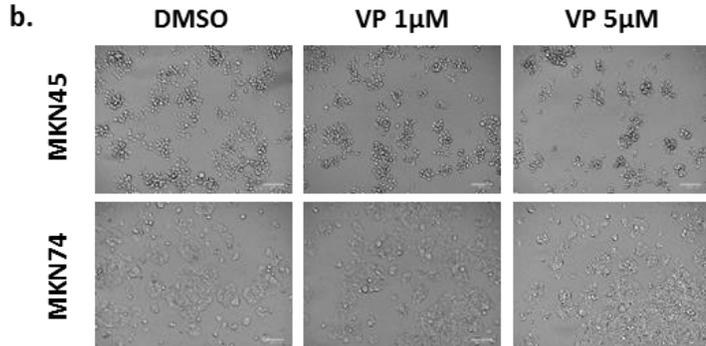
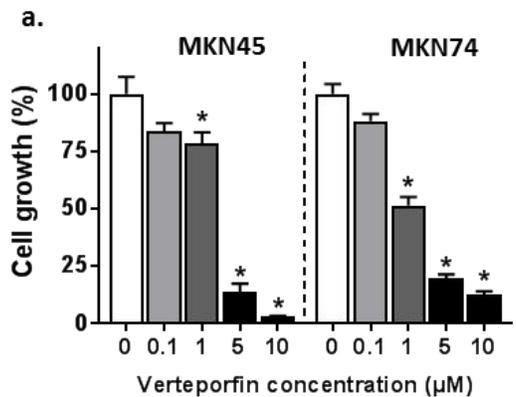
## References

1. Harvey K, Tapon N. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer*. 2007;7(3):182-191.
2. Harvey KF, Hariharan IK. The hippo pathway. *Cold Spring Harb Perspect Biol*. 2012;4(8):a011288.
3. Cancer Today. International Agency for Research on Cancer; 2016. <http://gco.iarc.fr/today>. Accessed 01/31/2017.
4. Rugge M, Fassan M, Graham DY. Epidemiology of Gastric Cancer. In: Strong VE, ed. *Gastric Cancer. Principles and practice*. Switzerland: Springer; 2015:23-34.
5. Keshava HB, Rosen JE, DeLuzio MR, Kim AW, Detterbeck FC, Boffa DJ. "What if I do nothing?" The natural history of operable cancer of the alimentary tract. *Eur J Surg Oncol*. 2017.
6. Xu ZP, Zhu JS, Zhang Q, Wang XY. A breakdown of the Hippo pathway in gastric cancer. *Hepatogastroenterology*. 2011;58(110-111):1611-1617.
7. Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. *Cancer Cell*. 2016;29(6):783-803.
8. Jiao S, Wang H, Shi Z, et al. A peptide mimicking VGLL4 function acts as a YAP antagonist therapy against gastric cancer. *Cancer Cell*. 2014;25(2):166-180.
9. Lim B, Park JL, Kim HJ, et al. Integrative genomics analysis reveals the multilevel dysregulation and oncogenic characteristics of TEAD4 in gastric cancer. *Carcinogenesis*. 2014;35(5):1020-1027.
10. Ziemssen F, Heimann H. Evaluation of verteporfin pharmacokinetics--redefining the need of photosensitizers in ophthalmology. *Expert Opin Drug Metab Toxicol*. 2012;8(8):1023-1041.
11. Akens MK, Hardisty MR, Wilson BC, et al. Defining the therapeutic window of vertebral photodynamic therapy in a murine pre-clinical model of breast cancer metastasis using the photosensitizer BPD-MA (Verteporfin). *Breast Cancer Res Treat*. 2010;119(2):325-333.
12. Huggett MT, Jermyn M, Gillams A, et al. Phase I/II study of verteporfin photodynamic therapy in locally advanced pancreatic cancer. *Br J Cancer*. 2014;110(7):1698-1704.
13. Liu-Chittenden Y, Huang B, Shim JS, et al. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev*. 2012;26(12):1300-1305.
14. Song S, Ajani JA, Honjo S, et al. Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer Res*. 2014;74(15):4170-4182.

15. Song S, Honjo S, Jin J, et al. The Hippo Coactivator YAP1 Mediates EGFR Overexpression and Confers Chemoresistance in Esophageal Cancer. *Clin Cancer Res.* 2015;21(11):2580-2590.
16. Zhang H, Ramakrishnan SK, Triner D, et al. Tumor-selective proteotoxicity of verteporfin inhibits colon cancer progression independently of YAP1. *Sci Signal.* 2015;8(397):ra98.
17. Aylon Y, Ofir-Rosenfeld Y, Yabuta N, et al. The Lats2 tumor suppressor augments p53-mediated apoptosis by promoting the nuclear proapoptotic function of ASPP1. *Genes Dev.* 2010;24(21):2420-2429.
18. Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes Dev.* 2006;20(19):2687-2700.
19. Aylon Y, Yabuta N, Besserglick H, et al. Silencing of the Lats2 tumor suppressor overrides a p53-dependent oncogenic stress checkpoint and enables mutant H-Ras-driven cell transformation. *Oncogene.* 2009;28(50):4469-4479.
20. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. *Nat Rev Cancer.* 2013;13(4):246-257.
21. Zhou Y, Huang T, Cheng AS, Yu J, Kang W, To KF. The TEAD Family and Its Oncogenic Role in Promoting Tumorigenesis. *Int J Mol Sci.* 2016;17(1).
22. Krupska I, Bruford EA, Chaqour B. Eyeing the Cyr61/CTGF/NOV (CCN) group of genes in development and diseases: highlights of their structural likenesses and functional dissimilarities. *Hum Genomics.* 2015;9:24.
23. Urtasun R, Latasa MU, Demartis MI, et al. Connective tissue growth factor autocriny in human hepatocellular carcinoma: oncogenic role and regulation by epidermal growth factor receptor/yes-associated protein-mediated activation. *Hepatology.* 2011;54(6):2149-2158.
24. Hiemer SE, Varelas X. Stem cell regulation by the Hippo pathway. *Biochim Biophys Acta.* 2013;1830(2):2323-2334.
25. Li N, Yu N, Wang J, et al. miR-222/VGLL4/YAP-TEAD1 regulatory loop promotes proliferation and invasion of gastric cancer cells. *Am J Cancer Res.* 2015;5(3):1158-1168.
26. Leung JY, Wilson HL, Voltzke KJ, et al. Sav1 Loss Induces Senescence and Stat3 Activation Coinciding with Tubulointerstitial Fibrosis. *Mol Cell Biol.* 2017.
27. Konstantinou EK, Notomi S, Kosmidou C, et al. Verteporfin-induced formation of protein cross-linked oligomers and high molecular weight complexes is mediated by light and leads to cell toxicity. *Sci Rep.* 2017;7:46581.
28. Bessède E, Staedel C, Acuña Amador LA, et al. Helicobacter pylori generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene.* 2014;33(32):4123-4131.
29. Nguyen PH, Giraud J, Chambonnier L, et al. Characterization of Biomarkers of Tumorigenic and Chemoresistant Cancer Stem Cells in Human Gastric Carcinoma. *Clin Cancer Res.* 2016.
30. Nguyen PH, Giraud J, Staedel C, et al. All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. *Oncogene.* 2016;35(43):5619-5628.
31. Dupont S, Morsut L, Aragona M, et al. Role of YAP/TAZ in mechanotransduction. *Nature.* 2011;474(7350):179-183.

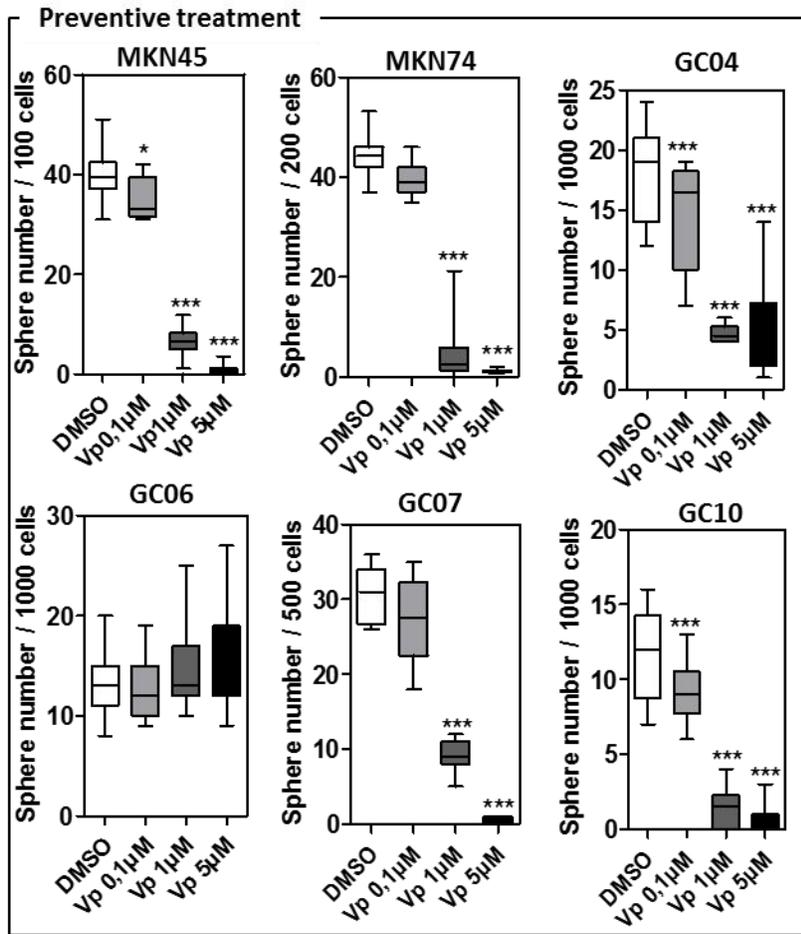


**Figure 1. YAP expression in gastric adenocarcinomas.** Immunohistochemistry staining for YAP was performed in tissue samples of gastric adenocarcinomas. YAP was mainly nuclear in 10-30% of positive cells (black arrows), and located in the invasive front of the tumors. Bars 20  $\mu$ m.

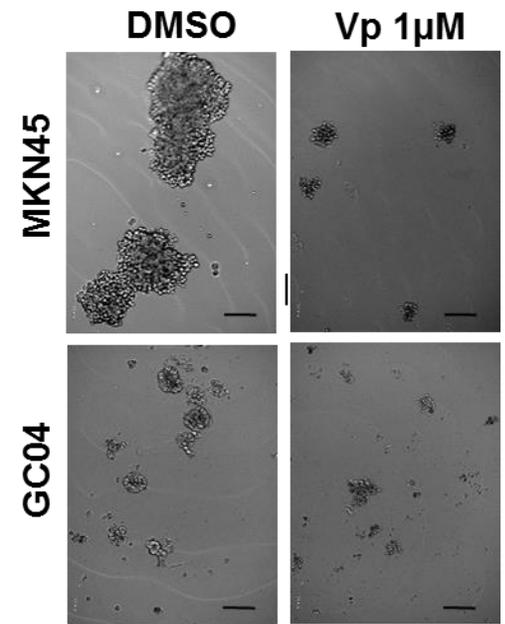


**Figure 2. In vitro effect of verteporfin in MKN45 and MKN74 gastric cancer cell lines.** Cell growth at 48h was decreased from 1  $\mu$ M concentration in both cell lines (a), without a change in cell morphology (b). Cell cycle analysis showed a cell cycle arrest (c), with accumulation of cells in G0/G1 phase, and decrease of cells in phase S (d). TEAD-luciferase reporter (e) and mRNA expression levels of CTGF, CYR61, IGF1BP3, JAGGED1, and LATS2 (f) were decreased upon treatment. \*  $p < 0.05$  vs DMSO-treated control in Kruskal-Wallis test.

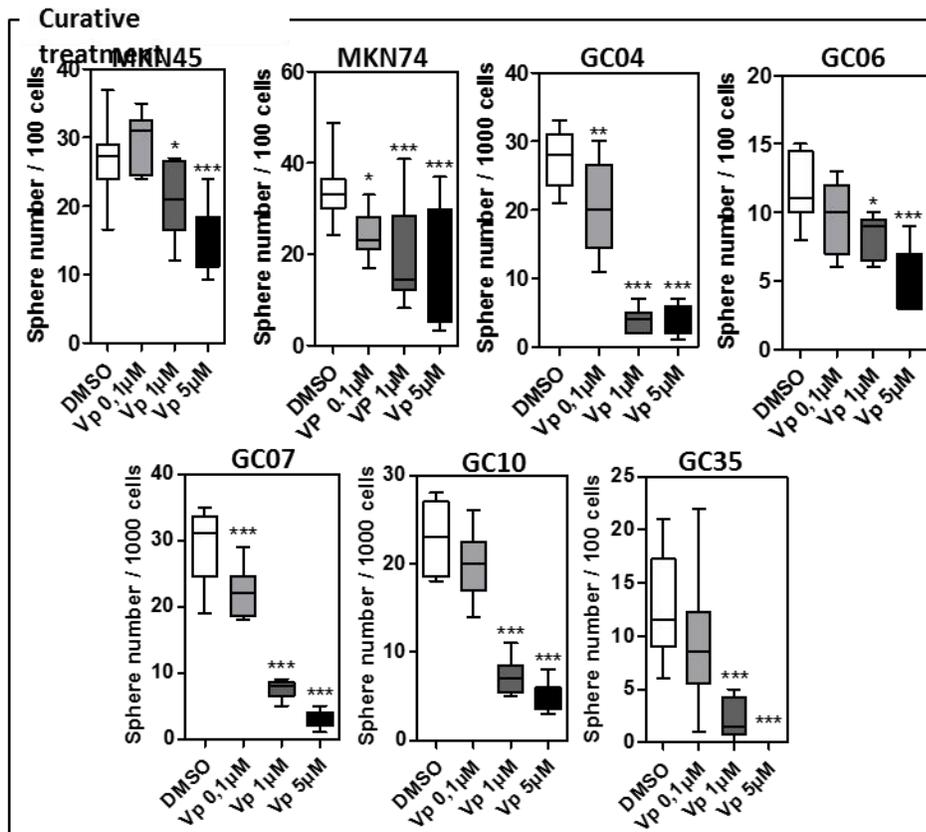
a.



b.

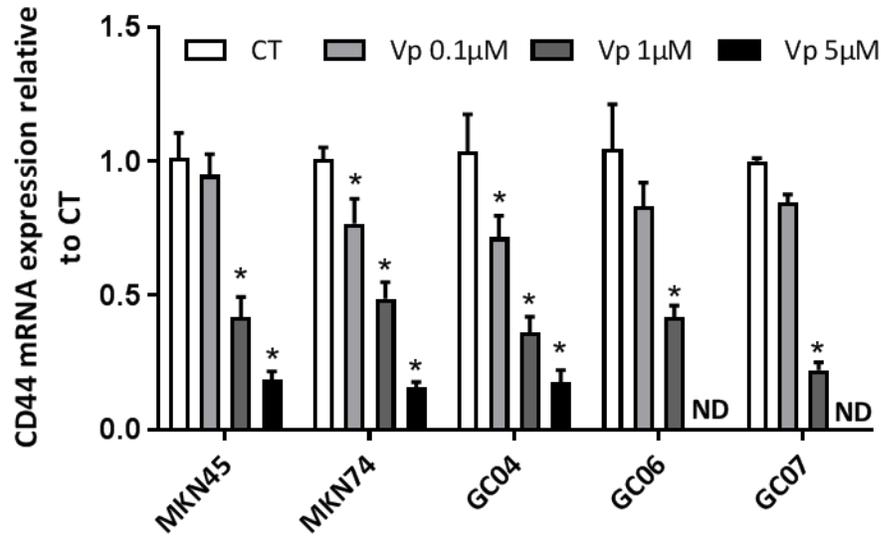


c.

