U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

THE ROLE OF CREB AND C/EBP TRANSCRIPTION FACTORS IN GASTRIC CARCINOGENESIS

Carlos Alberto Trindade Resende

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

ORIENTADOR: José Carlos Lemos Machado
Categoria: Investigador principal, Professor Associado
Afiliação: Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Faculdade de Medicina da Universidade do Porto

Porto, 2014

Financial Support:
PhD fellowship: SFRH / BD / 46504 /2008
Fundação para Ciência e Tecnologia (FCT)

PUBLICAÇÕES

Ao abrigo do disposto nº2, alínea a) do artigo 31º do Decreto-Lei nº 230/2009 fazem parte integrante desta tese de doutoramento os seguintes trabalhos já publicados ou submetidos para publicação:

Artigos que fazem parte integrante da tese de doutoramento

Artigo I – Regalo G, Resende C, Wen X, Gomes B, Durães C, Seruca R, Carneiro F, Machado JC. C/EBP alpha expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis. Laboratory Investigation. 2010; 90: 1132–1139

Artigo II – Regalo G, Förster S, Resende C, Bauer B, Fleige B, Kemmer W, Schlag PM, Ito K, Bae SC, Meyer TF, Ito Y, Machado JC, Leutz A. C/EBPβ/RUNX1t1 regulatory loop controls cell proliferation in gastric cancer. (Submitted to JCI)

Artigo III – Resende C, Regalo G, Durães C, Pinto MT, Wen X, Figueiredo C, Carneiro F, Machado JC. Interleukin-1B signalling leads to increased cell survival of gastric carcinoma cells through a CREB-C/EBPβ-associated mechanism (Accepted for publication in Gastric Cancer, 2014)

Artigos que não fazendo parte integrante da tese de doutoramento, complementam a introdução e discussão da mesma

Artigo IV – Resende C, Ristimäki A, Machado JC. Genetic and epigenetic alteration in gastric carcinogenesis. Helicobacter. 2010; 15 Suppl 1: 34–39

Artigo V – Resende C, Thiel A, Ristimäki A, Machado JC. Gastric cancer: basic aspects. Helicobacter. 2011; 16 Suppl 1: 38–44

AGRADECIMENTOS

Finda a labuta laboratorial e a escrita da tese, e como investigação é um trabalho de equipa, chegou o momento de agradecer a todos os que contribuíram para a minha chegada a este ponto.

Ao Professor José Carlos Machado fica o meu mais profundo agradecimento pela oportunidade que me concedeu de trabalhar no seu grupo. Dotado de uma inteligência acutilante, o Zé Carlos marcou-me pelo seu pragmatismo e profissionalismo. Desafiou-me sempre a sair da minha zona de conforto e a pensar "out-of-the-box", revelando-se um motivador permanente. Zé Carlos, obrigado pelas constantes tentativas em me fazer compreender o que é e como se faz Ciência!

À Professora Raquel Seruca agradeço o acolhimento e integração no Cancer Genetics. Tenho plena consciência do importante papel que as críticas e discussão dos resultados tiveram para o salutar desenvolvimento deste trabalho.

Ao Professor Sobrinho-Simões agradeço o estímulo pela curiosidade e espírito crítico que me conseguiu transmitir.

Ao Gonçalo Regalo agradeço a amizade, a boa companhia laboratorial e o contributo teórico-experimental fundamental nos trabalhos conducentes à elaboração desta tese. Gonçalo, foi contigo que comecei a minha "jornada" científica. Não podia ter tido melhor mentor "junior"!

Ao Luis Cirnes agradeço a amizade, o apoio técnico e o voto de confiança que depositou em mim. Tenho consciência que sem a tua ajuda, a minha chegada até aqui teria sido mais complicada.

À Cecília Durães agradeço a amizade e camaradagem nesta já longa trajetória profissional, assim como a ajuda na revisão desta tese.

À Maria João, Cheila, Márcia, Rute (as "meninas dos diagnósticos") à Madalena, Diana Martins, Bárbara Sousa, Rita Nobre e André Vieira (a malta da patologia mamária) quero agradecer o companheirismo, as trocas de ideias e o ambiente divertido mas responsável que partilharam comigo.

Aos elementos do Cancer Genetics e do Genetic Dynamics of Cancer Cells, quero agradecer a forma como me integraram como membro do grupo, assim como toda a ajuda e apoio técnico prestados.

Agradeço aos co-autores dos artigos apresentados nesta tese. Sem o vosso contributo nada disto seria possível.

Ao Nelson Ferreira, Nádia Gonçalves, João Vinagre, Ricardo Celestino e João Valente quero agradecer a amizade, os momentos divertidos e aventuras passadas, assim como as conversas paralelas que por várias vezes me elucidaram sobre o melhor caminho a seguir.

Ao pessoal do Population Genetics, principalmente Professor António Amorim, Leonor Gusmão, Sofia Quental, Rita Quental, Rui Pereira e Ana Moleirinho, quero agradecer a amizade, os momentos divertidos, as trocas de ideias e os conhecimentos científicos que partilharam comigo.