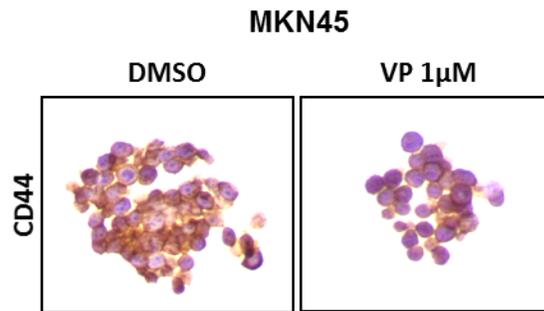


**Figure 3. *In vitro* effect of verteporfin in tumorspheres.** *In vitro* cultured tumorspheres from gastric cancer cell lines and PDXs were treated with verteporfin. Preventive treatment (a) was performed by adding verteporfin during the seeding of cells. Morphology and size changes of the tumorspheres upon treatment is shown for MKN45 and GC04(b). The curative effect of verteporfin, was studied by treating well established tumorspheres (c). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs DMSO-treated control in Kruskal-Wallis test

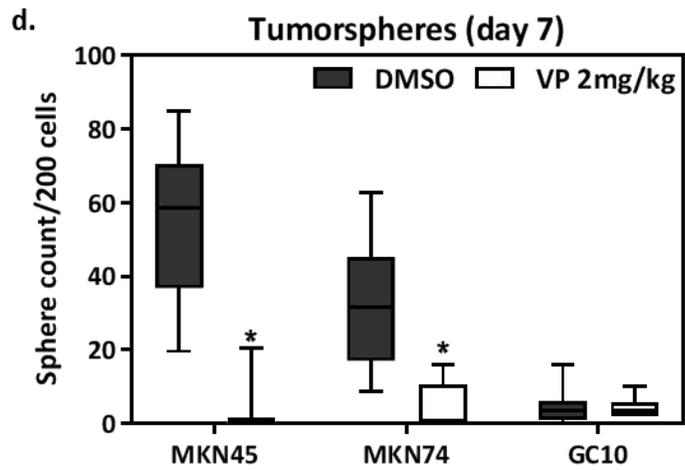
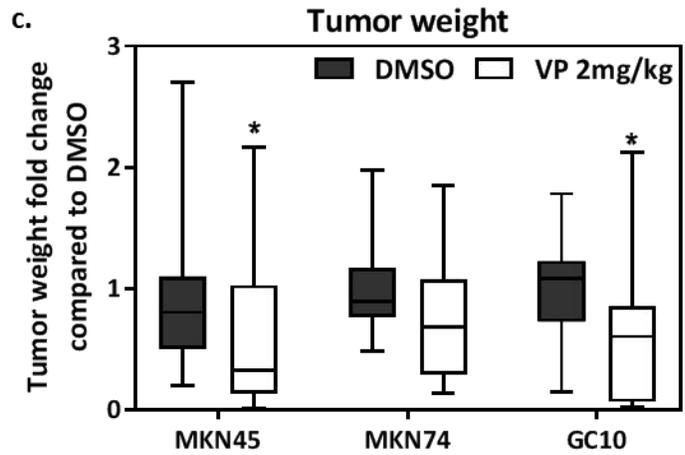
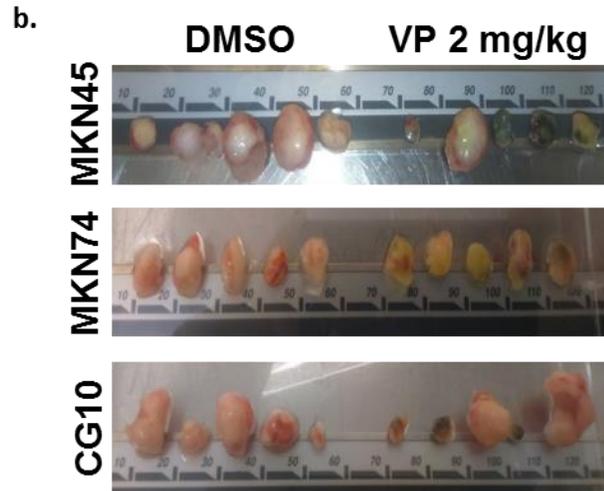
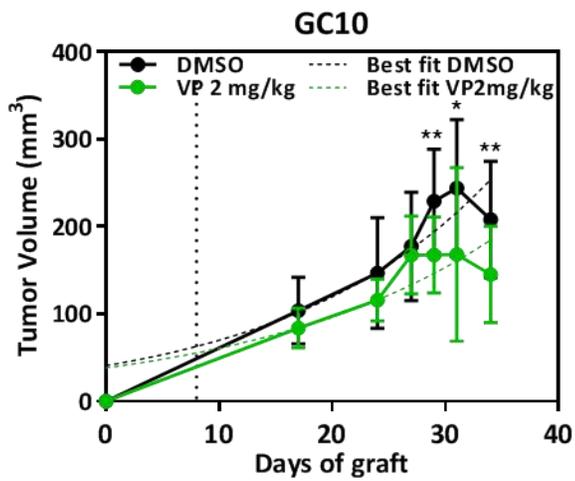
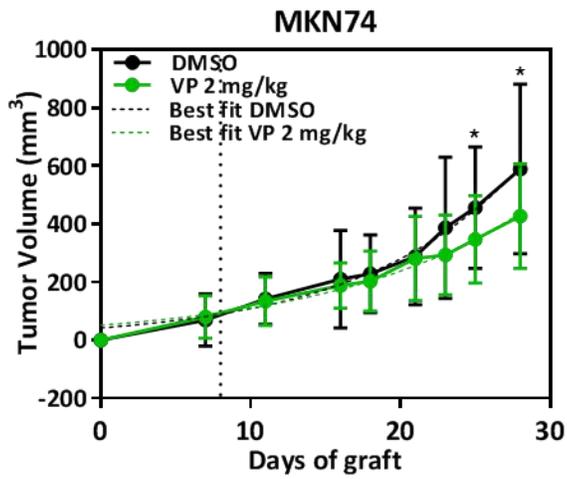
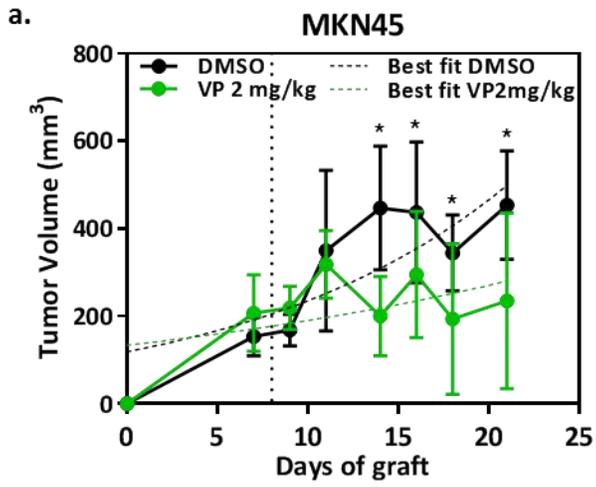
a.



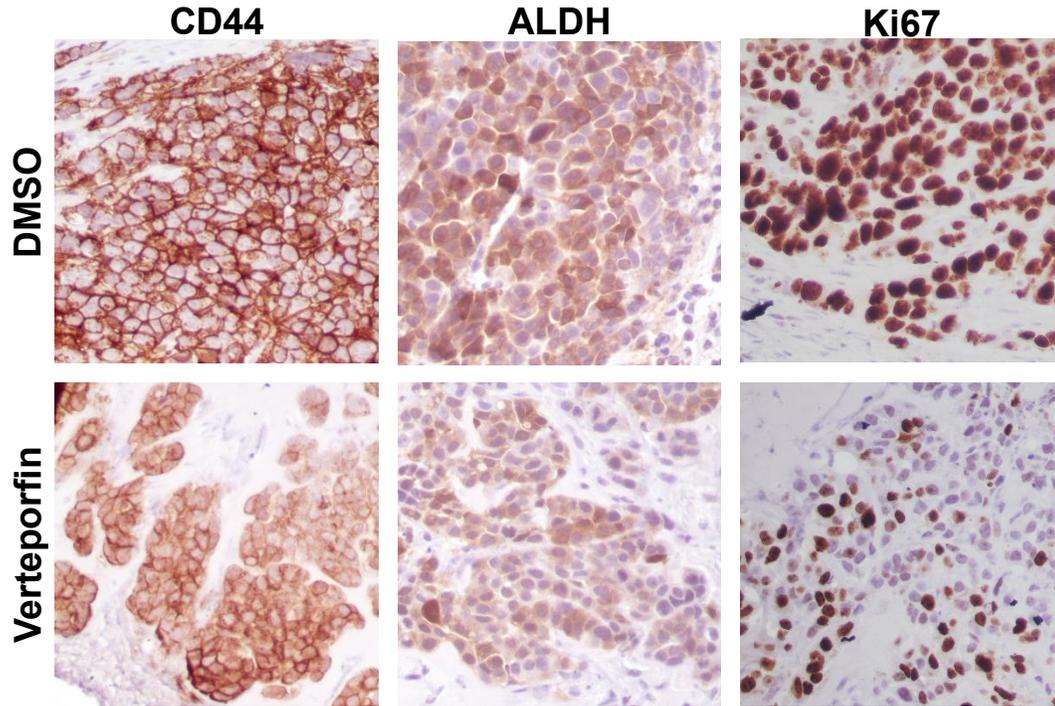
b.



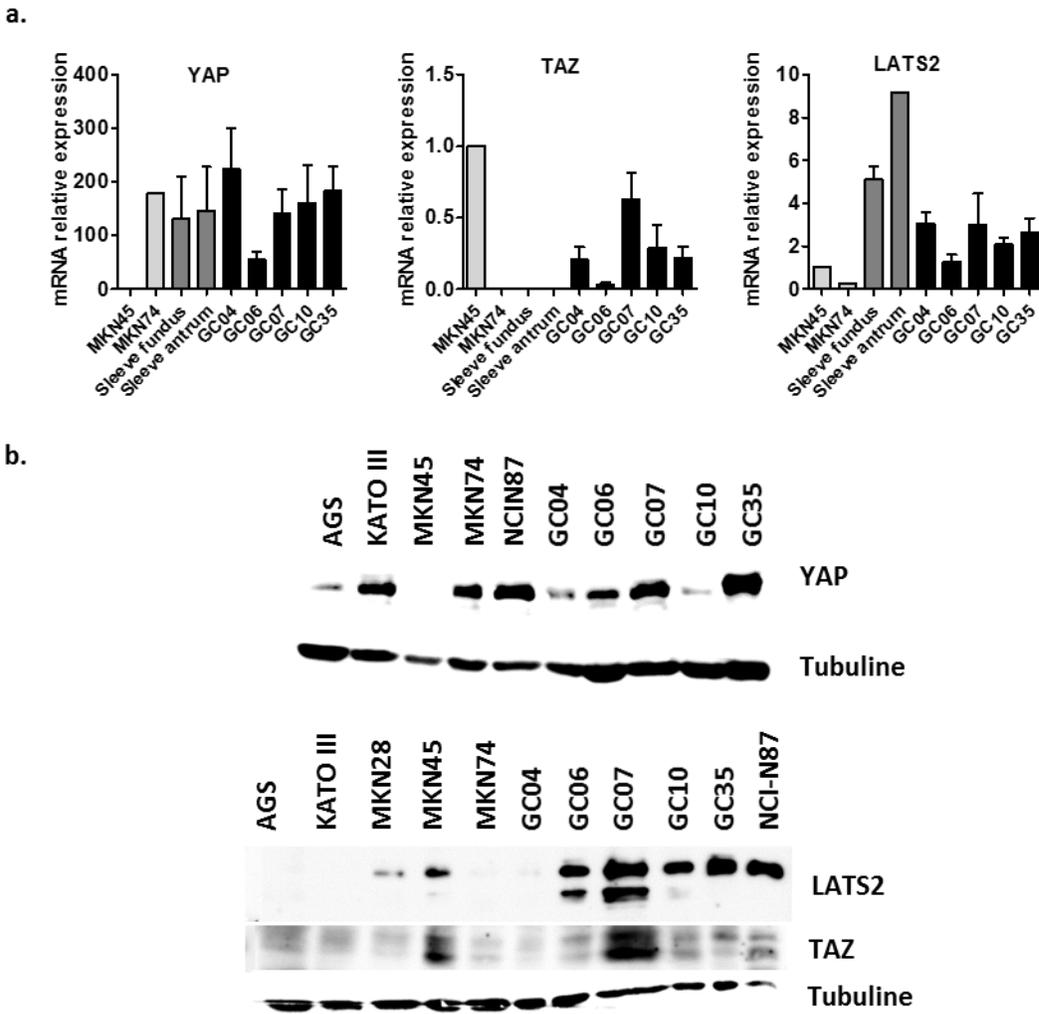
**Figure 4. Effect of verteporfin in CD44 expression on tumorspheres.** CD44 mRNA expression upon verteporfin treatment was studied in MKN45, MKN74, GC04, GC06, and GC07 (a). CD44 immunocytochemistry evaluation in tumorspheres from MKN45 confirmed the decrease of this marker upon treatment with verteporfin 1 µM (b). \*  $p < 0,05$  vs DMSO-treated control in Kruskal-Wallis test.



**Figure 5. Effect of verteporfin treatment on tumor growth in a murine xenograft model *in vivo*.** MKN45, MKN74, and GC10 cells were grafted in NSG mice and tumor growth was followed for DMSO-treated control and verteporfin-treated groups, where slower growth rates were observed (a). At the end of the treatment, tumors were extracted (b) tumor weight was determined (c) and tumors were dissociated to evaluate the tumorsphere forming capacity of residual cells (d). \*  $p < 0.05$ , \*\*  $p < 0.01$  vs DMSO-treated control in Mann-Whitney test.



**Figure 6. Effect of verteporfin treatment on CSC- and proliferation markers in a murine xenograft model *in vivo*.** MKN45-derived subcutaneous tumors extracted from DMSO-treated control (upper panel) and verteporfin-treated (lower panel) groups were processed for histology and stained for the expression of CSC markers CD44 (left) and ALDH (center), which were decreased on the intensity and percentage of positive cells. Proliferation marker Ki67 (right) was also strongly reduced in the treated tumors.



**Figure 1. Basal expression of Hippo pathway elements.** Expression of YAP, TAZ and LATS2 mRNAs (a) and proteins (b) were evaluated in gastric cancer cell lines, healthy stomach tissue, and gastric cancer samples.

### III. Discussion



The Hippo pathway is an evolutionary conserved signaling cascade regulating tissue growth and stem cell homeostasis. Previous studies of our group have shown that *H. pylori* upregulates the LATS2 kinase, which is a tumor suppressor. In the other hand, the infection gives rise to a population of CD44<sup>+</sup> cells with CSC-properties via an EMT. The present work describes for the first time two aspects of the Hippo pathway involvement in gastric carcinogenesis. In the first place, we describe the kinetics of Hippo pathway activation by *H. pylori* infection, and the role of the YAP1/LATS2/TEAD axis in EMT and the emergence of CSC-properties. Secondly, we show that targeting YAP1/TAZ/TEAD transcriptional program inhibits CSC-properties and tumor growth.

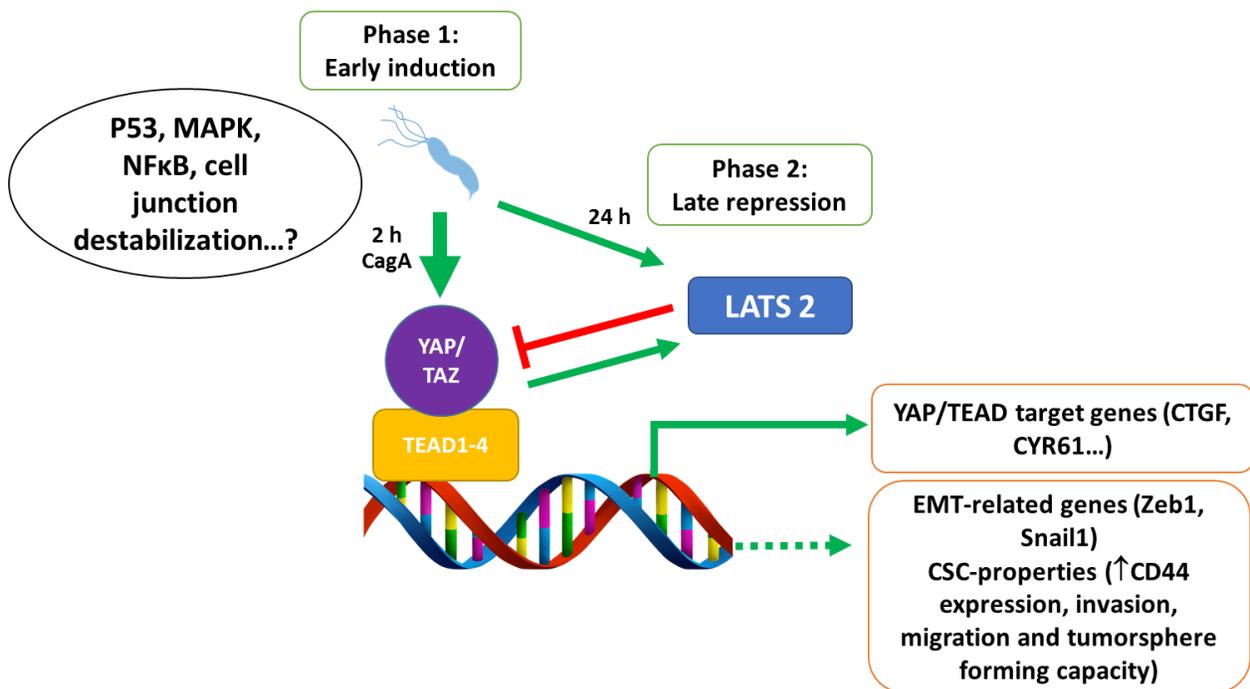
#### a. The LATS2/YAP1/TEAD axis controls EMT and CSC-properties

A co-upregulation of LATS2 and YAP1 was found in tissues sections from *H. pylori* infected patients compared to non-infected tissue samples, and this increase was maintained in gastritis and metaplasia. Our findings confirm the overexpression of YAP1 in association with malignant transformation and its role as a stemness factor. However, we did not find the inverse correlation between LATS and YAP1 usually described in the literature, but a positive correlation between the two proteins instead, along with a colocalization with CD44, that is, in the stem cell compartment of the gastric mucosa.

The *in vivo* findings led us to the study of the molecular regulation of the Hippo pathway in gastric epithelial cell lines challenged with *H. pylori*. We found that *H. pylori* infection modulates the Hippo pathway in biphasic pattern, where YAP1 transcriptional activity is stimulated transiently during the early stage of infection and then this activity is controlled by an activation of LATS2. LATS2 phosphorylates YAP1 inducing its exclusion from the nucleus and cytoplasmic retention and degradation, therefore blocking its co-transcriptional activity (figure 17). The modulation of the Hippo pathway by *H. pylori* infection is CagA dependent, confirming that this pathway may play a role in the carcinogenic process induced by infection. Repeated and chronic aggression of the gastric epithelium by *H. pylori in vivo* could lead to constant waves of YAP1/TEAD activation and LATS2 induction, resulting, as observed, in a co-overexpression of both and the nuclear localization of YAP1 reflecting its co-transcriptional activity.