Agradeço à Fundação para a Ciência e Tecnologia (FCT) por me conceder uma bolsa, de todo essencial para o desenvolvimento do meu projeto de doutoramento. Agradeço também ao Instituto de Ciências Biomédicas Abel Salazar (ICBAS) e Faculdade de Medicina da Universidade do Porto (FMUP) por me terem acolhido como aluno de doutoramento. Fica um especial agradecimento à Profª Fátima Gärtner, Diretora do Programa Doutoral em Patologia e Genética Molecular, por toda a ajuda e apoio prestados.

Finalmente à minha família agradeço nada menos que tudo: mãe e pai, obrigado pelo apoio incondicional que sempre me deram; Verónica e Rodrigo (Veni e Guigo), obrigado por serem quem são, pelo que representam e por criarem em mim a vontade de querer ser cada dia melhor do que fui no dia anterior!

CONTENTS

ABBREVIATIONS

3-MCA	3- Methylcholantrene
AKT	Protein Kinase B
ALL	Acute lymphoid leukemia
AML	Acute myelogenous leukemia
Ang	Angiotensin
APC	Adenomatous polyposis coli
AR	Androgen receptor
ATF1	Activating transcription factor 1
BCL2	Apoptosis regulator B-Cell CLL/Lymphoma 2
BO	Barrett's oesophagus
bZIP	Basic leucine zipper domain
C/EBP	CCAAT/enhancer binding protein
CagA	Cytotoxin-associated gene A
CAM	Chorioallantoic membrane
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CCS	Clear cell sarcoma
CDH1	Cadherin type 1 gene
CDK	Cyclin dependent kinase
c-JUN	Jun proto-oncogene
c-MYC	Avian Myelocytomatosis Viral Oncogene Homolog
COX2	Cyclooxygenase 2
CRC	Colorectal cancer
CRE	cAMP response elements
CREB	cAMP response element–binding protein
CREM	cAMP response element modulator
CRI	Cancer related inflammation
CUC	Chronic ulcerative colitis
CXCR4	Chemokine (C-X-C Motif) Receptor 4
DCAMKL1	Doublecortin-like kinase 1
DHMEQ	Dehydroxymethylepoxyquinomicin

DISC	Ductal in situ carcinoma
E2F	Eukaryotic 2 factor
E-cadherin	Epithelial cadherin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases 1 and 2
EWS	Ewing sarcoma RNA-binding protein
GC	Gastric cancer
GI	Gastrointestinal tract
H. pylori	*Helicobacter pylori*
HCV	Hepatitis C virus
HDGC	Hereditary diffuse gastric cancer
HER2	Epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HNSCC	Head and neck squamous cell carcinoma
Hp	Haptoglobin
HSC	Hematopoietic stem cell
IARC	International Agency for Research on Cancer
ICER	Inducible cAMP response element repressor
IFNc	Interferon C
IFNG	Interferon gamma
IFNGR1	Interferon gamma receptor 1
IGF1R	Insulin-like growth factor 1 (IGF-1) receptor
IL10	Interleukin 10
IL1B	Interleukin 1 beta
IL1RN	Interleukin 1 receptor antagonist
IL8	Interleukin 8
JUN	Jun Activation Domain Binding Protein
K1	Keratin 1
K10	Keratin 10
K19	Keratin 19
Kb	Kilo base pair
KDa	Kilodalton (protein molecular weight)
Ki67	Proliferation marker Ki67

KID	Kinase inducible domain
KIX	KID interaction domain
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAP	Liver-enriched activator protein
LIP	Liver-enriched inhibitor protein
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinases
MaSC	Mammary stem cells
MEC	Mammary epithelial cell
MEK	Mitogen-activated protein kinase kinase
MET	Hepatocyte growth factor receptor
MLH1	MutL homolog 1 protein
MMPs	Matrix metalloproteinases
MMR	Mismatch repair
MNU	N-methyl-N-nitrosourea
MSH2	MutS homolog 2 protein
MSH6	MutS homolog 6 protein
MSI	Microsatellite instability
MSS	Microsatellite stable
MUC5AC	Mucin 5 AC
MUC6	Mucin 6
MUC8	Mucin 8
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NSCLC	Non-small-cell lung cancer
OAC	Oesophageal adenocarcinoma
OPG	Osteoprotegerin
OR	Oestrogen receptor
P15	Cyclin-dependent kinase inhibitor 2B
P21	Cyclin-dependent kinase inhibitor 1
P27	Cyclin-dependent kinase inhibitor 1B
P300	E1A binding protein p300
P38 MAPK	Mitogen-activated protein kinase P38
PC	Prostate cancer

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
pCREB	Phosphorylated CREB
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMN	Polymorphonuclear leukocyte
PMS2	Mismatch repair endonuclease 2 protein
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RUNX1t1	Runt-related transcription factor 1; translocated to 1
RUNX3	Runt-related transcription factor 3
SMAD	Mothers against decapentaplegic homolog factor
SPEM	Spasmolytic polypeptide-expressing metaplasia
STAT	Signal transducer and activator of transcription
SWI/SNF	SWItch/Sucrose NonFermentable nucleosome remodeling complex
TCF/LEF	T-cell factor / Lymphoid enhancer-binding factor
TFF1	Trefoil factor 1
TFF2	Trefoil factor 2
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TORC	Transducer of regulated cAMP response element-binding protein
TP53	Transformation-Related Protein 53
VacA	Vacuolating cytotoxin A
WNT	Wingless-type mmtv integration site family
Wt	Wild type

ABSTRACT

Gastric cancer (GC) is an important cause of morbidity and mortality worldwide. GC is thought to result from the combined effects of three major players: environmental factors, susceptibility genetic variants, and deregulated signalling pathways associated with functionally relevant molecular aberrations. Over time, the combined effects of those players will alter the normal patterns of epithelial cell proliferation, apoptosis and differentiation programs, driving the gastric carcinogenesis process.

The most well-known association between environmental factors and genetic susceptibility in increasing the risk towards GC is found in the life-long infection with *Helicobacter pylori* and host chronic inflammatory response. In this scenario, individuals that due to their genetic makeup produce a more intense inflammatory response are at increased risk of GC. However, it remains unclear how inflammation drives gastric carcinogenesis.

It is thought that inflammatory mediators must be able to disrupt gastric homeostasis through the modulation of critical signalling pathways. At the cellular level, inflammatory stimuli induce the activation of signalling cascades that culminate in the activation and/or expression of transcription factors that will execute the biological functions triggered by those stimulatory signals. Furthermore, inflammation-activated transcription factors are also found to play relevant biological roles in malignancies. This observation is highly suggestive of a tight relation between inflammation and cancer, in which critical molecular effectors play crucial functions in the inflammatory and carcinogenic processes.

Based on the exposed, the main goal of this work was to get a deeper understanding of the signalling events and associated molecular mechanisms underlying inflammation-driven GC development. To accomplish our major goal, we focused on two specific aims: I) to determine the expression patterns of inflammation-associated molecular effectors on normal gastric mucosa, preneoplastic and GC lesions; II) to determine the inflammation-modulated signalling cascades responsible for the expression/activation status of molecular effectors and the underlying biological meaning through *in vitro* and *in vivo* approaches.

The results generated in this work indicate C/EBPα as a transcription factor specific of differentiated gastric foveolar epithelial cells. Additionally, C/EBPα was found downregulated in 30% of GC cases studied, and was found to act as a potent anti-proliferative effector on GC cells. Moreover, C/EBPα expression was found to be negatively regulated by p38 and ERK1/2 signalling, two critical MAPK branches previously reported as activated in gastric inflammation and GC. On the other hand, C/EBPβ which is frequently overexpressed in preneoplastic lesions

and GC, promotes *in vitro* and *in vivo* cell proliferation. Also, C/EBPβ expression was found to be necessary for the full tumorigenic ability of GC cells. Exploring the association between inflammatory mediators and C/EBPβ expression, we demonstrate that interleukin-1 beta (IL1B) is able to induce the expression of C/EBPβ through the activation of MAPK signalling cascades. Thus, our results suggest that these two members of the C/EBP-family of transcription factors play specific and non-overlapping functions in the maintenance of gastric homeostasis, and the deregulation of their normal pattern of expression can be an important step in gastric carcinogenesis. Based on other cell models, we observed CREB-dependent transcriptional activity as a mechanism underlying C/EBPβ expression in GC cells lines. We also demonstrate that in normal gastric mucosa C/EBPβ and CREB are expressed in a compartmentalized glandular region within which gastric progenitor cells are located, while in GC samples both proteins are associated and overexpressed in the majority of cases studied. Moreover, we demonstrate that CREB acts as a crucial effector in both basal and IL1B-induced GC cell proliferation and in *in vivo* tumorigenic ability of GC cells.

Our results provide further support to the hypothesis that the effect of chronic inflammation on gastric carcinogenesis, as seen in the context of genetically susceptible individuals infected with *H*. *pylori*, includes modulation of signalling pathways that regulate important biological mechanisms in epithelial cells. Furthermore, our results may help inform new strategies for prevention and treatment of GC, including the control of chronic inflammation and the identification of new therapy targets.

RESUMO

O cancro gástrico (CG) constitui uma das principais causas de morte por cancro em todo o mundo. Ao longo dos anos, vários estudos foram realizados com a finalidade de compreender os fatores subjacentes ao desenvolvimento desta neoplasia. Várias evidências sugerem que a combinação de fatores ambientais, suscetibilidade genética e ocorrência de mutações resulte na alteração dos padrões normais de proliferação e diferenciação celulares, desencadeando o processo de carcinogénese gástrica.

A mais relevante associação entre ambiente e suscetibilidade genética como fatores de risco para desenvolvimento de CG é encontrada na infeção por *Helicobacter pylori* e a resposta inflamatória crónica do hospedeiro. De facto, hospedeiros que devido à sua constituição genética desencadeiam uma resposta inflamatória mais intensa à infeção, estão em risco de desenvolver CG. No entanto, os mecanismos moleculares subjacentes à carcinogénese gástrica induzida pela inflamação são ainda pouco conhecidos.

Alguns estudos sugerem que os mediadores inflamatórios devem ser capazes de perturbar a homeostasia do epitélio gástrico através da modulação de importantes vias de sinalização. A nível celular, os estímulos inflamatórios são capazes de induzir a expressão e ativação de importantes fatores de transcrição que tomarão parte na execução das funções biológicas despoletadas por esses sinais. Além disso, os fatores de transcrição ativados por mediadores inflamatórios também atuam como importantes executores de funções biológicas nas células tumorais. Desta forma, tem sido sugerido a existência de uma interconexão entre inflamação e cancro, em que importantes fatores de transcrição desempenham funções cruciais em ambos os processos inflamatório e tumoral.