One of the key findings of our study is the reciprocal upregulation of YAP1 and LATS2 in gastric epithelial cells in both infected and non-infected conditions, as well as the identification of LATS2 as a potential YAP1 target gene. It has been reported that YAP1/TEAD directly induces *lats2* transcription upon binding to its promoter, and in turn, LATS2 inactivates YAP1 constituting a negative feedback loop. Supporting our findings, LATS2 was correlated with CYR61 and CTGF in the CCLE database. CYR61 and CTGF are the main YAP1/TEAD target genes and their expression reflects the transcriptional activity of the complex. LATS2 knockdown with siRNA was found to exacerbate the EMT process and the CSC-properties

induced by *H. pylori* in gastric epithelial cells, due to a failure to control YAP1/TEAD transcriptional activity evidenced by accumulation of nuclear, non-phosphorylated YAP1. The co-overexpression of LATS2/YAP1 appears at all stages of the gastric carcinogenesis cascade, in *H. pylori*-associated gastritis and metaplasia, and in GC. Therefore it constitutes a novel additional biomarker of *H. pylori*-triggered gastric stem cell expansion leading to CSC and GC.



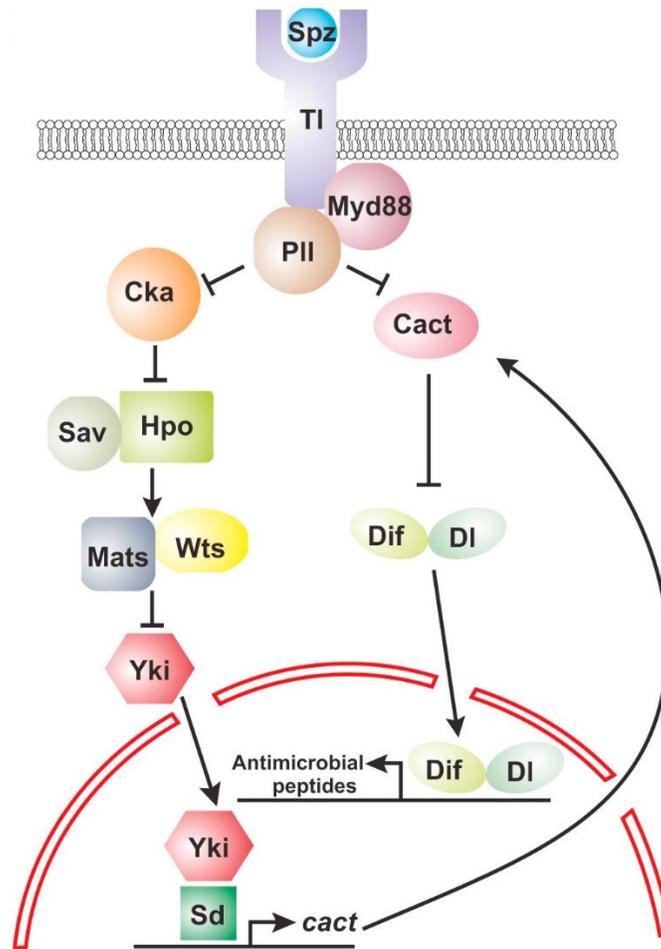
**Figure 17. Hippo pathway regulation by *H. pylori* infection.** *H. pylori* infection modulates the Hippo pathway in biphasic pattern in a CagA dependent manner. There is a first phase of early induction of YAP1/TEAD transcriptional activity, which is then repressed in a second phase by an activation of LATS2. The YAP1/TEAD transcriptional activity seems to be at least partially responsible for the induction of EMT and CSC-properties observed upon *H. pylori* infection of humans gastric epithelial cells. We identified LATS2 as a potential YAP1 target gene, which constitutes a negative feedback loop limiting YAP1 activity.

**i. What's next?**

**1. Is LATS2 linked with pro-inflammatory signaling pathways?**

NF-κB activation is a known major inducer of EMT, and also an effect of *H. pylori* infection. In the fruit fly, a recent study by Liu *et al*<sup>488</sup> described a role of Wts (LATS homologue) and Yki (YAP1

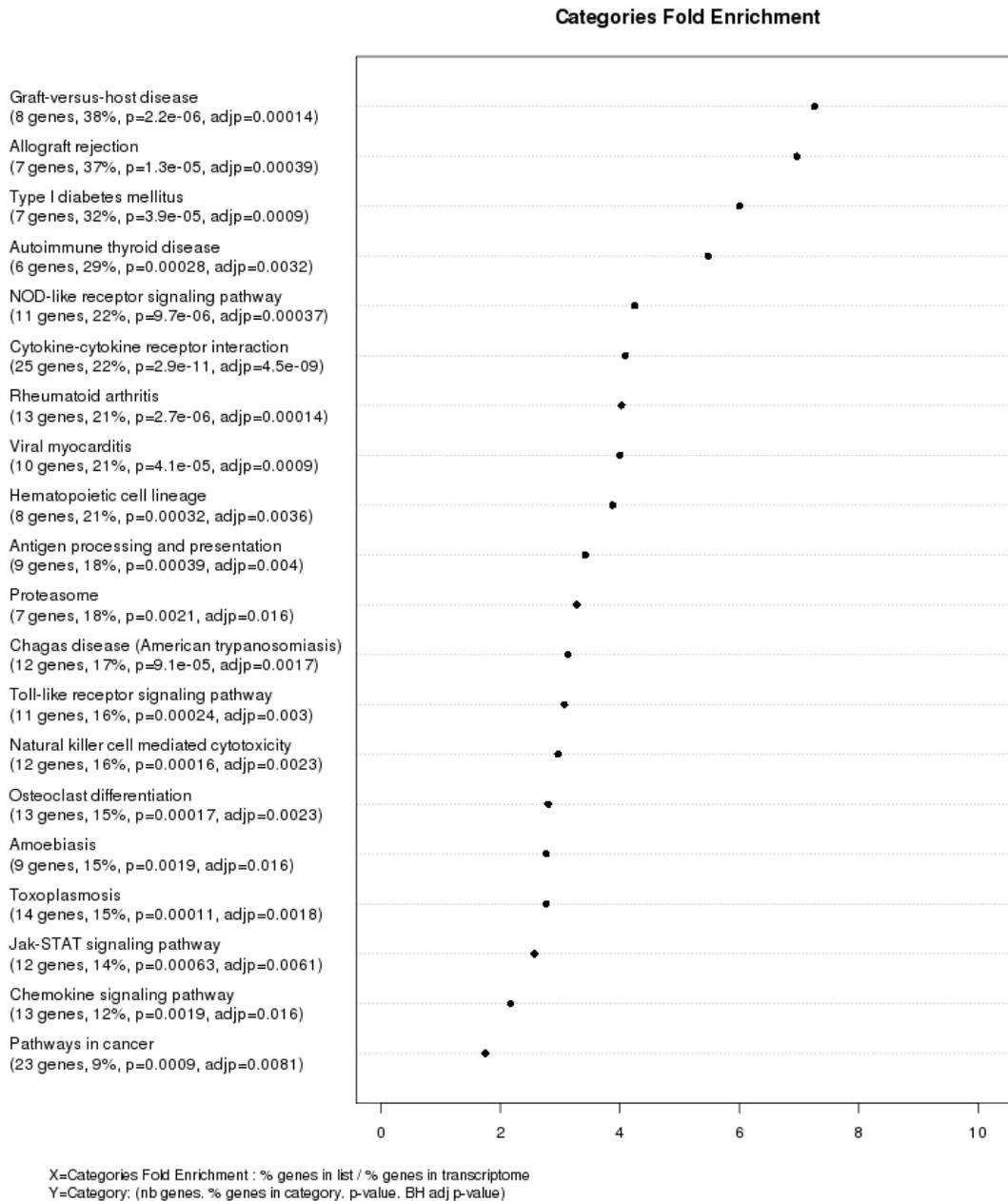
homologue) in the regulation of the antimicrobial peptide production, by an indirect repression of Cactus (the orthologue of the NF- $\kappa$ B inhibitor NF $\kappa$ BIA/B/C), as showed in figure 18.



**Figure 18. Regulation of anti-microbial peptide response in *Drosophila*<sup>488</sup>.** Upon activation of Toll (TI) pathway signaling by components of bacteria and fungi, the Myd88-Pelle (PII) complex induces the degradation of Cactus (Cact, orthologue of NF- $\kappa$ B inhibitor) and the consequent stimulation and nuclear translocation of the transcription factors Dif and DI (orthologues of NF- $\kappa$ B), which activate the production of antimicrobial peptides. Myd88-PII also suppresses the Hippo pathway inhibitor Cka, indirectly activating the Hippo pathway which in turns suppresses transcription of Yki (YAP1-homologue) target genes, including *cact*. This constitutes a coordinate regulation of the pro-inflammatory response between Toll and Hippo signaling.

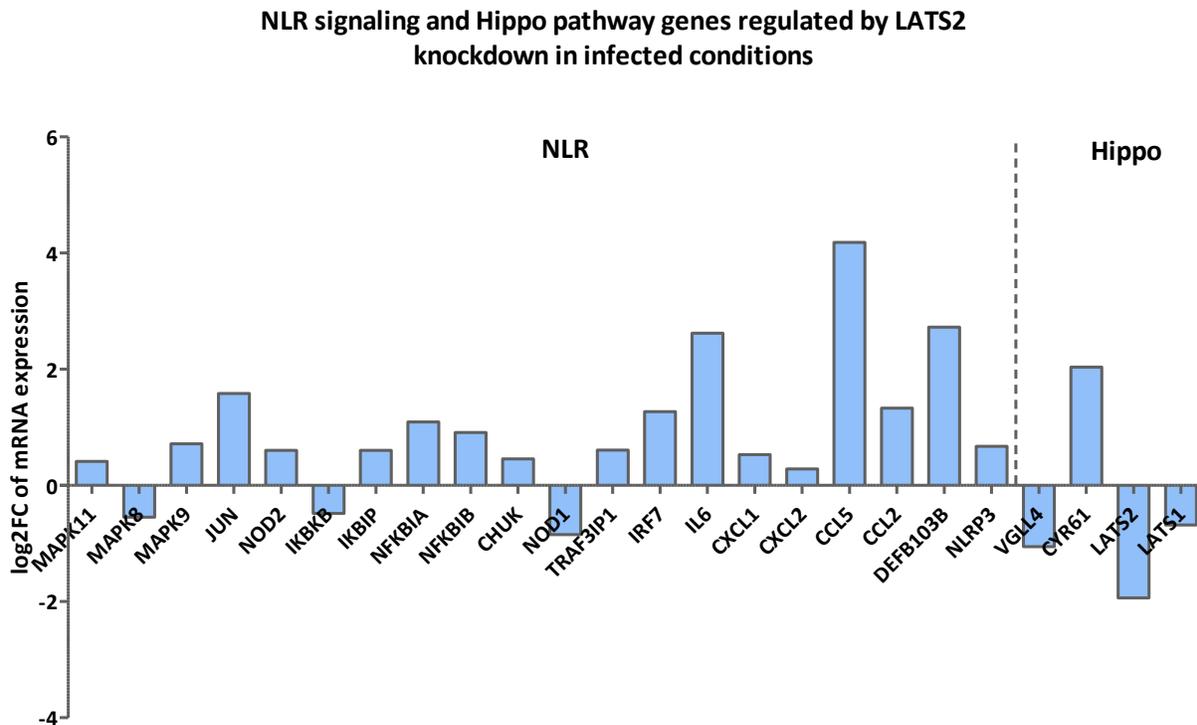
Preliminary results obtained in our MKN74 *H. pylori* infection model, showed an amplification of the inflammatory response upon knockdown of LATS2. The expression of  $\beta$ -defensin 3, one of the antimicrobial peptides most strongly induced by *H. pylori* infection in our transcriptomic study, was further increased by the inhibition of LATS2. To study if there were broader effects of LATS2 in the inflammatory

response and to get deeper insights in the mechanisms involved, a pilot transcriptomic study on the MKN74 cell line was performed, and various functional groups of genes were upregulated, most of them associated with inflammation or immune response (Figure 19). One of the categories that caught our attention was the NOD-like receptor (NRL) signaling pathway, which presented a four-fold increase.



**Figure 19. Functional categories enriched upon LATS2 inhibition in MKN74 cells.** Enrichment of genes groups according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), obtained by a transcriptomic analysis (Agilent Human Microarray 8x60K).

The NLR-signaling is activated by different pathogen-associated molecular patterns such as bacterial peptidoglycan and simple-stranded RNA virus, and its activation stimulates NF- $\kappa$ B transcriptional activity as well as other pro-inflammatory transcription factors such as AP-1 and the Interferon-responsive elements (IRFs). Individual evaluation of the expression of genes associated with these pathway shows at least 17 genes upregulated upon inhibition of LATS2 in the context of *H. pylori* infection, including cytokines, chemokines, and regulators of NF- $\kappa$ B activity. Interestingly, members of the MAPK family were also significantly increased (Figure 20).



**Figure 20. NLR signaling pathway associated genes differentially expressed upon LATS2 inhibition.** mRNA levels expressed as Log<sub>2</sub>(siLATS2/Control), obtained by a transcriptomic analysis. Only significantly regulated genes are shown ( $p < 0.05$ ).

The results are somehow unexpected based on the results obtained in the *Drosophila* model. However, other study by Yao *et al*<sup>489</sup>, describes a role of NF- $\kappa$ B inhibition by LATS2 mediated by a competition mechanism with the kinase TAK1, which is also regulated by NLR-signaling. LATS2 inhibits the phosphorylation and activation of the NF- $\kappa$ B inhibitor IKB, indirectly releasing NF- $\kappa$ B to go to the nucleus and exert its transcriptional activity<sup>489</sup>. These and our preliminary findings open the possibility of a novel, YAP1-independent role of LATS2 in the induction of EMT and CSC-properties via NF- $\kappa$ B, most likely in a coordinated fashion with the oncogenic MAPK signaling and induced by the inflammatory response to

bacterial infection, which has never been described before. Therefore, it constitutes a new and original perspective of research that will be developed in our group in a near future.

## **2. *Is the Hippo pathway activation status correlated with the histological subtype of gastric carcinomas?***

Here we reported a positive correlation between LATS2 expression nuclear YAP1 expressions on GC tissue sections. However, when the cases were separated according to the histological classification of the tumor in intestinal and diffuse, a positive trend was observed in the intestinal category but no significant correlation was found. This is probably due to the small number of cases in each group after stratification in smaller categories. Nevertheless, it may be worth to explore if such relation exists, not only to further elucidate the molecular mechanism of regulation of the Hippo pathway in each scenario, but also to increase the value of LATS2 and YAP1 expression and localization as a biomarker for gastric cancer. In order to carry on this study, our group is currently working in the characterization of LATS2 and YAP1 expression in tissue microarrays containing samples of 178 cases of gastric cancer fully characterized for clinic, pathologic, and epidemiologic features and paired non-cancerous distant gastric mucosa (healthy tissue, gastritis, metaplasia, and dysplasia). This should allow us to determine from which lesion of the Correa cascade the markers are overexpressed and to establish them as a potential biomarker of early stages in the carcinogenic sequence.

Another possibility to approach this question lays in the use of *in silico* databases, such as the Cancer Cell Line Encyclopedia (CCLE) or The Cancer Genome Atlas (TCGA). In the present study, the 38 gastric cancer cell lines present in the CCLE were analyzed for the expression levels and correlation of LATS2, YAP1, CTGF, CYR61, and CD44. However, when we stratified the cell lines according to the histological type, the scenario was exactly the same we found with the tissue samples. The evaluation of the Hippo pathway genes in the TCGA database needs to be done, maybe resulting in interesting data, as a validation of YAP1 and LATS2 as biomarkers of gastric cancer, and also in the exploration of potential relations between their expression and the histological or molecular subtypes of gastric cancer.

### **b. *Verteporfin targeting of the YAP1/TAZ/TEAD transcriptional program inhibits CSC-properties and tumor growth***

Guided by the results highlighting the role of YAP1/TEAD signaling in gastric carcinogenesis and CSC properties, the second part of the study consisted in the evaluation of anti-tumor properties of verteporfin, which disrupts the YAP1/TEAD interaction<sup>482</sup>, inhibiting its transcriptional activity. We showed that treatment with verteporfin decreased cell growth, YAP1/TEAD transcriptional activity, and

tumorsphere formation *in vitro* and tumor growth and CSC-properties and markers *in vivo*. These results show the CSC-inhibitory activity of verteporfin both *in vitro* and *in vivo*. We corroborated the results obtained by various groups in models of liver, colorectal, and esophageal carcinogenesis<sup>482-485</sup>, and to our knowledge, this is the first description of the anti-tumor activity of verteporfin in gastric cancer. Other mechanisms of tumor growth inhibition have been described for verteporfin, for example the capacity to inhibit autophagosome accumulation (reducing cell viability in nutrient-deprived conditions)<sup>481</sup> and STAT3/IL-6 signaling<sup>486,487</sup>, as well as the capacity to induce oligomerization of high molecular weight proteins, which increases apoptotic cell death<sup>487</sup>. All these mechanisms could also have a role in the effect found in our model, and are not exclusive. However, even if they contribute to the effect, our results confirm a role for the YAP1/TEAD disrupting action of verteporfin, reflected in the decrease of YAP1/TEAD target genes expression.

Among the advantages of verteporfin use as anti-tumor treatment, are a selectivity for cancerous cells with high YAP1/TEAD activity and the fact that it is an FDA-approved molecule for the routine treatment of ophthalmologic pathologies (age-related macular degeneration, choroidal neovascularization, and chronic central serous chorioretinopathy). But, despite the observed significant action of verteporfin treatment in tumor reduction, the insolubility of the molecule was a significant challenge. As it is not water soluble, the use of DMSO as a carrier which could introduce a bias caused by the toxic activity of DMSO. This inconvenience was, however, overcome using the appropriate control. The other important disadvantage was also related to the product solubility, which limits its bioavailability and the possibility of reaching the adequate concentration in the tumor when administered in a systemic way, unless using very high quantities of the product which could carry systemic toxicity and increase the financial burden of the treatment. As an alternative, we used a peritumoral injection, which proved to fail in the homogeneous distribution of verteporfin around the tumor and in some cases, caused the tumor to escape the treatment, invading towards the opposite side. However, the positive results obtained despite the flaws of the model, open the possibility of exploring new alternatives for verteporfin formulation that make it more suitable for implementing the treatment in patients. More importantly, this work represents a proof of concept, in which the disruption of the YAP1/TAZ/TEAD transcriptional activity represents a strategy of great potential in the development of antitumor drugs for gastric cancer.