Com base no exposto, este trabalho teve como objetivo principal a obtenção de uma compreensão mais profunda dos eventos de sinalização e mecanismos moleculares subjacentes, despoletados pela inflamação, no desenvolvimento de CG. De forma a atingir este objetivo, focámos o trabalho em dois objetivos específicos: I) determinar os padrões de expressão de fatores de transcrição associados a inflamação em estômago normal, lesões pré-neoplásicas e GC; II) determinar os eventos de sinalização celular responsáveis pela modulação da expressão dos fatores de transcrição, assim como determinar o significado biológico destes fatores através de ensaios *in vitro* e *in vivo*.

Neste trabalho observámos que o C/EBPα, além de ser um marcador de diferenciação do epitélio gástrico, é também um possível supressor tumoral gástrico, dada a perda de expressão em 30% dos casos de CG estudados e o papel anti-proliferativo que desempenha a nível celular. Em oposição, o C/EBPβ, justificando a sobre-expressão em lesões pre-

neoplásicas e CG, desempenha um papel crucial na proliferação *in vivo* do epitélio gástrico normal e no potencial tumorigénico de linhas celulares de CG. Estes resultados sugerem que estes dois membros da família C/EBP desempenham funções específicas e não sobreponíveis na manutenção da homeostasia gástrica, e que a desregulação da expressão normal de cada um deles pode desempenhar um papel relevante na carcinogénese gástrica. Mecanisticamente relevante, e dado a inflamação e a ativação oncogénica culminarem na expressão/ativação de fatores de transcrição, os nossos resultados demonstram que o mediador pro-inflamatório IL1B induz a expressão de C/EBPβ, possivelmente através da ação transcricional do CREB, que se mostrou ser fundamental para a transcrição e manutenção dos níveis celulares basais de C/EBPβ. Para além da regulação do gene *CEBPB*, e da sobre-expressão na maioria dos casos de CG analisados, o CREB revelou desempenhar um importante papel na proliferação celular induzida pela IL1B. Adicionalmente, foi possível demonstrar que o CREB desempenha um papel tumorigénico nas células de CG. Os resultados obtidos fornecem suporte à hipótese de que os efeitos da inflamação crónica na carcinogénese gástrica, como observado em indivíduos geneticamente suscetíveis infetados com *H. pylori*, incluiu a modulação de vias de sinalização que regulam importantes mecanismos biológicos nas células epiteliais gástricas. Além disso, os resultados obtidos neste trabalho poderão ser utilizados no desenvolvimento de novas estratégias para prevenção e tratamento de CG, incluindo o controlo da inflamação crónica e a identificação de novos alvos terapêuticos.

INTRODUCTION

1 – Gastric cancer

Gastric cancer (GC) is a worldwide health burden that, for the majority of cases, is thought to result from the combined effects of three major players: environmental factors, susceptibility genetic variants, and the accumulation of specific (epi)genetic alterations. The long-term effects caused by these players will alter the epithelial cell proliferative, apoptotic, and differentiation programs, culminating in the initiation and progression of the gastric carcinogenesis process.

1.1 – Epidemiological data

GC incidence and mortality decreased significantly over the past 70 years [1, 2]. However, a global cancer statistics analysis places GC as one of the most prevalent and deadly forms of cancer worldwide [3, 4]. By gender, GC is the fourth most common cause of cancer in men and the fifth in women, and incidence rates are about twice in males as in females. The predominance of GC in males may be related to hormonal factors, namely the lack of a protective effect induced by oestrogens, as suggested by some studies [5, 6].

The worldwide distribution of GC is marked by geographical variations, with the highest incidence rates detected in Eastern Asia, Eastern Europe, and South America [4]. Because GC incidence in Japan is one of the highest in the world [7], the implementation of population-applied mass screening programs for early GC detection is responsible for the significant decline in mortality [8]. Outside Japan, where no such mass screening is implemented, only a minor percentage of GCs are discovered at an early stage, ending in a 5-year survival rate of less than 20% in United States and 10 - 20% in European countries [9]. In Portugal, GC is the fourth most common cancer type and the second cause of cancer death in men and the third in women. Moreover, Portugal exhibits the highest GC incidence in Western Europe [10].

Due to the different histopathological characteristics of GC, there was an urgent need to organize GC in different categories or types. The Laurén classification is the most commonly used and describes two main histological types with different clinical and pathological characteristics: the diffuse and intestinal [11]. Diffuse GC is marked by the presence of isolated poorly cohesive clusters of cells dispersed through the stroma, it can be multifocal [12], can have a hereditary basis, and occurs more commonly in young patients [13]. Furthermore, no preceding steps have been identified for diffuse type other than chronic gastritis [6, 12]. The

intestinal type is the most common form of GC, it is constituted by well-differentiated cells that form glandular structures with an intestinal-like pattern, and is more frequently observed at older ages [12]. The pathological evolution of normal gastric mucosa into intestinal type GC has been characterized as a progressive multistep process. The process begins with chronic gastritis, which progresses to atrophic gastritis followed by intestinal metaplasia, dysplasia, and at last cancer with subsequent metastatic dissemination [14]

1.2 – Environmental risk factors

A large amount of data suggests that environmental and lifestyle factors are pivotal contributors to GC aetiology [3, 4]. An argument that strengthens the association between environmental factors and GC incidence comes from the observation that migrant populations from high risk areas exhibit a marked reduction in risk when they move to low-incidence areas [15]. Furthermore, subsequent generations acquire risk levels similar to the background risk of the host country [7].

1.2.1 – Diet and lifestyle

A relevant factor in the aetiology of GC is diet, especially for development of the intestinal histological type. Consumption of adequate amounts of fruit and vegetables seems to lower the risk of GC, with antioxidants proposed as having protective effects by reducing the cellular damage caused by free radicals [6, 16, 17]. The high intake of salt and salt-preserved foods was found to be significantly associated with increased risk to develop GC [1, 17, 18]. Indeed, not only the ingestion of salt induced gastritis in animal models but also enhanced the effects of gastric carcinogens [19, 20]. It has been suggested that the worldwide decrease in GC incidence during the last decades can be attributed to the introduction of refrigeration, which led to a reduction in consumption of salt preserved foods and allowed the increase intake of fresh vegetables and fruits [1].

A lifestyle factor causally associated with the development of GC is tobacco smoking [21]. The results obtained in independent studies enrolling populations from distinct geographical origins and distinct genetic backgrounds strongly suggested the positive association between smoking and GC [22, 23].

1.2.2 – *Helicobacter pylori* infection and gastric pathogenesis

Helicobacter pylori (*H. pylori*) is a Gram-negative, urease-positive bacterium that specifically colonizes the human stomach, and is responsible for one of the most common chronic bacterial infections worldwide [24]. This bacterium is normally found in the superficial gastric mucus layer, with only a small percentage found adherent to gastric epithelial cells. The infection is mainly acquired during early childhood, predominantly by the gastric-oral route. However, other modes of transmission such as the faecal–oral or the oral–oral route together with indirect transmission through contaminated food or water may also be possible [25]. Until 2002, the overall estimate of the prevalence of *H. pylori* infection in middle-aged adults was calculated as 74% in developing countries and 58% in developed countries [26].

Even before the discovery of *H. pylori* it was well recognized that gastric inflammation and decreased production of gastric acid (hypochlorhydria) were conditions strongly associated with GC. Long-term follow-up studies in high-risk populations demonstrated the slow development of atrophic gastritis over years and identified the importance of gastric atrophy and intestinal metaplasia as risk factors for GC [27]. Correa *et al.* (1992) proposed the hypothesis that GC development, namely intestinal type GC, was a slow and complex multistep process, in which *H. pylori*-induced gastritis was the initial trigger [14]. Since then, the link between *H. pylori* infection and the risk for GC became well established [28]. Noteworthy, in a long-term study enrolling more than 1500 participants, GC developed in 2.9% of *H. pylori* infected individuals but in none of the uninfected subjects, strengthening the association between *H. pylori* and GC [29]. Therefore, due to the long-term effects of the bacteria over gastric epithelial cells, *H. pylori* was recognized as a type 1 carcinogen for GC by the International Agency for Research on Cancer (IARC) [30].

H. pylori as a species is quite heterogeneous, being composed by various strains. The strains differ in terms of genetic background and, as a direct consequence, the corresponding bacterial proteins produced. Some of the proteins synthesized are virulence factors that have profound damaging effects on gastric epithelial cells. The two best characterized virulence factors are the cytotoxin VacA and the cag pathogenicity island and its effector CagA [31].

Although *H. pylori* infection is the initial cause of gastric inflammation, only a small percentage of infected individuals will ultimately progress to cancer. The pathological basis for this phenomenon resides in the interaction of *H. pylori* virulence factors with the underlying inflammatory response of the host towards the infection [32]. Support for the importance of host response was observed in early animal experiments when different mouse strains were infected with *H. pylori*. The ability of *H. pylori* to colonize the gastric mucosa and to induce

histological lesions, such as gastritis and glandular atrophy, was strongly dependent on the mouse strain [33, 34]. Those results revealed the critical importance of the host genetic background for the development of gastric lesions.

1.3 – Host genetic susceptibility

Gastric colonization by *H. pylori* is followed by a mucosa infiltration of different immune cells such as lymphocytes, polymorphonuclear leukocytes (PMNs), and monocytes/macrophages [35], which are responsible for the increased mucosal expression of many inflammatory mediators, including several interleukins (such as IL1B, IL6, and IL8), tumour necrosis factor alpha (TNFα), and interferon-c (IFNc) [36-38]. Genetic polymorphisms in inflammation-related genes influence inter-individual variation in the magnitude of inflammatory response, and this contributes to the individual ultimate clinical outcome [6, 39].