#### **i. Other small molecules that modulate the Hippo pathway**

Besides verteporfin, several other small molecules have been identified as modulators of the Hippo pathway with a potential use in cancer treatment and regenerative medicine, thoroughly reviewed in Johnson et al<sup>490</sup>. In the exploratory phase of the project we were interested in testing some of these molecules as well. One of them was the Src/Abl family tyrosine kinase inhibitor Dasatinib, which has been

shown to possess antitumor properties in preclinical assays in hematological malignancies<sup>491</sup> and also the capacity of suppressing the proliferation of cells dependent on  $\beta$ -catenin signaling, by inhibiting YES1 and the YAP1/ $\beta$ -catenin/TBX5 transcriptional complex<sup>492</sup>. However, the *in vitro* results in gastric cancer cell lines were less marked than those showed by verteporfin, with a broader theoretical mechanism of action more indirectly associated with YAP1/TEAD activity, hence we decide not to pursue this line of research but it remains a possibility to explore in the future.

As mentioned before, other groups have addressed the subject, notably in the study by Jiao *et al*<sup>474</sup> where an inhibitor peptide termed “Super-TDU”, targeting YAP1/TEAD4 interaction, was used successfully both *in vitro* and *in vivo*.

## ii. What’s next?

### 1. TAZ role in gastric cancer

For these work, we were focused on YAP1 which is the most studied of the two oncogenes in the cancer field. However, there is also TAZ, which has never been studied in the context of gastric cancer. To study the regulation of the Hippo pathway by *H. pylori* infection, two cell lines were used in this work, AGS and MKN74. In these cell lines, YAP1 predominates and TAZ is expressed in a low level or not at all, therefore they do not constitute an appropriate model for the study of TAZ. However, we have identified two other gastric cancer cell lines, MKN45 and NCI-N87, with a high and intermediate expression of TAZ and a less important role of YAP1, which constitute an adequate model to study TAZ. Despite the fact that they are normally treated as identical, preliminary results indicate that the kinetics of activation in response to *H. pylori* maybe different between YAP1 and TAZ, but it seems that the effect of its inhibition on CSC-properties are similar. Further studies on the subject are been carried on by our group to shed more light on TAZ regulation during gastric carcinogenesis.

### 2. Hippo pathway status in response to ATRA treatment

Nguyen *et al*<sup>158</sup> recently described an antitumor and anti-CSC activity of all-*trans* retinoic acid (ATRA) in gastric cancer. The results are similar to those observed with verteporfin in this work, i.e. an inhibition of cell proliferation with a cell cycle arrest in G0/G1 and a decrease of tumorsphere formation *in vitro*, as well as an impairment of tumor growth *in vivo*, even if the efficacy of verteporfin seems to be slightly lower. Given the availability of biological material from the ATRA research, the evaluation of the response of the Hippo pathway to ATRA treatment would be of great interest in the cells of the residual tumors and more so, in the relapsing tumors. The relapsing tumors originate from residual CSC that where

resistant to ATRA, and as the Hippo pathway deregulation has been associated with the CSC phenotype, the study of its activation status is very relevant.

In summary, this work shows for the first time that the Hippo pathway and particularly the LATS2/YAP1/TEAD axis is early activated during the carcinogenesis process induced by chronic *H. pylori* infection and controls the subsequent EMT and CSC-like features. Targeting this pathway was shown to be efficient in preventing tumor growth in a PDX model, therefore highlighting the potential of its inhibition to be implemented in gastric cancer therapy.



## IV. References



1. Cancer Today. International Agency for Research on Cancer; 2016. <http://gco.iarc.fr/today>. Accessed 01/31/2017.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;65(2):87-108.
3. Ruge M, Fassan M, Graham DY. Epidemiology of Gastric Cancer. In: Strong VE, ed. *Gastric Cancer. Principles and practice*. Switzerland: Springer; 2015:23-34.
4. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends-- An Update. *Cancer Epidemiol Biomarkers Prev*. 2016;25(1):16-27.
5. Sierra MS, Cueva P, Bravo LE, Forman D. Stomach cancer burden in Central and South America. *Cancer Epidemiol*. 2016;44 Suppl 1:S62-S73.
6. Wu X, Chen VW, Andrews PA, Ruiz B, Correa P. Incidence of esophageal and gastric cancers among Hispanics, non-Hispanic whites and non-Hispanic blacks in the United States: subsite and histology differences. *Cancer Causes Control*. 2007;18(6):585-593.
7. Jin H, Pinheiro PS, Xu J, Amei A. Cancer incidence among Asian American populations in the United States, 2009-2011. *Int J Cancer*. 2016;138(9):2136-2145.
8. Stern MC, Fejerman L, Das R, et al. Variability in Cancer Risk and Outcomes Within US Latinos by National Origin and Genetic Ancestry. *Curr Epidemiol Rep*. 2016;3:181-190.
9. Keshava HB, Rosen JE, DeLuzio MR, Kim AW, Detterbeck FC, Boffa DJ. "What if I do nothing?" The natural history of operable cancer of the alimentary tract. *Eur J Surg Oncol*. 2017.
10. Glória L, Bossard N, Bouvier AM, et al. Trends in net survival from stomach cancer in six European Latin countries: results from the SUDCAN population-based study. *Eur J Cancer Prev*. 2017;26 Trends in cancer net survival in six European Latin Countries: the SUDCAN study:S32-S39.
11. Veisani Y, Delpisheh A. Survival rate of gastric cancer in Iran; a systematic review and meta-analysis. *Gastroenterol Hepatol Bed Bench*. 2016;9(2):78-86.
12. González CA, Megraud F, Buissonniere A, et al. Helicobacter pylori infection assessed by ELISA and by immunoblot and noncardia gastric cancer risk in a prospective study: the Eurgast-EPIC project. *Ann Oncol*. 2012;23(5):1320-1324.
13. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health*. 2016;4(9):e609-616.
14. Correa P. Gastric cancer: overview. *Gastroenterol Clin North Am*. 2013;42(2):211-217.
15. Piazuolo MB, Correa P. Gastric cáncer: Overview. *Colomb Med (Cali)*. 2013;44(3):192-201.
16. Henson DE, Dittus C, Younes M, Nguyen H, Albores-Saavedra J. Differential trends in the intestinal and diffuse types of gastric carcinoma in the United States, 1973-2000: increase in the signet ring cell type. *Arch Pathol Lab Med*. 2004;128(7):765-770.
17. Barber M, Murrell A, Ito Y, et al. Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer. *J Pathol*. 2008;216(3):295-306.
18. Colquhoun A, Arnold M, Ferlay J, Goodman KJ, Forman D, Soerjomataram I. Global patterns of cardia and non-cardia gastric cancer incidence in 2012. *Gut*. 2015;64(12):1881-1888.
19. Kyburz A, Müller A. Helicobacter pylori and Extragastric Diseases. *Curr Top Microbiol Immunol*. 2017;400:325-347.
20. Tang LH, Selby LV. Pathology of Gastric Cancer. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:57-76.
21. Hu B, El Hajj N, Sittler S, Lammert N, Barnes R, Meloni-Ehrig A. Gastric cancer: Classification, histology and application of molecular pathology. *J Gastrointest Oncol*. 2012;3(3):251-261.
22. LAUREN P. THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. *Acta Pathol Microbiol Scand*. 1965;64:31-49.
23. Bosman FT, Carneiro F, Hruban RH, Theise ND. *WHO Classification of Tumours of the Digestive System*. Vol 3. 4 ed: IARC; 2010.

24. Polk DB, Peek RM. Helicobacter pylori: gastric cancer and beyond. *Nat Rev Cancer*. 2010;10(6):403-414.
25. Liu C, Crawford JM. Tracto gastrointestinal. In: Kumar V, Abbas A, Fausto N, eds. *Patología estructural y funcional*. España: Elsevier; 2005:1517.
26. Piazzuelo MB, Epplein M, Correa P. Gastric cancer: an infectious disease. *Infect Dis Clin North Am*. 2010;24(4):853-869, vii.
27. Correa P, Haenszel W, Cuello C, Tannenbaum S, Archer M. A model for gastric cancer epidemiology. *Lancet*. 1975;2(7924):58-60.
28. Correa P, Piazzuelo MB. The gastric precancerous cascade. *J Dig Dis*. 2012;13(1):2-9.
29. Network CGAR. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*. 2014;513(7517):202-209.
30. Cancer AJCo. [www.cancerstaging.org](http://www.cancerstaging.org). Accessed February 22th, 2017.
31. Washington K. 7th Edition of the AJCC Cancer Staging Manual: Stomach. *Annals of Surgical Oncology*. 2010;17(12):3077-3079.
32. Setia N, Clark JW, Duda DG, et al. Familial Gastric Cancers. *Oncologist*. 2015;20(12):1365-1377.
33. Yoon H, Kim N. Diagnosis and management of high risk group for gastric cancer. *Gut Liver*. 2015;9(1):5-17.
34. Cheng XJ, Lin JC, Tu SP. Etiology and Prevention of Gastric Cancer. *Gastrointest Tumors*. 2016;3(1):25-36.
35. Lu L, Chen J, Tang H, et al. EGCG Suppresses ERK5 Activation to Reverse Tobacco Smoke-Triggered Gastric Epithelial-Mesenchymal Transition in BALB/c Mice. *Nutrients*. 2016;8(7).
36. Kim J, Cho YA, Choi WJ, Jeong SH. Gene-diet interactions in gastric cancer risk: a systematic review. *World J Gastroenterol*. 2014;20(28):9600-9610.
37. Kulnigg-Dabsch S. Autoimmune gastritis. *Wien Med Wochenschr*. 2016;166(13-14):424-430.
38. Coati I, Fassan M, Farinati F, Graham DY, Genta RM, Rugge M. Autoimmune gastritis: Pathologist's viewpoint. *World J Gastroenterol*. 2015;21(42):12179-12189.
39. Mukaiho K, Nakayama T, Hagiwara T, Hattori T, Sugihara H. Two distinct etiologies of gastric cardia adenocarcinoma: interactions among pH, Helicobacter pylori, and bile acids. *Front Microbiol*. 2015;6:412.
40. Derakhshan MH, Malekzadeh R, Watabe H, et al. Combination of gastric atrophy, reflux symptoms and histological subtype indicates two distinct aetiologies of gastric cardia cancer. *Gut*. 2008;57(3):298-305.
41. Dijksterhuis JP, Petersen J, Schulte G. WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3. *Br J Pharmacol*. 2014;171(5):1195-1209.
42. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17(1):9-26.
43. Willert K, Nusse R. Wnt proteins. *Cold Spring Harb Perspect Biol*. 2012;4(9):a007864.
44. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127(3):469-480.
45. Chiurillo MA. Role of the Wnt/ $\beta$ -catenin pathway in gastric cancer: An in-depth literature review. *World J Exp Med*. 2015;5(2):84-102.
46. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J*. 1997;16(13):3797-3804.
47. Liu C, Li Y, Semenov M, et al. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell*. 2002;108(6):837-847.
48. Sugimura R, Li L. Noncanonical Wnt signaling in vertebrate development, stem cells, and diseases. *Birth Defects Res C Embryo Today*. 2010;90(4):243-256.
49. Duchartre Y, Kim YM, Kahn M. The Wnt signaling pathway in cancer. *Crit Rev Oncol Hematol*. 2016;99:141-149.

50. Gencer S, Şen G, Doğusoy G, Belli AK, Paksoy M, Yazicioğlu MB.  $\beta$ -Catenin-independent noncanonical Wnt pathway might be induced in gastric cancers. *Turk J Gastroenterol*. 2010;21(3):224-230.
51. Kolligs FT, Bommer G, Göke B. Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion*. 2002;66(3):131-144.
52. Ebert MP, Fei G, Kahmann S, et al. Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene are present in intestinal-type gastric cancer. *Carcinogenesis*. 2002;23(1):87-91.
53. Cai C, Zhu X. The Wnt/ $\beta$ -catenin pathway regulates self-renewal of cancer stem-like cells in human gastric cancer. *Mol Med Rep*. 2012;5(5):1191-1196.
54. Clements WM, Wang J, Sarnaik A, et al. beta-Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. *Cancer Res*. 2002;62(12):3503-3506.
55. Fang DC, Luo YH, Yang SM, Li XA, Ling XL, Fang L. Mutation analysis of APC gene in gastric cancer with microsatellite instability. *World J Gastroenterol*. 2002;8(5):787-791.
56. Franco AT, Israel DA, Washington MK, et al. Activation of beta-catenin by carcinogenic *Helicobacter pylori*. *Proc Natl Acad Sci U S A*. 2005;102(30):10646-10651.
57. Gnad T, Feoktistova M, Leverkus M, Lendeckel U, Naumann M. *Helicobacter pylori*-induced activation of beta-catenin involves low density lipoprotein receptor-related protein 6 and Dishevelled. *Mol Cancer*. 2010;9:31.
58. Yong X, Tang B, Xiao YF, et al. *Helicobacter pylori* upregulates Nanog and Oct4 via Wnt/ $\beta$ -catenin signaling pathway to promote cancer stem cell-like properties in human gastric cancer. *Cancer Lett*. 2016;374(2):292-303.
59. Concha-Benavente F, Ferris RL. Oncogenic growth factor signaling mediating tumor escape from cellular immunity. *Curr Opin Immunol*. 2017;45:52-59.
60. Kunath T, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development*. 2007;134(16):2895-2902.
61. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer*. 2010;10(2):116-129.
62. Knights V, Cook SJ. De-regulated FGF receptors as therapeutic targets in cancer. *Pharmacol Ther*. 2010;125(1):105-117.
63. Dudley AT, Tabin CJ. Deconstructing phosphatases in limb development. *Nat Cell Biol*. 2003;5(6):499-501.
64. Hattori Y, Odagiri H, Nakatani H, et al. K-sam, an amplified gene in stomach cancer, is a member of the heparin-binding growth factor receptor genes. *Proc Natl Acad Sci U S A*. 1990;87(15):5983-5987.
65. Jang JH, Shin KH, Park JG. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res*. 2001;61(9):3541-3543.
66. Katoh M. FGF signaling network in the gastrointestinal tract (review). *Int J Oncol*. 2006;29(1):163-168.
67. Hodkinson PS, Mackinnon A, Sethi T. Targeting growth factors in lung cancer. *Chest*. 2008;133(5):1209-1216.
68. Galizia G, Lieto E, Orditura M, et al. Epidermal growth factor receptor (EGFR) expression is associated with a worse prognosis in gastric cancer patients undergoing curative surgery. *World J Surg*. 2007;31(7):1458-1468.
69. Kim J, Kim N, Park JH, et al. The Effect of *Helicobacter pylori* on Epidermal Growth Factor Receptor-Induced Signal Transduction and the Preventive Effect of Celecoxib in Gastric Cancer Cells. *Gut Liver*. 2013;7(5):552-559.

70. Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H, Ullrich A. Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. *Biochem Biophys Res Commun.* 2002;295(3):695-701.
71. Yan F, Cao H, Chaturvedi R, et al. Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. *Gastroenterology.* 2009;136(4):1297-1307, e1291-1293.
72. Borggreffe T, Oswald F. The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol Life Sci.* 2009;66(10):1631-1646.
73. Ntziachristos P, Lim JS, Sage J, Aifantis I. From fly wings to targeted cancer therapies: a centennial for notch signaling. *Cancer Cell.* 2014;25(3):318-334.
74. Xiao YF, Yong X, Tang B, et al. Notch and Wnt signaling pathway in cancer: Crucial role and potential therapeutic targets (Review). *Int J Oncol.* 2016;48(2):437-449.
75. Yuan X, Wu H, Xu H, et al. Notch signaling: an emerging therapeutic target for cancer treatment. *Cancer Lett.* 2015;369(1):20-27.
76. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y. Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. *Carcinogenesis.* 2010;31(5):777-784.
77. Sun Y, Gao X, Liu J, et al. Differential Notch1 and Notch2 expression and frequent activation of Notch signaling in gastric cancers. *Arch Pathol Lab Med.* 2011;135(4):451-458.
78. Yeh TS, Wu CW, Hsu KW, et al. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res.* 2009;69(12):5039-5048.
79. Li GG, Li L, Li C, et al. Influence of up-regulation of Notch ligand DLL4 on biological behaviors of human gastric cancer cells. *World J Gastroenterol.* 2013;19(28):4486-4494.
80. Piazzini G, Fini L, Selgrad M, et al. Epigenetic regulation of Delta-Like1 controls Notch1 activation in gastric cancer. *Oncotarget.* 2011;2(12):1291-1301.
81. Tseng YC, Tsai YH, Tseng MJ, et al. Notch2-induced COX-2 expression enhancing gastric cancer progression. *Mol Carcinog.* 2012;51(12):939-951.
82. Katoh M. Dysregulation of stem cell signaling network due to germline mutation, SNP, Helicobacter pylori infection, epigenetic change and genetic alteration in gastric cancer. *Cancer Biol Ther.* 2007;6(6):832-839.
83. Liu T, He W, Li Y. Helicobacter pylori Infection of Gastric Epithelial Cells Affects NOTCH Pathway In Vitro. *Dig Dis Sci.* 2016;61(9):2516-2521.
84. Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J Cell Biol.* 1982;95(1):333-339.
85. Hay ED. Role of cell-matrix contacts in cell migration and epithelial-mesenchymal transformation. *Cell Differ Dev.* 1990;32(3):367-375.
86. Hay ED, Zuk A. Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis.* 1995;26(4):678-690.
87. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009;119(6):1420-1428.
88. Mongroo PS, Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol Ther.* 2010;10(3):219-222.
89. Sun D, Mcalmon KR, Davies JA, Bernfield M, Hay ED. Simultaneous loss of expression of syndecan-1 and E-cadherin in the embryonic palate during epithelial-mesenchymal transformation. *Int J Dev Biol.* 1998;42(5):733-736.
90. Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. *Biochim Biophys Acta.* 2012;1826(1):23-31.
91. Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene.* 2007;26(5):711-724.
92. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer.* 2007;7(6):415-428.