One of the most biologically relevant pro-inflammatory cytokines in the context of *H. pylori* infection is interleukin-1beta (IL1B), due to two main reasons: the protein levels are strongly up-regulated in response to infection and this cytokine is the most powerful known gastric acid inhibitor – contributing to the development of gastric atrophy and hypochlorhydria [36, 38]. In an associative study using a Caucasian population, El-Omar *et al.* (2000) demonstrated that polymorphisms in the pro-inflammatory *IL1* gene cluster (*IL1B* encoding IL1B and *IL1RN* encoding the endogenous receptor antagonist IL1RA) increased the risk of developing neoplastic gastric changes in response to *H. pylori* infection [40]. Specifically, the individuals that carry the *IL1B*-31C or -511T and *IL1RN**2/*2 genotypes were significantly associated with risk of developing neoplastic gastric lesions. Functionally relevant, the aforementioned alleles were positively associated with increased production of IL1B [41-43]. Further, the carriers for the combination of *IL1B*-511T and *IL1RN**2 alleles exhibited the highest levels of IL1B in a context of *H. pylori* infection [44]. Validating the first associative studies, the correlation for *IL1* gene cluster polymorphisms and GC were also observed in independent studies using patient samples from different geographical areas and ethnic groups [45-47]. Additionally, supporting the association between *H. pylori* and host response, Figueiredo *et al.* (2003), reported that the combination of high-risk bacterial virulence factors and host genotype conferred the greatest risk of developing gastric malignancy [32].

The existence of polymorphisms in other inflammation-related genes, such as *TNFA* and *IL10* were also reported as independent additional risk factors for development of GC [48]. The cytokine TNFα has a powerful pro-inflammatory effect, and is produced in the gastric

mucosa in response to *H. pylori* infection [49]. Machado *et al.* (2003) reported the *TNFA*-308 G>A polymorphism as an independent factor for increased risk of GC [50]. On the other hand, IL10 functions as a potent anti-inflammatory cytokine that is able to downregulate IL1B and TNFα protein levels. In this manner, decreased levels or deficiency of IL10 may result in a hyper-inflammatory response to *H. pylori*. In this scenario, individuals homozygous for the *IL10* ATA haplotype (based on three promoter polymorphisms at positions -592, -819, and -1082) were identified at increased risk GC. Some studies suggested that the presence of at least one of the three risk alleles was directly associated with the risk of GC, but only among Asians [51-53]. Noteworthy, the risk towards GC increased progressively with the number of risk alleles in inflammation-associated genes (*IL1B*-511T, *IL1RN**2/2, *TNFA*-308*A, and *IL10* ATA) [48]. Also, a promoter polymorphism on *IL8* (-251 T>A), for which the A allele was described as being associated with increased production of the interleukin-8 in *H*. *pylori*-infected gastric mucosa, was described to Increase the risk of developing premalignant gastric lesions [54]. Functionally, IL8 is a cytokine that functions as a potent chemo-attractant factor for immune cells, and in the induction of pro-inflammatory cytokines, and by this way acts as an important pro-inflammatory signal. Taken together, those results clearly demonstrate that inflammation is biologically a functional driver force in the process of gastric carcinogenesis.

In summary, individuals that possess a particular inflammation-related genetic makeup react naturally against *H. pylori* with a more intense inflammatory response. The cumulative stress caused by the feedback between bacteria, the genetic constitution of the host, and environmental factors will eventually drive preneoplastic transformation from an atrophic gastritis to a full-developed GC (Figure 1).

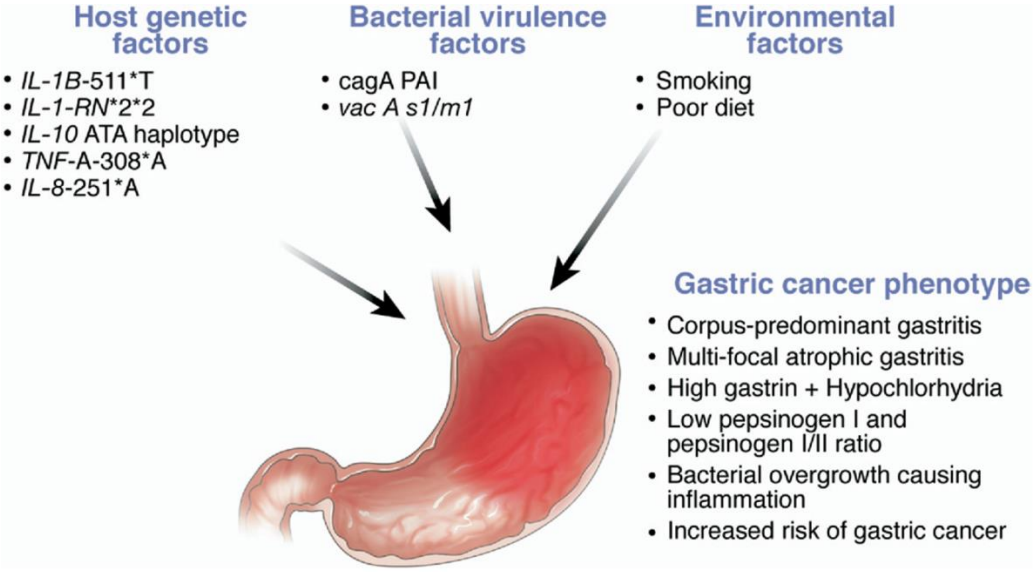


Figure 1. Effects of bacterial, host genetics, and environmental factors on the pathogenesis of *H. pylori*-induced gastric cancer. The combination of risk factors induces chronic inflammation, hypochlorhydria, and sustained genotoxic stress. Gastric cancer arises as the long-term end product of the combinatorial effects of those factors over the gastric epithelium (adapted from Amieva & El-Omar, 2008 [31]).