93. Lin Y, Dong C, Zhou BP. Epigenetic regulation of EMT: the Snail story. *Curr Pharm Des.* 2014;20(11):1698-1705.
94. Theys J, Jutten B, Habets R, et al. E-Cadherin loss associated with EMT promotes radioresistance in human tumor cells. *Radiother Oncol.* 2011;99(3):392-397.
95. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene.* 2005;24(50):7443-7454.
96. Ye X, Weinberg RA. Epithelial-Mesenchymal Plasticity: A Central Regulator of Cancer Progression. *Trends Cell Biol.* 2015;25(11):675-686.
97. Nakaya Y, Sheng G. Epithelial to mesenchymal transition during gastrulation: an embryological view. *Dev Growth Differ.* 2008;50(9):755-766.
98. Kerosuo L, Bronner-Fraser M. What is bad in cancer is good in the embryo: importance of EMT in neural crest development. *Semin Cell Dev Biol.* 2012;23(3):320-332.
99. Nakaya Y, Sheng G. EMT in developmental morphogenesis. *Cancer Lett.* 2013;341(1):9-15.
100. Vićovac L, Aplin JD. Epithelial-mesenchymal transition during trophoblast differentiation. *Acta Anat (Basel).* 1996;156(3):202-216.
101. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol.* 2006;172(7):973-981.
102. Stone RC, Pastar I, Ojeh N, et al. Epithelial-mesenchymal transition in tissue repair and fibrosis. *Cell Tissue Res.* 2016;365(3):495-506.
103. Gilles C, Polette M, Zahm JM, et al. Vimentin contributes to human mammary epithelial cell migration. *J Cell Sci.* 1999;112 ( Pt 24):4615-4625.
104. You S, Avidan O, Tariq A, et al. Role of epithelial-mesenchymal transition in repair of the lacrimal gland after experimentally induced injury. *Invest Ophthalmol Vis Sci.* 2012;53(1):126-135.
105. Lepilina A, Coon AN, Kikuchi K, et al. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell.* 2006;127(3):607-619.
106. Kumar V, Abbas AK, Aster JC. *Robbins and Cotran: Pathologic Basis of Disease.* 9th ed. Philadelphia, PA: Elsevier Saunders; 2015.
107. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell.* 2008;14(6):818-829.
108. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer.* 2002;2(6):442-454.
109. Jung HY, Fattet L, Yang J. Molecular pathways: linking tumor microenvironment to epithelial-mesenchymal transition in metastasis. *Clin Cancer Res.* 2015;21(5):962-968.
110. Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes Dev.* 2013;27(20):2192-2206.
111. Wei SC, Yang J. Forcing through Tumor Metastasis: The Interplay between Tissue Rigidity and Epithelial-Mesenchymal Transition. *Trends Cell Biol.* 2016;26(2):111-120.
112. Li L, Li W. Epithelial-mesenchymal transition in human cancer: comprehensive reprogramming of metabolism, epigenetics, and differentiation. *Pharmacol Ther.* 2015;150:33-46.
113. Lombaerts M, van Wezel T, Philippo K, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br J Cancer.* 2006;94(5):661-671.
114. Wang Y, Shang Y. Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. *Exp Cell Res.* 2013;319(2):160-169.
115. Bolós V, Peinado H, Pérez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J Cell Sci.* 2003;116(Pt 3):499-511.
116. Cano A, Pérez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol.* 2000;2(2):76-83.
117. Micalizzi DS, Ford HL. Epithelial-mesenchymal transition in development and cancer. *Future Oncol.* 2009;5(8):1129-1143.

118. Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell*. 2009;15(3):195-206.
119. Chen L, Gibbons DL, Goswami S, et al. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. *Nat Commun*. 2014;5:5241.
120. Du B, Shim JS. Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. *Molecules*. 2016;21(7).
121. Fischer KR, Durrans A, Lee S, et al. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature*. 2015;527(7579):472-476.
122. Huang J, Li H, Ren G. Epithelial-mesenchymal transition and drug resistance in breast cancer (Review). *Int J Oncol*. 2015;47(3):840-848.
123. !!! INVALID CITATION !!! {}.
124. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):704-715.
125. Morel AP, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One*. 2008;3(8):e2888.
126. Scheel C, Weinberg RA. Cancer stem cells and epithelial-mesenchymal transition: concepts and molecular links. *Semin Cancer Biol*. 2012;22(5-6):396-403.
127. Yang L, Ping YF, Yu X, et al. Gastric cancer stem-like cells possess higher capability of invasion and metastasis in association with a mesenchymal transition phenotype. *Cancer Lett*. 2011;310(1):46-52.
128. Ryu HS, Park DJ, Kim HH, Kim WH, Lee HS. Combination of epithelial-mesenchymal transition and cancer stem cell-like phenotypes has independent prognostic value in gastric cancer. *Hum Pathol*. 2012;43(4):520-528.
129. Kanzawa M, Semba S, Hara S, Itoh T, Yokozaki H. WNT5A is a key regulator of the epithelial-mesenchymal transition and cancer stem cell properties in human gastric carcinoma cells. *Pathobiology*. 2013;80(5):235-244.
130. Guo J, Wang B, Fu Z, Wei J, Lu W. Hypoxic Microenvironment Induces EMT and Upgrades Stem-Like Properties of Gastric Cancer Cells. *Technol Cancer Res Treat*. 2016;15(1):60-68.
131. Bessède E, Staedel C, Acuña Amador LA, et al. Helicobacter pylori generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene*. 2014;33(32):4123-4131.
132. Sougleri IS, Papadakos KS, Zadik MP, Mavri-Vavagianni M, Mentis AF, Sgouras DN. Helicobacter pylori CagA protein induces factors involved in the epithelial to mesenchymal transition (EMT) in infected gastric epithelial cells in an EPIYA- phosphorylation-dependent manner. *FEBS J*. 2016;283(2):206-220.
133. Choi YJ, Kim N, Chang H, et al. Helicobacter pylori-induced epithelial-mesenchymal transition, a potential role of gastric cancer initiation and an emergence of stem cells. *Carcinogenesis*. 2015;36(5):553-563.
134. Wang K, Yuen ST, Xu J, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. *Nat Genet*. 2014;46(6):573-582.
135. Cools-Lartigue J, Baker L, Ferri LE. Molecular Mechanisms in Gastric Carcinogenesis. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:35-56.
136. Boku N. HER2-positive gastric cancer. *Gastric Cancer*. 2014;17(1):1-12.
137. Sukawa Y, Yamamoto H, Nosho K, et al. HER2 expression and PI3K-Akt pathway alterations in gastric cancer. *Digestion*. 2014;89(1):12-17.
138. Teng L, Lu J. cMET as a potential therapeutic target in gastric cancer (Review). *Int J Mol Med*. 2013;32(6):1247-1254.
139. Churin Y. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol*. 2003;161:249-255.

140. Kang C, Song JJ, Lee J, Kim MY. Epigenetics: an emerging player in gastric cancer. *World J Gastroenterol*. 2014;20(21):6433-6447.
141. Tsai KW, Liao YL, Wu CW, et al. Aberrant hypermethylation of miR-9 genes in gastric cancer. *Epigenetics*. 2011;6(10):1189-1197.
142. Zheng L, Qi T, Yang D, et al. microRNA-9 suppresses the proliferation, invasion and metastasis of gastric cancer cells through targeting cyclin D1 and Ets1. *PLoS One*. 2013;8(1):e55719.
143. Xie SS, Jin J, Xu X, Zhuo W, Zhou TH. Emerging roles of non-coding RNAs in gastric cancer: Pathogenesis and clinical implications. *World J Gastroenterol*. 2016;22(3):1213-1223.
144. Sabesan A, Bennett JJ. Diagnosis, Staging, and Workup of Gastric Cancer. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:127-142.
145. Sumiyama K. Past and current trends in endoscopic diagnosis for early stage gastric cancer in Japan. *Gastric Cancer*. 2017;20(Suppl 1):20-27.
146. Yamaguchi Y, Nagata Y, Hiratsuka R, et al. Gastric Cancer Screening by Combined Assay for Serum Anti-Helicobacter pylori IgG Antibody and Serum Pepsinogen Levels--The ABC Method. *Digestion*. 2016;93(1):13-18.
147. Rosero-Bixby L, Sierra R. X-ray screening seems to reduce gastric cancer mortality by half in a community-controlled trial in Costa Rica. *Br J Cancer*. 2007;97(7):837-843.
148. Huang YK, Yu JC, Kang WM, et al. Significance of Serum Pepsinogens as a Biomarker for Gastric Cancer and Atrophic Gastritis Screening: A Systematic Review and Meta-Analysis. *PLoS One*. 2015;10(11):e0142080.
149. !!! INVALID CITATION !!!
150. Syrjänen K. A Panel of Serum Biomarkers (GastroPanel®) in Non-invasive Diagnosis of Atrophic Gastritis. Systematic Review and Meta-analysis. *Anticancer Res*. 2016;36(10):5133-5144.
151. Komatsu Y, Jobe BA. Endoscopic Resection for Gastric Cancer. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:163-173.
152. Badgwell B, Mansfield PF. Open Methods of Resection and Reconstruction for Subtotal and Total Gastrectomy. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:199-209.
153. Ku GY, Ilson DH. Neoadjuvant and Adjuvant Treatment—Strategies and Clinical Trials—Western Perspective. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:297-302.
154. Hajj C, Goodman KA. Radiation Treatment for Gastric Cancer. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:307-315.
155. Jácome AA, Coutinho AK, Lima EM, Andrade AC, Dos Santos JS. Personalized medicine in gastric cancer: Where are we and where are we going? *World J Gastroenterol*. 2016;22(3):1160-1171.
156. Lianos GD, Mangano A, Rausei S, et al. Targeted Therapy and Novel Agents for the Treatment of Gastric Cancer: A View Toward the Future. In: Strong VE, ed. *Gastric Cancer: Principles and Practice*. Cham: Springer International Publishing; 2015:317-330.
157. Song H, Zhu J, Lu D. Molecular-targeted first-line therapy for advanced gastric cancer. *Cochrane Database Syst Rev*. 2016;7:CD011461.
158. Nguyen PH, Giraud J, Staedel C, et al. All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. *Oncogene*. 2016;35(43):5619-5628.
159. Hayakawa Y, Fox JG, Gonda T, Worthley DL, Muthupalani S, Wang TC. Mouse models of gastric cancer. *Cancers (Basel)*. 2013;5(1):92-130.
160. Treuting PM, Valasek MA, Dintzis SM. 11 - Upper Gastrointestinal Tract. In: *Comparative Anatomy and Histology*. San Diego: Academic Press; 2012:155-175.
161. Sausville EA, Burger AM. Contributions of human tumor xenografts to anticancer drug development. *Cancer Res*. 2006;66(7):3351-3354, discussion 3354.
162. Shultz LD, Goodwin N, Ishikawa F, Hosur V, Lyons BL, Greiner DL. Human cancer growth and therapy in immunodeficient mouse models. *Cold Spring Harb Protoc*. 2014;2014(7):694-708.

163. Choi YY, Lee JE, Kim H, et al. Establishment and characterisation of patient-derived xenografts as preclinical models for gastric cancer. *Sci Rep*. 2016;6:22172.
164. Zhu Y, Tian T, Li Z, et al. Establishment and characterization of patient-derived tumor xenograft using gastroscopic biopsies in gastric cancer. *Sci Rep*. 2015;5:8542.
165. Hoffman RM. Patient-derived orthotopic xenografts: better mimic of metastasis than subcutaneous xenografts. *Nat Rev Cancer*. 2015;15(8):451-452.
166. Siolas D, Hannon GJ. Patient-derived tumor xenografts: transforming clinical samples into mouse models. *Cancer Res*. 2013;73(17):5315-5319.
167. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.
168. Eckfeldt CE, Mendenhall EM, Verfaillie CM. The molecular repertoire of the 'almighty' stem cell. *Nat Rev Mol Cell Biol*. 2005;6(9):726-737.
169. He S, Nakada D, Morrison SJ. Mechanisms of stem cell self-renewal. *Annu Rev Cell Dev Biol*. 2009;25:377-406.
170. Huang G, Ye S, Zhou X, Liu D, Ying QL. Molecular basis of embryonic stem cell self-renewal: from signaling pathways to pluripotency network. *Cell Mol Life Sci*. 2015;72(9):1741-1757.
171. Fuchs E, Chen T. A matter of life and death: self-renewal in stem cells. *EMBO Rep*. 2013;14(1):39-48.
172. Wang Z, Ema H. Mechanisms of self-renewal in hematopoietic stem cells. *Int J Hematol*. 2016;103(5):498-509.
173. Simons BD, Clevers H. Stem cell self-renewal in intestinal crypt. *Exp Cell Res*. 2011;317(19):2719-2724.
174. López-Arribillaga E, Rodilla V, Pellegrinet L, et al. Bmi1 regulates murine intestinal stem cell proliferation and self-renewal downstream of Notch. *Development*. 2015;142(1):41-50.
175. Fink J, Koo BK. Clonal Evolution of Stem Cells in the Gastrointestinal Tract. *Adv Exp Med Biol*. 2016;908:11-25.
176. Lee ER, Leblond CP. Dynamic histology of the antral epithelium in the mouse stomach: II. Ultrastructure and renewal of isthmal cells. *Am J Anat*. 1985;172(3):205-224.
177. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. *Anat Rec*. 1993;236(2):259-279.
178. Bjerknes M, Cheng H. Multipotential stem cells in adult mouse gastric epithelium. *Am J Physiol Gastrointest Liver Physiol*. 2002;283(3):G767-777.
179. O'Brien CA, Kreso A, Dick JE. Cancer stem cells in solid tumors: an overview. *Semin Radiat Oncol*. 2009;19(2):71-77.
180. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell*. 2014;14(3):275-291.
181. Song Y, Wang Y, Tong C, Xi H, Zhao X, Chen L. A unified model of the hierarchical and stochastic theories of gastric cancer. *Br J Cancer*. 2017.
182. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3(7):730-737.
183. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;100(7):3983-3988.
184. Takaishi S, Okumura T, Tu S, et al. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells*. 2009;27(5):1006-1020.
185. Fukuda K, Saikawa Y, Ohashi M, et al. Tumor initiating potential of side population cells in human gastric cancer. *Int J Oncol*. 2009;34(5):1201-1207.
186. Sugihara E, Shimizu T, Kojima K, et al. Ink4a and Arf are crucial factors in the determination of the cell of origin and the therapeutic sensitivity of Myc-induced mouse lymphoid tumor. *Oncogene*. 2012;31(23):2849-2861.

187. Fischbach NA, Rozenfeld S, Shen W, et al. HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo. *Blood*. 2005;105(4):1456-1466.
188. Huntly BJ, Shigematsu H, Deguchi K, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell*. 2004;6(6):587-596.
189. Krivtsov AV, Feng Z, Armstrong SA. Transformation from committed progenitor to leukemia stem cells. *Ann N Y Acad Sci*. 2009;1176:144-149.
190. Barker N, Ridgway RA, van Es JH, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature*. 2009;457(7229):608-611.
191. Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. *Science*. 2004;306(5701):1568-1571.
192. Varon C, Dubus P, Mazurier F, et al. Helicobacter pylori infection recruits bone marrow-derived cells that participate in gastric preneoplasia in mice. *Gastroenterology*. 2012;142(2):281-291.
193. Barker N, Huch M, Kujala P, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell*. 2010;6(1):25-36.
194. Li XB, Yang G, Zhu L, et al. Gastric Lgr5(+) stem cells are the cellular origin of invasive intestinal-type gastric cancer in mice. *Cell Res*. 2016;26(7):838-849.
195. Li Q, Jia Z, Wang L, et al. Disruption of Klf4 in villin-positive gastric progenitor cells promotes formation and progression of tumors of the antrum in mice. *Gastroenterology*. 2012;142(3):531-542.
196. Kirchner T, Müller S, Hattori T, et al. Metaplasia, intraepithelial neoplasia and early cancer of the stomach are related to dedifferentiated epithelial cells defined by cytokeratin-7 expression in gastritis. *Virchows Arch*. 2001;439(4):512-522.
197. Nam KT, Lee HJ, Sousa JF, et al. Mature chief cells are cryptic progenitors for metaplasia in the stomach. *Gastroenterology*. 2010;139(6):2028-2037.e2029.
198. Schatton T, Frank NY, Frank MH. Identification and targeting of cancer stem cells. *Bioessays*. 2009;31(10):1038-1049.
199. Yan Y, Zuo X, Wei D. Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med*. 2015;4(9):1033-1043.
200. Naor D, Sionov RV, Ish-Shalom D. CD44: structure, function, and association with the malignant process. *Adv Cancer Res*. 1997;71:241-319.
201. Zöller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer*. 2011;11(4):254-267.
202. Williams K, Motiani K, Giridhar PV, Kasper S. CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches. *Exp Biol Med (Maywood)*. 2013;238(3):324-338.
203. Borowicz S, Van Scoyk M, Avasarala S, et al. The soft agar colony formation assay. *J Vis Exp*. 2014(92):e51998.
204. Tirino V, Desiderio V, Paino F, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J*. 2013;27(1):13-24.
205. Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A*. 2004;101(39):14228-14233.
206. Nguyen PH, Giraud J, Chambonnier L, et al. Characterization of Biomarkers of Tumorigenic and Chemoresistant Cancer Stem Cells in Human Gastric Carcinoma. *Clin Cancer Res*. 2016.
207. Marshall B. Helicobacter pylori: 20 years on. *Clin Med*. 2002;2(2):147-152.
208. Marshall B. Gastric spirochaetes: 100 years of discovery before and after Kobayashi. *Keio J Med*. 2002;51 Suppl 2:33-37.
209. Luck JM, Seth TN. Gastric Urease. *Biochem J*. 1924;18(6):1227-1231.
210. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet*. 1984;1(8390):1311-1315.

211. GOODWIN CS, ARMSTRONG JA, CHILVERS T, et al. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov. Respectively. *International Journal of Systematic Bacteriology*. 1989;39(4):397-405.
212. Andersen LP, Holck S, Povlsen CO, Elsborg L, Justesen T. Campylobacter pyloridis in peptic ulcer disease. I. Gastric and duodenal infection caused by C. pyloridis: histopathologic and microbiologic findings. *Scand J Gastroenterol*. 1987;22(2):219-224.
213. Morris A, Nicholson G. Ingestion of Campylobacter pyloridis causes gastritis and raised fasting gastric pH. *Am J Gastroenterol*. 1987;82(3):192-199.
214. Buck GE, Gourley WK, Lee WK, Subramanyam K, Latimer JM, DiNuzzo AR. Relation of Campylobacter pyloridis to gastritis and peptic ulcer. *J Infect Dis*. 1986;153(4):664-669.
215. Goodwin CS, Armstrong JA, Marshall BJ. Campylobacter pyloridis, gastritis, and peptic ulceration. *J Clin Pathol*. 1986;39(4):353-365.
216. An international association between Helicobacter pylori infection and gastric cancer. The EUROGAST Study Group. *Lancet*. 1993;341(8857):1359-1362.
217. Hansson LE, Engstrand L, Nyrén O, et al. Helicobacter pylori infection: independent risk indicator of gastric adenocarcinoma. *Gastroenterology*. 1993;105(4):1098-1103.
218. Blaser MJ, Kobayashi K, Cover TL, Cao P, Feurer ID, Pérez-Pérez GI. Helicobacter pylori infection in Japanese patients with adenocarcinoma of the stomach. *Int J Cancer*. 1993;55(5):799-802.
219. Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum*. 1994;61:1-241.
220. Khalifa MM, Sharaf RR, Aziz RK. Helicobacter pylori: a poor man's gut pathogen? *Gut Pathog*. 2010;2(1):2.
221. Peleteiro B, Bastos A, Ferro A, Lunet N. Prevalence of Helicobacter pylori infection worldwide: a systematic review of studies with national coverage. *Dig Dis Sci*. 2014;59(8):1698-1709.
222. Bruce MG, Maarros HI. Epidemiology of Helicobacter pylori infection. *Helicobacter*. 2008;13 Suppl 1:1-6.
223. Banatvala N, Mayo K, Megraud F, Jennings R, Deeks JJ, Feldman RA. The cohort effect and Helicobacter pylori. *J Infect Dis*. 1993;168(1):219-221.
224. Harvey RF, Spence RW, Lane JA, et al. Relationship between the birth cohort pattern of Helicobacter pylori infection and the epidemiology of duodenal ulcer. *QJM*. 2002;95(8):519-525.
225. Roosendaal R, Kuipers EJ, Buitenwerf J, et al. Helicobacter pylori and the birth cohort effect: evidence of a continuous decrease of infection rates in childhood. *Am J Gastroenterol*. 1997;92(9):1480-1482.
226. Grad YH, Lipsitch M, Aiello AE. Secular trends in Helicobacter pylori seroprevalence in adults in the United States: evidence for sustained race/ethnic disparities. *Am J Epidemiol*. 2012;175(1):54-59.
227. Muhammad JS, Zaidi SF, Sugiyama T. Epidemiological ins and outs of helicobacter pylori: a review. *J Pak Med Assoc*. 2012;62(9):955-959.
228. Wen S, Moss SF. Helicobacter pylori virulence factors in gastric carcinogenesis. *Cancer Lett*. 2009;282(1):1-8.
229. Mentis A, Lehours P, Mégraud F. Epidemiology and Diagnosis of Helicobacter pylori infection. *Helicobacter*. 2015;20 Suppl 1:1-7.
230. Malaty HM. Epidemiology of Helicobacter pylori infection. *Best Pract Res Clin Gastroenterol*. 2007;21(2):205-214.
231. Osaki T, Okuda M, Ueda J, et al. Multilocus sequence typing of DNA from faecal specimens for the analysis of intra-familial transmission of Helicobacter pylori. *J Med Microbiol*. 2013;62(Pt 5):761-765.
232. van Duynhoven YT, de Jonge R. Transmission of Helicobacter pylori: a role for food? *Bull World Health Organ*. 2001;79(5):455-460.

233. Vaira D, Holton J, Ricci C, et al. Review article: the transmission of *Helicobacter pylori* from stomach to stomach. *Aliment Pharmacol Ther.* 2001;15 Suppl 1:33-42.
234. Gião MS, Azevedo NF, Wilks SA, Vieira MJ, Keevil CW. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. *Appl Environ Microbiol.* 2008;74(19):5898-5904.
235. Ahmed KS, Khan AA, Ahmed I, et al. Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Med J.* 2007;48(6):543-549.
236. Bellack NR, Koehoorn MW, MacNab YC, Morshed MG. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. *Epidemiol Infect.* 2006;134(3):439-449.
237. Schwarz S, Morelli G, Kusecek B, et al. Horizontal versus familial transmission of *Helicobacter pylori*. *PLoS Pathog.* 2008;4(10):e1000180.
238. Garrity GM, Bell JA, Lilburn T. Class V. Epsilonproteobacteria class. nov. In: Brenner DJ, Krieg NR, Staley JT, eds. *Bergey's Manual® of Systematic Bacteriology: Volume Two The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria.* Boston, MA: Springer US; 2005:1145-1194.
239. Euzéby JP. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *Int J Syst Bacteriol.* 1997;47(2):590-592.
240. O'Rourke J, Bode G. Morphology and Ultrastructure. In: Mobley H, Mendz G, Hazell S, eds. *Helicobacter pylori: Physiology and Genetics.* Washington (DC): ASM Press; 2001.
241. Owen RJ. *Helicobacter*--species classification and identification. *Br Med Bull.* 1998;54(1):17-30.
242. Wen Y, Marcus EA, Matrubutham U, Gleeson MA, Scott DR, Sachs G. Acid-adaptive genes of *Helicobacter pylori*. *Infect Immun.* 2003;71(10):5921-5939.
243. Dunn BE, Campbell GP, Perez-Perez GI, Blaser MJ. Purification and characterization of urease from *Helicobacter pylori*. *J Biol Chem.* 1990;265(16):9464-9469.
244. Ha NC, Oh ST, Sung JY, Cha KA, Lee MH, Oh BH. Supramolecular assembly and acid resistance of *Helicobacter pylori* urease. *Nat Struct Biol.* 2001;8(6):505-509.
245. Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun.* 1991;59(7):2470-2475.
246. Tsuda M, Karita M, Mizote T, Morshed MG, Okita K, Nakazawa T. Essential role of *Helicobacter pylori* urease in gastric colonization: definite proof using a urease-negative mutant constructed by gene replacement. *Eur J Gastroenterol Hepatol.* 1994;6 Suppl 1:S49-52.
247. Kuwahara H, Miyamoto Y, Akaike T, et al. *Helicobacter pylori* urease suppresses bactericidal activity of peroxyntrite via carbon dioxide production. *Infect Immun.* 2000;68(8):4378-4383.
248. Fan X, Gunasena H, Cheng Z, et al. *Helicobacter pylori* urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J Immunol.* 2000;165(4):1918-1924.
249. Malfertheiner P, Megraud F, O'Morain CA, et al. Management of *Helicobacter pylori* infection--the Maastricht V/Florence Consensus Report. *Gut.* 2017;66(1):6-30.
250. Yu XD, Zheng RB, Xie JH, et al. Biological evaluation and molecular docking of baicalin and scutellarin as *Helicobacter pylori* urease inhibitors. *J Ethnopharmacol.* 2015;162:69-78.
251. Ardekani LS, Gargari SL, Rasooli I, et al. A novel nanobody against urease activity of *Helicobacter pylori*. *Int J Infect Dis.* 2013;17(9):e723-728.
252. Scott DR, Marcus EA, Wen Y, Oh J, Sachs G. Gene expression in vivo shows that *Helicobacter pylori* colonizes an acidic niche on the gastric surface. *Proc Natl Acad Sci U S A.* 2007;104(17):7235-7240.
253. Akada JK, Shirai M, Takeuchi H, Tsuda M, Nakazawa T. Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Mol Microbiol.* 2000;36(5):1071-1084.
254. Fong YH, Wong HC, Yuen MH, Lau PH, Chen YW, Wong KB. Structure of UreG/UreF/UreH complex reveals how urease accessory proteins facilitate maturation of *Helicobacter pylori* urease. *PLoS Biol.* 2013;11(10):e1001678.

255. McGowan CC, Necheva A, Thompson SA, Cover TL, Blaser MJ. Acid-induced expression of an LPS-associated gene in *Helicobacter pylori*. *Mol Microbiol.* 1998;30(1):19-31.
256. Pflock M, Kennard S, Finsterer N, Beier D. Acid-responsive gene regulation in the human pathogen *Helicobacter pylori*. *J Biotechnol.* 2006;126(1):52-60.
257. Pflock M, Finsterer N, Joseph B, Mollenkopf H, Meyer TF, Beier D. Characterization of the ArsRS regulon of *Helicobacter pylori*, involved in acid adaptation. *J Bacteriol.* 2006;188(10):3449-3462.
258. Loh JT, Gupta SS, Friedman DB, Krezel AM, Cover TL. Analysis of protein expression regulated by the *Helicobacter pylori* ArsRS two-component signal transduction system. *J Bacteriol.* 2010;192(8):2034-2043.
259. Kavermann H, Burns BP, Angermuller K, et al. Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. *J Exp Med.* 2003;197(7):813-822.
260. Baldwin DN, Shepherd B, Kraemer P, et al. Identification of *Helicobacter pylori* genes that contribute to stomach colonization. *Infect Immun.* 2007;75(2):1005-1016.
261. Eaton KA, Morgan DR, Krakowka S. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. *J Med Microbiol.* 1992;37(2):123-127.
262. Kao CY, Sheu BS, Sheu SM, et al. Higher motility enhances bacterial density and inflammatory response in dyspeptic patients infected with *Helicobacter pylori*. *Helicobacter.* 2012;17(6):411-416.
263. Aihara E, Closson C, Matthis AL, et al. Motility and chemotaxis mediate the preferential colonization of gastric injury sites by *Helicobacter pylori*. *PLoS Pathog.* 2014;10(7):e1004275.
264. Martínez LE, Hardcastle JM, Wang J, et al. *Helicobacter pylori* strains vary cell shape and flagellum number to maintain robust motility in viscous environments. *Mol Microbiol.* 2016;99(1):88-110.
265. Geis G, Leying H, Suerbaum S, Mai U, Opferkuch W. Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. *J Clin Microbiol.* 1989;27(3):436-441.
266. Josenhans C, Labigne A, Suerbaum S. Comparative ultrastructural and functional studies of *Helicobacter pylori* and *Helicobacter mustelae* flagellin mutants: both flagellin subunits, FlaA and FlaB, are necessary for full motility in *Helicobacter* species. *J Bacteriol.* 1995;177(11):3010-3020.
267. Kostrzynska M, Betts JD, Austin JW, Trust TJ. Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. *J Bacteriol.* 1991;173(3):937-946.
268. Geis G, Suerbaum S, Forsthoff B, Leying H, Opferkuch W. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. *J Med Microbiol.* 1993;38(5):371-377.
269. Soleimani N, Mohabati Mobarez A, Farhangi B. Cloning, expression and purification flagellar sheath adhesion of *Helicobacter pylori* in *Escherichia coli* host as a vaccination target. *Clin Exp Vaccine Res.* 2016;5(1):19-25.
270. Yoshiyama H, Nakazawa T. Unique mechanism of *Helicobacter pylori* for colonizing the gastric mucus. *Microbes Infect.* 2000;2(1):55-60.
271. Qin Z, Lin WT, Zhu S, Franco AT, Liu J. Imaging the motility and chemotaxis machineries in *Helicobacter pylori* by cryo-electron tomography. *J Bacteriol.* 2016.
272. Sycuro LK, Pincus Z, Gutierrez KD, et al. Peptidoglycan crosslinking relaxation promotes *Helicobacter pylori*'s helical shape and stomach colonization. *Cell.* 2010;141(5):822-833.
273. Sycuro LK, Wyckoff TJ, Biboy J, et al. Multiple peptidoglycan modification networks modulate *Helicobacter pylori*'s cell shape, motility, and colonization potential. *PLoS Pathog.* 2012;8(3):e1002603.
274. Sycuro LK, Rule CS, Petersen TW, et al. Flow cytometry-based enrichment for cell shape mutants identifies multiple genes that influence *Helicobacter pylori* morphology. *Mol Microbiol.* 2013;90(4):869-883.
275. Bonis M, Ecobichon C, Guadagnini S, Prévost MC, Boneca IG. A M23B family metallopeptidase of *Helicobacter pylori* required for cell shape, pole formation and virulence. *Mol Microbiol.* 2010;78(4):809-819.

276. Keilberg D, Ottemann KM. How *Helicobacter pylori* senses, targets and interacts with the gastric epithelium. *Environ Microbiol.* 2016;18(3):791-806.
277. Lam KH, Ling TK, Au SW. Crystal structure of activated CheY1 from *Helicobacter pylori*. *J Bacteriol.* 2010;192(9):2324-2334.
278. Foynes S, Dorrell N, Ward SJ, et al. *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infect Immun.* 2000;68(4):2016-2023.
279. Pittman MS, Goodwin M, Kelly DJ. Chemotaxis in the human gastric pathogen *Helicobacter pylori*: different roles for CheW and the three CheV paralogues, and evidence for CheV2 phosphorylation. *Microbiology.* 2001;147(Pt 9):2493-2504.
280. Lertsethtakarn P, Ottemann KM, Hendrixson DR. Motility and chemotaxis in *Campylobacter* and *Helicobacter*. *Annu Rev Microbiol.* 2011;65:389-410.
281. Rolig AS, Shanks J, Carter JE, Ottemann KM. *Helicobacter pylori* requires TlpD-driven chemotaxis to proliferate in the antrum. *Infect Immun.* 2012;80(10):3713-3720.
282. Andermann TM, Chen YT, Ottemann KM. Two predicted chemoreceptors of *Helicobacter pylori* promote stomach infection. *Infect Immun.* 2002;70(10):5877-5881.
283. Croxen MA, Sisson G, Melano R, Hoffman PS. The *Helicobacter pylori* chemotaxis receptor TlpB (HP0103) is required for pH taxis and for colonization of the gastric mucosa. *J Bacteriol.* 2006;188(7):2656-2665.
284. Cerda OA, Núñez-Villena F, Soto SE, Ugalde JM, López-Solís R, Toledo H. tlpA gene expression is required for arginine and bicarbonate chemotaxis in *Helicobacter pylori*. *Biol Res.* 2011;44(3):277-282.
285. Huang JY, Sweeney EG, Sigal M, et al. Chemodetection and Destruction of Host Urea Allows *Helicobacter pylori* to Locate the Epithelium. *Cell Host Microbe.* 2015;18(2):147-156.
286. Sanders L, Andermann TM, Ottemann KM. A supplemented soft agar chemotaxis assay demonstrates the *Helicobacter pylori* chemotactic response to zinc and nickel. *Microbiology.* 2013;159(Pt 1):46-57.
287. Schreiber S, Konradt M, Groll C, et al. The spatial orientation of *Helicobacter pylori* in the gastric mucus. *Proc Natl Acad Sci U S A.* 2004;101(14):5024-5029.
288. Rader BA, Wreden C, Hicks KG, Sweeney EG, Ottemann KM, Guillemin K. *Helicobacter pylori* perceives the quorum-sensing molecule AI-2 as a chemorepellent via the chemoreceptor TlpB. *Microbiology.* 2011;157(Pt 9):2445-2455.
289. Odenbreit S. Adherence properties of *Helicobacter pylori*: impact on pathogenesis and adaptation to the host. *Int J Med Microbiol.* 2005;295(5):317-324.
290. Kirschner DE, Blaser MJ. The dynamics of *Helicobacter pylori* infection of the human stomach. *J Theor Biol.* 1995;176(2):281-290.
291. Voss BJ, Gaddy JA, McDonald WH, Cover TL. Analysis of surface-exposed outer membrane proteins in *Helicobacter pylori*. *J Bacteriol.* 2014;196(13):2455-2471.
292. Alm RA, Bina J, Andrews BM, Doig P, Hancock RE, Trust TJ. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. *Infect Immun.* 2000;68(7):4155-4168.
293. Oleastro M, Ménard A. The Role of *Helicobacter pylori* Outer Membrane Proteins in Adherence and Pathogenesis. *Biology (Basel).* 2013;2(3):1110-1134.
294. Sheu BS, Yang HB, Yeh YC, Wu JJ. *Helicobacter pylori* colonization of the human gastric epithelium: a bug's first step is a novel target for us. *J Gastroenterol Hepatol.* 2010;25(1):26-32.
295. Moore ME, Borén T, Solnick JV. Life at the margins: modulation of attachment proteins in *Helicobacter pylori*. *Gut Microbes.* 2011;2(1):42-46.
296. Oleastro M, Cordeiro R, Ferrand J, et al. Evaluation of the clinical significance of homB, a novel candidate marker of *Helicobacter pylori* strains associated with peptic ulcer disease. *J Infect Dis.* 2008;198(9):1379-1387.