1.4 – Gastric epithelial progenitor cells – putative target cells for neoplastic transformation

It is generally accepted that cancer arises from a single cell in which a series of molecular aberrations are responsible for continued clonal evolution and heterogeneity [55, 56]. In this respect, cancer onset is dependent on two fundamental processes: the ongoing acquisition of heritable (epi)genetic aberrations by individual cells, and the selection acting on the resultant phenotypic diversity. By the combined action of mutational occurrence and selection, foci of cells with an altered pattern of proliferation versus differentiation will give rise to neoplastic lesions [57].

The multistep tumourigenesis theory focuses mainly on the nature and number of mutations, leaving behind the intrinsic properties of the cell in which those mutations occur. Nonetheless, the identification of the cell types that, by the effect of mutations, are able to initiate and sustain growth of the cancer clone is a major issue in cancer research [56]. All cells in a tissue are derived from tissue-specific progenitor/stem cells able to undergo self-renewal as well as to give rise to all cells that will differentiate into the mature cells that compose each tissue. Progenitor cells live longer than their derived differentiated cells and, for that particular

reason, may be exposed to mutagenic agents for a longer time, ending in the accumulation of an advantageous set of aberrations that will drive carcinogenesis [58].

1.4.1 – Gastric gland unit

The gastrointestinal (GI) tract plays crucial roles in the processes of digestion and nutrient absorption, and also creates a functional physical and chemical barrier against pathogens. In addition, GI epithelia exhibits the highest cellular turnover [59]. Histologically, the endoderm-derived gastric mucosa is classically divided into four regions: cardia (most proximal), fundus, corpus (intermediate, encompassing the majority of gastric area), and antrum or pylorum (most distal region). The gastric mucosa is formed by glandular units composed by a pit or foveolar zone and a long tubular structure further subdivided into the isthmus, neck, and base zones. In the corpus region, the gastric gland is composed of four types of terminally differentiated cells that are replaced at different rates: parietal cells, zymogenic chief cells, surface mucous foveolar cells, and hormone-secreting enterorendocrine cells. The gastric antrum has fewer parietal cells but possesses a unique population of mucus-producing cells located near the base of the gland units that resemble corpus mucous neck cells [60]. Interestingly, mucous neck cells can perform two functions: secreting mucins and small peptides, and also give rise to chief cells (Figure 2) [60, 61].

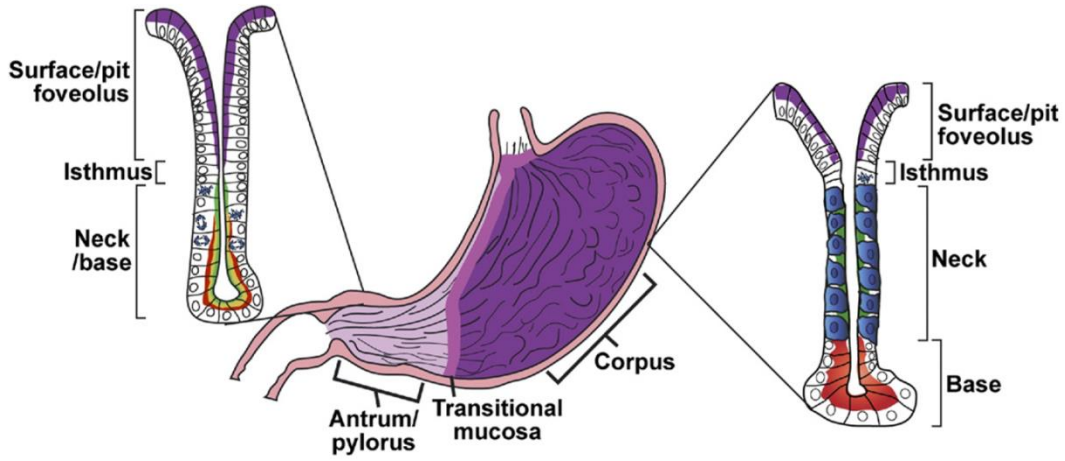


Figure 2. Anatomy and histology of a mammalian stomach. The gastric prominent regions in most mammals are a proximal corpus and a distal antrum or pylorus. The corpus epithelium is organized into repeating gastric units that are invaginations from the surface and contain multiple cell lineages in four distinct zones. Acid-secreting parietal cells are represented in blue, digestive enzyme secreting zymogenic chief cells in red, mucous neck cells in green, and the mucus-secreting pit cells nearest the surface in *purple*. In the antrum, the gastric units are simpler, with few parietal or zymogenic cells. Antral units contain two distinct types of mucous cells: the ones lining the surface (in

purple) are similar to the surface cells of the corpus, and those nearer to the base have properties intermediate between zymogenic cells and mucous neck cells of the corpus (in red-yellow) (adapted from Mills & Shivdasani, 2011 [60]).

The common origin of all gastric epithelial cells is well established, although stem cell properties seem to differ between corpus and antrum [62]. Knowing that gastric progenitor/stem cells originate all the resident gastric-specific cell types and that aberrant differentiation of the gastric epithelium occurs during tumourigenesis, understanding normal and abnormal gastric epithelial stem cell biology may be of the outmost relevance to uncover the origins of GC.