297. Yeh YC, Chang WL, Yang HB, Cheng HC, Wu JJ, Sheu BS. H. pylori cagL amino acid sequence polymorphism Y58E59 induces a corpus shift of gastric integrin  $\alpha 5\beta 1$  related with gastric carcinogenesis. *Mol Carcinog.* 2011;50(10):751-759.
298. Yamaoka Y, Ojo O, Fujimoto S, et al. Helicobacter pylori outer membrane proteins and gastroduodenal disease. *Gut.* 2006;55(6):775-781.
299. Covacci A, Censini S, Bugnoli M, et al. Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A.* 1993;90(12):5791-5795.
300. Wroblewski LE, Peek RM, Wilson KT. Helicobacter pylori and gastric cancer: factors that modulate disease risk. *Clin Microbiol Rev.* 2010;23(4):713-739.
301. Weel JF, van der Hulst RW, Gerrits Y, et al. The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and Helicobacter pylori-related diseases. *J Infect Dis.* 1996;173(5):1171-1175.
302. Selbach M, Moese S, Hauck CR, Meyer TF, Backert S. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. *J Biol Chem.* 2002;277(9):6775-6778.
303. Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol.* 2002;43(4):971-980.
304. Poppe M, Feller SM, Römer G, Wessler S. Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. *Oncogene.* 2007;26(24):3462-3472.
305. Tammer I, Brandt S, Hartig R, König W, Backert S. Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology.* 2007;132(4):1309-1319.
306. Higashi H, Tsutsumi R, Muto S, et al. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. *Science.* 2002;295(5555):683-686.
307. Yamazaki S, Yamakawa A, Ito Y, et al. The CagA protein of Helicobacter pylori is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. *J Infect Dis.* 2003;187(2):334-337.
308. Higashi H, Nakaya A, Tsutsumi R, et al. Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem.* 2004;279(17):17205-17216.
309. Neel BG, Gu H, Pao L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci.* 2003;28(6):284-293.
310. Tsutsumi R, Takahashi A, Azuma T, Higashi H, Hatakeyama M. Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. *Mol Cell Biol.* 2006;26(1):261-276.
311. Murata-Kamiya N. Pathophysiological functions of the CagA oncoprotein during infection by Helicobacter pylori. *Microbes Infect.* 2011;13(10):799-807.
312. Selbach M, Moese S, Hurwitz R, Hauck CR, Meyer TF, Backert S. The Helicobacter pylori CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J.* 2003;22(3):515-528.
313. Saadat I, Higashi H, Obuse C, et al. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature.* 2007;447(7142):330-333.
314. Kikuchi K, Murata-Kamiya N, Kondo S, Hatakeyama M. Helicobacter pylori stimulates epithelial cell migration via CagA-mediated perturbation of host cell signaling. *Microbes Infect.* 2012;14(5):470-476.
315. Higashi H, Tsutsumi R, Fujita A, et al. Biological activity of the Helicobacter pylori virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci U S A.* 2002;99(22):14428-14433.
316. Azuma T, Yamakawa A, Yamazaki S, et al. Correlation between variation of the 3' region of the cagA gene in Helicobacter pylori and disease outcome in Japan. *J Infect Dis.* 2002;186(11):1621-1630.

317. Hatakeyama M. Linking epithelial polarity and carcinogenesis by multitasking *Helicobacter pylori* virulence factor CagA. *Oncogene*. 2008;27(55):7047-7054.
318. Handa O, Naito Y, Yoshikawa T. CagA protein of *Helicobacter pylori*: a hijacker of gastric epithelial cell signaling. *Biochem Pharmacol*. 2007;73(11):1697-1702.
319. Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. *Gastroenterology*. 2004;127(2):514-523.
320. Nagase L, Hayashi T, Senda T, Hatakeyama M. Dramatic increase in SHP2 binding activity of *Helicobacter pylori* Western CagA by EPIYA-C duplication: its implications in gastric carcinogenesis. *Sci Rep*. 2015;5:15749.
321. Ferreira RM, Machado JC, Leite M, Carneiro F, Figueiredo C. The number of *Helicobacter pylori* CagA EPIYA C tyrosine phosphorylation motifs influences the pattern of gastritis and the development of gastric carcinoma. *Histopathology*. 2012;60(6):992-998.
322. Mimuro H, Suzuki T, Tanaka J, Asahi M, Haas R, Sasakawa C. Grb2 is a key mediator of *Helicobacter pylori* CagA protein activities. *Mol Cell*. 2002;10(4):745-755.
323. Churin Y, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol*. 2003;161(2):249-255.
324. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science*. 2003;300(5624):1430-1434.
325. Bagnoli F, Buti L, Tompkins L, Covacci A, Amieva MR. *Helicobacter pylori* CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci U S A*. 2005;102(45):16339-16344.
326. Censini S, Lange C, Xiang Z, et al. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A*. 1996;93(25):14648-14653.
327. Olbermann P, Josenhans C, Moodley Y, et al. A global overview of the genetic and functional diversity in the *Helicobacter pylori* *cag* pathogenicity island. *PLoS Genet*. 2010;6(8):e1001069.
328. Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. *Helicobacter pylori* virulence and genetic geography. *Science*. 1999;284(5418):1328-1333.
329. Akopyants NS, Clifton SW, Kersulyte D, et al. Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Mol Microbiol*. 1998;28(1):37-53.
330. Maeda S, Yoshida H, Ikenoue T, et al. Structure of *cag* pathogenicity island in Japanese *Helicobacter pylori* isolates. *Gut*. 1999;44(3):336-341.
331. Nilsson C, Sillén A, Eriksson L, et al. Correlation between *cag* pathogenicity island composition and *Helicobacter pylori*-associated gastroduodenal disease. *Infect Immun*. 2003;71(11):6573-6581.
332. Salama N, Guillemin K, McDaniel TK, Sherlock G, Tompkins L, Falkow S. A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. *Proc Natl Acad Sci U S A*. 2000;97(26):14668-14673.
333. Israel DA, Salama N, Arnold CN, et al. *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J Clin Invest*. 2001;107(5):611-620.
334. Hsu PI, Hwang IR, Cittelly D, et al. Clinical presentation in relation to diversity within the *Helicobacter pylori* *cag* pathogenicity island. *Am J Gastroenterol*. 2002;97(9):2231-2238.
335. Ikenoue T, Maeda S, Ogura K, et al. Determination of *Helicobacter pylori* virulence by simple gene analysis of the *cag* pathogenicity island. *Clin Diagn Lab Immunol*. 2001;8(1):181-186.
336. Ta LH, Hansen LM, Sause WE, et al. Conserved Transcriptional Unit Organization of the Cag Pathogenicity Island among *Helicobacter pylori* Strains. *Front Cell Infect Microbiol*. 2012;2:46.

337. Peek RM, Fiske C, Wilson KT. Role of innate immunity in Helicobacter pylori-induced gastric malignancy. *Physiol Rev.* 2010;90(3):831-858.
338. Tegtmeyer N, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in Helicobacter pylori pathogenesis. *FEBS J.* 2011;278(8):1190-1202.
339. Keates S, Keates AC, Warny M, Peek RM, Murray PG, Kelly CP. Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by cag+ and cag- Helicobacter pylori. *J Immunol.* 1999;163(10):5552-5559.
340. Naumann M, Wessler S, Bartsch C, et al. Activation of activator protein 1 and stress response kinases in epithelial cells colonized by Helicobacter pylori encoding the cag pathogenicity island. *J Biol Chem.* 1999;274(44):31655-31662.
341. Crabtree JE. Role of cytokines in pathogenesis of Helicobacter pylori-induced mucosal damage. *Dig Dis Sci.* 1998;43(9 Suppl):46S-55S.
342. Yamaoka Y, Kita M, Kodama T, Sawai N, Imanishi J. Helicobacter pylori cagA gene and expression of cytokine messenger RNA in gastric mucosa. *Gastroenterology.* 1996;110(6):1744-1752.
343. Yamaoka Y, Kodama T, Kita M, Imanishi J, Kashima K, Graham DY. Relation between cytokines and Helicobacter pylori in gastric cancer. *Helicobacter.* 2001;6(2):116-124.
344. Sánchez-Zauco NA, Torres J, Pérez-Figueroa GE, et al. Impact of cagPAI and T4SS on the inflammatory response of human neutrophils to Helicobacter pylori infection. *PLoS One.* 2014;8(6):e64623.
345. Backert S, Selbach M. Role of type IV secretion in Helicobacter pylori pathogenesis. *Cell Microbiol.* 2008;10(8):1573-1581.
346. Hatakeyama M. Helicobacter pylori CagA--a potential bacterial oncoprotein that functionally mimics the mammalian Gab family of adaptor proteins. *Microbes Infect.* 2003;5(2):143-150.
347. Cover TL, Blaser MJ. Purification and characterization of the vacuolating toxin from Helicobacter pylori. *J Biol Chem.* 1992;267(15):10570-10575.
348. Lu H, Yamaoka Y, Graham DY. Helicobacter pylori virulence factors: facts and fantasies. *Curr Opin Gastroenterol.* 2005;21(6):653-659.
349. Cover TL. The vacuolating cytotoxin of Helicobacter pylori. *Mol Microbiol.* 1996;20(2):241-246.
350. Szabò I, Brutsche S, Tombola F, et al. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of Helicobacter pylori is required for its biological activity. *EMBO J.* 1999;18(20):5517-5527.
351. Tombola F, Carlesso C, Szabò I, et al. Helicobacter pylori vacuolating toxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophys J.* 1999;76(3):1401-1409.
352. Lanzavecchia S, Bellon PL, Lupetti P, Dallai R, Rappuoli R, Telford JL. Three-dimensional reconstruction of metal replicas of the Helicobacter pylori vacuolating cytotoxin. *J Struct Biol.* 1998;121(1):9-18.
353. Atherton JC. The pathogenesis of Helicobacter pylori-induced gastro-duodenal diseases. *Annu Rev Pathol.* 2006;1:63-96.
354. Molinari M, Galli C, Norais N, et al. Vacuoles induced by Helicobacter pylori toxin contain both late endosomal and lysosomal markers. *J Biol Chem.* 1997;272(40):25339-25344.
355. Molinari M, Galli C, de Bernard M, et al. The acid activation of Helicobacter pylori toxin VacA: structural and membrane binding studies. *Biochem Biophys Res Commun.* 1998;248(2):334-340.
356. Sommi P, Ricci V, Fiocca R, et al. Persistence of Helicobacter pylori VacA toxin and vacuolating potential in cultured gastric epithelial cells. *Am J Physiol.* 1998;275(4 Pt 1):G681-688.
357. de Bernard M, Burrone D, Papini E, Rappuoli R, Telford J, Montecucco C. Identification of the Helicobacter pylori VacA toxin domain active in the cell cytosol. *Infect Immun.* 1998;66(12):6014-6016.
358. Smoot DT, Resau JH, Earlington MH, Simpson M, Cover TL. Effects of Helicobacter pylori vacuolating cytotoxin on primary cultures of human gastric epithelial cells. *Gut.* 1996;39(6):795-799.

359. Li H, Kalies I, Mellgård B, Helander HF. A rat model of chronic *Helicobacter pylori* infection. Studies of epithelial cell turnover and gastric ulcer healing. *Scand J Gastroenterol.* 1998;33(4):370-378.
360. Cover TL, Krishna US, Israel DA, Peek RM. Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Res.* 2003;63(5):951-957.
361. Peek RM, Blaser MJ, Mays DJ, et al. *Helicobacter pylori* strain-specific genotypes and modulation of the gastric epithelial cell cycle. *Cancer Res.* 1999;59(24):6124-6131.
362. Galmiche A, Rassow J, Doye A, et al. The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J.* 2000;19(23):6361-6370.
363. Willhite DC, Cover TL, Blanke SR. Cellular vacuolation and mitochondrial cytochrome c release are independent outcomes of *Helicobacter pylori* vacuolating cytotoxin activity that are each dependent on membrane channel formation. *J Biol Chem.* 2003;278(48):48204-48209.
364. Boncristiano M, Paccani SR, Barone S, et al. The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med.* 2003;198(12):1887-1897.
365. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R. *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science.* 2003;301(5636):1099-1102.
366. Molinari M, Salio M, Galli C, et al. Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *J Exp Med.* 1998;187(1):135-140.
367. Papini E, Satin B, Norais N, et al. Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *J Clin Invest.* 1998;102(4):813-820.
368. Supajatura V, Ushio H, Wada A, et al. Cutting edge: VacA, a vacuolating cytotoxin of *Helicobacter pylori*, directly activates mast cells for migration and production of proinflammatory cytokines. *J Immunol.* 2002;168(6):2603-2607.
369. Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem.* 1995;270(30):17771-17777.
370. Cover TL, Tummuru MK, Cao P, Thompson SA, Blaser MJ. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J Biol Chem.* 1994;269(14):10566-10573.
371. Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem.* 1995;270(30):17771-17777.
372. Letley DP, Rhead JL, Twells RJ, Dove B, Atherton JC. Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *J Biol Chem.* 2003;278(29):26734-26741.
373. Pagliaccia C, de Bernard M, Lupetti P, et al. The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc Natl Acad Sci U S A.* 1998;95(17):10212-10217.
374. Ji X, Fernandez T, Burrone D, et al. Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infect Immun.* 2000;68(6):3754-3757.
375. Atherton JC, Peek RM, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology.* 1997;112(1):92-99.
376. Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol.* 2010;7(11):629-641.
377. Rhead JL, Letley DP, Mohammadi M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology.* 2007;133(3):926-936.
378. Maldonado RF, Sá-Correia I, Valvano MA. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. *FEMS Microbiol Rev.* 2016;40(4):480-493.
379. Seeley JJ, Ghosh S. Molecular mechanisms of innate memory and tolerance to LPS. *J Leukoc Biol.* 2017;101(1):107-119.

380. Cullen TW, Giles DK, Wolf LN, Ecobichon C, Boneca IG, Trent MS. Helicobacter pylori versus the host: remodeling of the bacterial outer membrane is required for survival in the gastric mucosa. *PLoS Pathog*. 2011;7(12):e1002454.
381. Appelmek BJ, Monteiro MA, Martin SL, Moran AP, Vandenbroucke-Grauls CM. Why Helicobacter pylori has Lewis antigens. *Trends Microbiol*. 2000;8(12):565-570.
382. Monteiro MA, Zheng P, Ho B, et al. Expression of histo-blood group antigens by lipopolysaccharides of Helicobacter pylori strains from asian hosts: the propensity to express type 1 blood-group antigens. *Glycobiology*. 2000;10(7):701-713.
383. Wirth HP, Yang M, Peek RM, Tham KT, Blaser MJ. Helicobacter pylori Lewis expression is related to the host Lewis phenotype. *Gastroenterology*. 1997;113(4):1091-1098.
384. Appelmek BJ, Martino MC, Veenhof E, et al. Phase variation in H type I and Lewis a epitopes of Helicobacter pylori lipopolysaccharide. *Infect Immun*. 2000;68(10):5928-5932.
385. Du MQ, Isaccson PG. Gastric MALT lymphoma: from aetiology to treatment. *Lancet Oncol*. 2002;3(2):97-104.
386. Kapadia CR. Gastric atrophy, metaplasia, and dysplasia: a clinical perspective. *J Clin Gastroenterol*. 2003;36(5 Suppl):S29-36; discussion S61-22.
387. Lauwers GY. Defining the pathologic diagnosis of metaplasia, atrophy, dysplasia, and gastric adenocarcinoma. *J Clin Gastroenterol*. 2003;36(5 Suppl):S37-43; discussion S61-32.
388. Jackson CB, Judd LM, Menheniott TR, et al. Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression. *J Pathol*. 2007;213(2):140-151.
389. Ferreira AC, Isomoto H, Moriyama M, Fujioka T, Machado JC, Yamaoka Y. Helicobacter and gastric malignancies. *Helicobacter*. 2008;13 Suppl 1:28-34.
390. Kato I, Tominaga S, Ito Y, et al. Atrophic gastritis and stomach cancer risk: cross-sectional analyses. *Jpn J Cancer Res*. 1992;83(10):1041-1046.
391. Lindén SK, Driessen KM, McGuckin MA. Improved in vitro model systems for gastrointestinal infection by choice of cell line, pH, microaerobic conditions, and optimization of culture conditions. *Helicobacter*. 2007;12(4):341-353.
392. Jia Q, Feng M, Wang Y, Xue S. Gastric cancer cells in collagen gel matrix: three-dimensional growth and differential expression of adhesion molecules (CD44s, CD54, E-cadherin). *J Biomed Mater Res A*. 2008;84(4):917-925.
393. Kim YJ, Bae HI, Kwon OK, Choi MS. Three-dimensional gastric cancer cell culture using nanofiber scaffold for chemosensitivity test. *Int J Biol Macromol*. 2009;45(1):65-71.
394. Pompaiah M, Bartfeld S. Gastric Organoids: An Emerging Model System to Study Helicobacter pylori Pathogenesis. *Curr Top Microbiol Immunol*. 2017;400:149-168.
395. Bartfeld S, Bayram T, van de Wetering M, et al. In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology*. 2015;148(1):126-136.e126.
396. McCracken KW, Catá EM, Crawford CM, et al. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. *Nature*. 2014;516(7531):400-404.
397. Schlaermann P, Toelle B, Berger H, et al. A novel human gastric primary cell culture system for modelling Helicobacter pylori infection in vitro. *Gut*. 2016;65(2):202-213.
398. Kim DH, Kim SW, Song YJ, et al. Long-term evaluation of mice model infected with Helicobacter pylori: focus on gastric pathology including gastric cancer. *Aliment Pharmacol Ther*. 2003;18 Suppl 1:14-23.
399. Wang X, Willén R, Svensson M, Ljungh A, Wadström T. Two-year follow-up of Helicobacter pylori infection in C57BL/6 and Balb/cA mice. *APMIS*. 2003;111(4):514-522.
400. Rogers AB, Taylor NS, Whary MT, Stefanich ED, Wang TC, Fox JG. Helicobacter pylori but not high salt induces gastric intraepithelial neoplasia in B6129 mice. *Cancer Res*. 2005;65(23):10709-10715.

401. Hahm KB, Lee KM, Kim YB, et al. Conditional loss of TGF-beta signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice. *Aliment Pharmacol Ther.* 2002;16 Suppl 2:115-127.
402. Fox JG, Rogers AB, Whary MT, et al. Accelerated progression of gastritis to dysplasia in the pyloric antrum of TFF2 -/- C57BL6 x Sv129 Helicobacter pylori-infected mice. *Am J Pathol.* 2007;171(5):1520-1528.
403. Kuzushita N, Rogers AB, Monti NA, et al. p27kip1 deficiency confers susceptibility to gastric carcinogenesis in Helicobacter pylori-infected mice. *Gastroenterology.* 2005;129(5):1544-1556.
404. Fox JG, Wang TC, Rogers AB, et al. Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. *Gastroenterology.* 2003;124(7):1879-1890.
405. Tu S, Bhagat G, Cui G, et al. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell.* 2008;14(5):408-419.
406. Yokota K, Kurebayashi Y, Takayama Y, et al. Colonization of Helicobacter pylori in the gastric mucosa of Mongolian gerbils. *Microbiol Immunol.* 1991;35(6):475-480.
407. Hirayama F, Takagi S, Yokoyama Y, Iwao E, Ikeda Y. Establishment of gastric Helicobacter pylori infection in Mongolian gerbils. *J Gastroenterol.* 1996;31 Suppl 9:24-28.
408. Hirayama F, Takagi S, Kusuhara H, Iwao E, Yokoyama Y, Ikeda Y. Induction of gastric ulcer and intestinal metaplasia in mongolian gerbils infected with Helicobacter pylori. *J Gastroenterol.* 1996;31(5):755-757.
409. Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology.* 1998;115(3):642-648.
410. Hirayama F, Takagi S, Iwao E, Yokoyama Y, Haga K, Hanada S. Development of poorly differentiated adenocarcinoma and carcinoid due to long-term Helicobacter pylori colonization in Mongolian gerbils. *J Gastroenterol.* 1999;34(4):450-454.
411. Zheng Q, Chen XY, Shi Y, Xiao SD. Development of gastric adenocarcinoma in Mongolian gerbils after long-term infection with Helicobacter pylori. *J Gastroenterol Hepatol.* 2004;19(10):1192-1198.
412. Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of Helicobacter pylori-induced gastric carcinoma in Mongolian gerbils. *Cancer Res.* 1998;58(19):4255-4259.
413. Honda S, Fujioka T, Tokieda M, Gotoh T, Nishizono A, Nasu M. Gastric ulcer, atrophic gastritis, and intestinal metaplasia caused by Helicobacter pylori infection in Mongolian gerbils. *Scand J Gastroenterol.* 1998;33(5):454-460.
414. Franco AT, Johnston E, Krishna U, et al. Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors. *Cancer Res.* 2008;68(2):379-387.
415. Romero-Gallo J, Harris EJ, Krishna U, Washington MK, Perez-Perez GI, Peek RM. Effect of Helicobacter pylori eradication on gastric carcinogenesis. *Lab Invest.* 2008;88(3):328-336.
416. Elfvin A, Bölin I, Von Bothmer C, et al. Helicobacter pylori induces gastritis and intestinal metaplasia but no gastric adenocarcinoma in Mongolian gerbils. *Scand J Gastroenterol.* 2005;40(11):1313-1320.
417. Harvey K, Tapon N. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer.* 2007;7(3):182-191.
418. Harvey KF, Hariharan IK. The hippo pathway. *Cold Spring Harb Perspect Biol.* 2012;4(8):a011288.
419. Sebio A, Lenz HJ. Molecular Pathways: Hippo Signaling, a Critical Tumor Suppressor. *Clin Cancer Res.* 2015;21(22):5002-5007.
420. Moleirinho S, Tilston-Lunel A, Angus L, Gunn-Moore F, Reynolds PA. The expanding family of FERM proteins. *Biochem J.* 2013;452(2):183-193.
421. Yi C, Kissil J. Merlin and Angiomotin in Hippo-Yap Signaling. In: Oren M, Aylon Y, eds. *The Hippo Signaling Pathway and Cancer.* New York, NY: Springer New York; 2013:11-25.

422. Angus L, Moleirinho S, Herron L, et al. Willin/FRMD6 expression activates the Hippo signaling pathway kinases in mammals and antagonizes oncogenic YAP. *Oncogene*. 2012;31(2):238-250.
423. Kremerskothen J, Plaas C, Büther K, et al. Characterization of KIBRA, a novel WW domain-containing protein. *Biochem Biophys Res Commun*. 2003;300(4):862-867.
424. Baumgartner R, Poernbacher I, Buser N, Hafen E, Stocker H. The WW domain protein Kibra acts upstream of Hippo in *Drosophila*. *Dev Cell*. 2010;18(2):309-316.
425. Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. *Genes Dev*. 2016;30(1):1-17.
426. Slavotinek AM. The Family of Crumbs Genes and Human Disease. *Mol Syndromol*. 2016;7(5):274-281.
427. Thompson BJ, Pichaud F, Röper K. Sticking together the Crumbs - an unexpected function for an old friend. *Nat Rev Mol Cell Biol*. 2013;14(5):307-314.
428. Boggiano JC, Fehon RG. Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. *Dev Cell*. 2012;22(4):695-702.
429. Szymaniak AD, Mahoney JE, Cardoso WV, Varelas X. Crumbs3-Mediated Polarity Directs Airway Epithelial Cell Fate through the Hippo Pathway Effector Yap. *Dev Cell*. 2015;34(3):283-296.
430. Elbediwy A, Vincent-Mistiaen ZI, Thompson BJ. YAP and TAZ in epithelial stem cells: A sensor for cell polarity, mechanical forces and tissue damage. *Bioessays*. 2016;38(7):644-653.
431. Martin-Belmonte F, Perez-Moreno M. Epithelial cell polarity, stem cells and cancer. *Nat Rev Cancer*. 2011;12(1):23-38.
432. Mohseni M, Sun J, Lau A, et al. A genetic screen identifies an LKB1-MARK signalling axis controlling the Hippo-YAP pathway. *Nat Cell Biol*. 2014;16(1):108-117.
433. Gumbiner BM, Kim NG. The Hippo-YAP signaling pathway and contact inhibition of growth. *J Cell Sci*. 2014;127(Pt 4):709-717.
434. Yu FX, Guan KL. The Hippo pathway: regulators and regulations. *Genes Dev*. 2013;27(4):355-371.
435. Kim NG, Koh E, Chen X, Gumbiner BM. E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. *Proc Natl Acad Sci U S A*. 2011;108(29):11930-11935.
436. Reddy P, Deguchi M, Cheng Y, Hsueh AJ. Actin cytoskeleton regulates Hippo signaling. *PLoS One*. 2013;8(9):e73763.
437. Regué L, Mou F, Avruch J. G protein-coupled receptors engage the mammalian Hippo pathway through F-actin: F-Actin, assembled in response to Galpha12/13 induced RhoA-GTP, promotes dephosphorylation and activation of the YAP oncogene. *Bioessays*. 2013;35(5):430-435.
438. Wu S, Huang J, Dong J, Pan D. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*. 2003;114(4):445-456.
439. Harvey KF, Pflieger CM, Hariharan IK. The *Drosophila* Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell*. 2003;114(4):457-467.
440. Galan JA, Avruch J. MST1/MST2 Protein Kinases: Regulation and Physiologic Roles. *Biochemistry*. 2016;55(39):5507-5519.
441. Fitamant J, Zhou D, Mou F, Barrufet LR, Bardeesy N, Avruch J. MST1/2 and Other Upstream Signaling that Affect Hippo Pathway Function. In: Oren M, Aylon Y, eds. *The Hippo Signaling Pathway and Cancer*. New York, NY: Springer New York; 2013:27-49.
442. Justice RW, Zilian O, Woods DF, Noll M, Bryant PJ. The *Drosophila* tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev*. 1995;9(5):534-546.
443. Nishiyama Y, Hirota T, Morisaki T, et al. A human homolog of *Drosophila* warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. *FEBS Lett*. 1999;459(2):159-165.
444. Tao W, Zhang S, Turenchalk GS, et al. Human homologue of the *Drosophila melanogaster* lats tumour suppressor modulates CDC2 activity. *Nat Genet*. 1999;21(2):177-181.

445. Yabuta N, Fujii T, Copeland NG, et al. Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the *Drosophila* tumor suppressor gene *lats/warts*. *Genomics*. 2000;63(2):263-270.
446. Li Y, Pei J, Xia H, Ke H, Wang H, Tao W. Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene*. 2003;22(28):4398-4405.
447. Visser S, Yang X. LATS tumor suppressor: a new governor of cellular homeostasis. *Cell Cycle*. 2010;9(19):3892-3903.
448. Chan EH, Nousiainen M, Chalamalasetty RB, Schäfer A, Nigg EA, Silljé HH. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene*. 2005;24(12):2076-2086.
449. Hoa L, Kulaberoglu Y, Gundogdu R, et al. The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. *Cell Signal*. 2016;28(5):488-497.
450. Hergovich A. Regulation and functions of mammalian LATS/NDR kinases: looking beyond canonical Hippo signalling. *Cell Biosci*. 2013;3(1):32.
451. Lee KH, Goan YG, Hsiao M, et al. MicroRNA-373 (miR-373) post-transcriptionally regulates large tumor suppressor, homolog 2 (LATS2) and stimulates proliferation in human esophageal cancer. *Exp Cell Res*. 2009;315(15):2529-2538.
452. Belair C, Baud J, Chabas S, et al. *Helicobacter pylori* interferes with an embryonic stem cell micro RNA cluster to block cell cycle progression. *Silence*. 2011;2(1):7.
453. Zhang K, Rodriguez-Aznar E, Yabuta N, et al. Lats2 kinase potentiates Snail1 activity by promoting nuclear retention upon phosphorylation. *EMBO J*. 2012;31(1):29-43.
454. Suzuki H, Yabuta N, Okada N, et al. Lats2 phosphorylates p21/CDKN1A after UV irradiation and regulates apoptosis. *J Cell Sci*. 2013;126(Pt 19):4358-4368.
455. Sudol M, Bork P, Einbond A, et al. Characterization of the mammalian YAP (Yes-associated protein) gene and its role in defining a novel protein module, the WW domain. *J Biol Chem*. 1995;270(24):14733-14741.
456. Kanai F, Marignani PA, Sarbassova D, et al. TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *EMBO J*. 2000;19(24):6778-6791.
457. Piccolo S, Dupont S, Cordenonsi M. The biology of YAP/TAZ: hippo signaling and beyond. *Physiol Rev*. 2014;94(4):1287-1312.
458. Hansen CG, Moroishi T, Guan KL. YAP and TAZ: a nexus for Hippo signaling and beyond. *Trends Cell Biol*. 2015;25(9):499-513.
459. Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev*. 2001;15(10):1229-1241.
460. Zhao B, Ye X, Yu J, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev*. 2008;22(14):1962-1971.
461. Bandura JL, Edgar BA. Yorkie and Scalloped: partners in growth activation. *Dev Cell*. 2008;14(3):315-316.
462. Pobbati AV, Hong W. Emerging roles of TEAD transcription factors and its coactivators in cancers. *Cancer Biol Ther*. 2013;14(5):390-398.
463. Krupska I, Bruford EA, Chaqour B. Eyeing the Cyr61/CTGF/NOV (CCN) group of genes in development and diseases: highlights of their structural likenesses and functional dissimilarities. *Hum Genomics*. 2015;9:24.
464. Hall-Glenn F, Lyons KM. Roles for CCN2 in normal physiological processes. *Cell Mol Life Sci*. 2011;68(19):3209-3217.
465. Urtasun R, Latasa MU, Demartis MI, et al. Connective tissue growth factor autocriny in human hepatocellular carcinoma: oncogenic role and regulation by epidermal growth factor receptor/yes-associated protein-mediated activation. *Hepatology*. 2011;54(6):2149-2158.
466. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. *Nat Rev Cancer*. 2013;13(4):246-257.

467. Aylon Y, Ofir-Rosenfeld Y, Yabuta N, et al. The Lats2 tumor suppressor augments p53-mediated apoptosis by promoting the nuclear proapoptotic function of ASPP1. *Genes Dev.* 2010;24(21):2420-2429.
468. Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes Dev.* 2006;20(19):2687-2700.
469. Aylon Y, Yabuta N, Besserglick H, et al. Silencing of the Lats2 tumor suppressor overrides a p53-dependent oncogenic stress checkpoint and enables mutant H-Ras-driven cell transformation. *Oncogene.* 2009;28(50):4469-4479.
470. Zhou Y, Huang T, Cheng AS, Yu J, Kang W, To KF. The TEAD Family and Its Oncogenic Role in Promoting Tumorigenesis. *Int J Mol Sci.* 2016;17(1).
471. Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. *Cancer Cell.* 2016;29(6):783-803.
472. Hiemer SE, Varelas X. Stem cell regulation by the Hippo pathway. *Biochim Biophys Acta.* 2013;1830(2):2323-2334.
473. Xu ZP, Zhu JS, Zhang Q, Wang XY. A breakdown of the Hippo pathway in gastric cancer. *Hepatogastroenterology.* 2011;58(110-111):1611-1617.
474. Jiao S, Wang H, Shi Z, et al. A peptide mimicking VGLL4 function acts as a YAP antagonist therapy against gastric cancer. *Cancer Cell.* 2014;25(2):166-180.
475. Li N, Yu N, Wang J, et al. miR-222/VGLL4/YAP-TEAD1 regulatory loop promotes proliferation and invasion of gastric cancer cells. *Am J Cancer Res.* 2015;5(3):1158-1168.
476. Lim B, Park JL, Kim HJ, et al. Integrative genomics analysis reveals the multilevel dysregulation and oncogenic characteristics of TEAD4 in gastric cancer. *Carcinogenesis.* 2014;35(5):1020-1027.
477. Moroishi T, Hansen CG, Guan KL. The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer.* 2015;15(2):73-79.
478. Ziemssen F, Heimann H. Evaluation of verteporfin pharmacokinetics--redefining the need of photosensitizers in ophthalmology. *Expert Opin Drug Metab Toxicol.* 2012;8(8):1023-1041.
479. Akens MK, Hardisty MR, Wilson BC, et al. Defining the therapeutic window of vertebral photodynamic therapy in a murine pre-clinical model of breast cancer metastasis using the photosensitizer BPD-MA (Verteporfin). *Breast Cancer Res Treat.* 2010;119(2):325-333.
480. Huggett MT, Jermyn M, Gillams A, et al. Phase I/II study of verteporfin photodynamic therapy in locally advanced pancreatic cancer. *Br J Cancer.* 2014;110(7):1698-1704.
481. Donohue E, Tovey A, Vogl AW, et al. Inhibition of autophagosome formation by the benzoporphyrin derivative verteporfin. *J Biol Chem.* 2011;286(9):7290-7300.
482. Liu-Chittenden Y, Huang B, Shim JS, et al. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev.* 2012;26(12):1300-1305.
483. Song S, Ajani JA, Honjo S, et al. Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer Res.* 2014;74(15):4170-4182.
484. Song S, Honjo S, Jin J, et al. The Hippo Coactivator YAP1 Mediates EGFR Overexpression and Confers Chemoresistance in Esophageal Cancer. *Clin Cancer Res.* 2015;21(11):2580-2590.
485. Zhang H, Ramakrishnan SK, Triner D, et al. Tumor-selective proteotoxicity of verteporfin inhibits colon cancer progression independently of YAP1. *Sci Signal.* 2015;8(397):ra98.
486. Leung JY, Wilson HL, Voltzke KJ, et al. Sav1 Loss Induces Senescence and Stat3 Activation Coinciding with Tubulointerstitial Fibrosis. *Mol Cell Biol.* 2017.
487. Konstantinou EK, Notomi S, Kosmidou C, et al. Verteporfin-induced formation of protein cross-linked oligomers and high molecular weight complexes is mediated by light and leads to cell toxicity. *Sci Rep.* 2017;7:46581.
488. Liu B, Zheng Y, Yin F, Yu J, Silverman N, Pan D. Toll Receptor-Mediated Hippo Signaling Controls Innate Immunity in *Drosophila*. *Cell.* 2016;164(3):406-419.
489. Yao F, Zhou W, Zhong C, Fang W. LATS2 inhibits the activity of NF- $\kappa$  B signaling by disrupting the interaction between TAK1 and IKK $\beta$ . *Tumour Biol.* 2015;36(10):7873-7879.

490. Johnson R, Halder G. The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nat Rev Drug Discov.* 2014;13(1):63-79.
491. Lombardo LJ, Lee FY, Chen P, et al. Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem.* 2004;47(27):6658-6661.
492. Rosenbluh J, Nijhawan D, Cox AG, et al.  $\beta$ -Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell.* 2012;151(7):1457-1473.



**V. Annexes**



a. **Deletion of IQGAP1 promotes *Helicobacter pylori*-induced gastric dysplasia in mice and acquisition of cancer stem cell properties *in vitro***

Published in Oncotarget, 2016, Vol. 7, (No. 49), pp: 80688-80699