In the late 1940s Leblond *et al.* (1948), using nucleotides marked with radioisotopes, identified in the gastric epithelium the cells that incorporated marked nucleotides into their nucleus – an obvious indication of DNA replication [63]. A narrowing region in the gastric gland (isthmus) where the resident cells incorporated marked nucleotides, was identified. Furthermore, those authors also observed one or only a few cells in the isthmus constantly regenerating cells that migrate bi-directionally, differentiating along the migration into the mature cell types that constitute the gastric glands [63].

In 1974, Cheng *et al.* proposed the Unitarian Theory stating that all mature gastrointestinal epithelial cells derive from a common progenitor/stem cell [64]. Nevertheless, in a series of *in vivo* chemical mutagenesis experiments, Bjerknes and Cheng (2002) observed that while most glands arose from a single stem cell, some gastric glands carried mutant cells of only a single lineage, suggesting new cells had arisen continually along that lineage [65]. This observation suggested that some gastric units might keep progenitor cells that are committed to replenishing cells of only a single lineage.

1.4.2 – Response of gastric epithelial progenitor cells to injury

According to Mills *et al.* (2011), gastric mucosal injury can be grouped into two categories: focal (characterized by repairable damage that does not change the cell differentiation pattern) and diffuse (characterized by chronic damage that change cellular differentiation) [60]. The focal damage, caused by e.g. toxin ingestion, is rapidly repaired by restitution from expansion of surface epithelial cells and by increased cell proliferation in neighbouring gastric units [66]. The diffuse category of injury results in abnormal cell differentiation (metaplasia), most usually caused from chronic inflammation in response to infection with the bacterium *H. pylori* [14] or by autoimmune gastritis [67]. Metaplasia is normally

associated with cancer risk development and seem to reflect a permanent alteration in the biological conduct of the progenitor cells [60].

Regarding *H. pylori* infection, it is well known that the permanent colonization of gastric mucosa and associated inflammation leads to the expansion of the proliferative cell zone and development of the preneoplastic lesion spasmolytic polypeptide-expressing metaplasia (SPEM), caused by the chronic inflammatory response of the host (Figure 3). The parietal cells are lost, and the zymogenic cell lineage is reprogrammed so that genes expressed exclusively in mucous neck cells, such as trefoil factor 2 (TFF2), are expressed in cells at the base of the gland [68]. Furthermore, the increased number of proliferating cells observed in gastric metaplasia can enhance the probability of occurrence of tumour-driver mutational events.

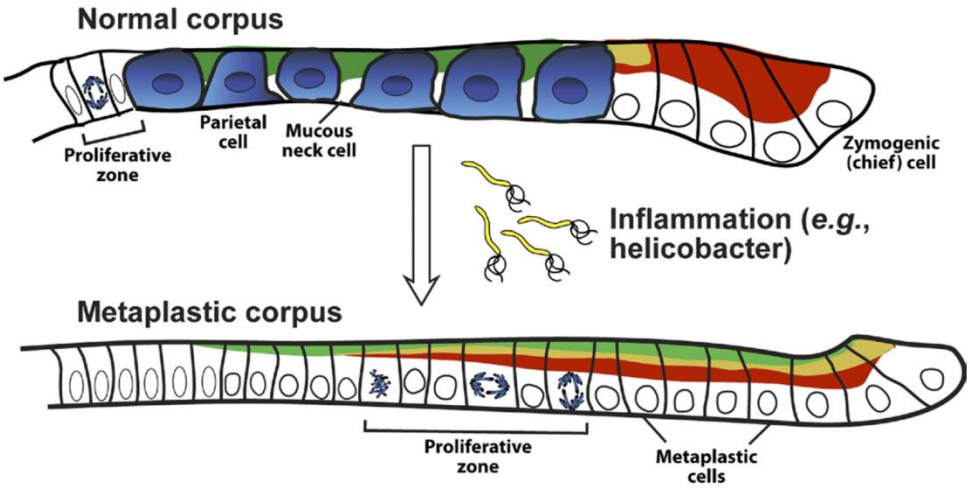


Figure 3. *H. pylori* induces development of SPEM. Chronic inflammation of the corpus leads to changes in epithelial differentiation within the gastric unit. Gastric atrophy arises, and the zymogenic chief cell lineage is reprogrammed so that genes that are normally expressed only in mucous neck cells, such as TFF2 (represented in green), are expressed at high levels in cells at the base of the gland. Proliferation is increased and occurs more basally in the unit (adapted from Mills & Shivdasani, 2011 [60]).

1.5 – Molecular biology of gastric cancer

The combinatorial effects of long-term gastric bacteria colonization and associated chronic inflammation create the proper conditions for the occurrence and fixation of molecular alterations. Genetic aberrations in a panel of genes have been described in GC [39, 69]. However, the mutational landscape of GC remains largely unknown because the described

mutations are found at low frequencies and, except some cases, there is a general lack of knowledge about their biological role in the process of gastric carcinogenesis.

1.5.1 – The *CDH1* gene

Germline mutations in *CDH1* (encoding cell adhesion E-cadherin) are found in 30–40% of all hereditary diffuse gastric cancer (HDGC) families [70], with the remaining 60–70% of cases lacking a known genetic culprit. Additionally, *CDH1* mutations have also been found in sporadic diffuse type GC with a frequency of 50–70%, but not in intestinal type GC [71]. Nonetheless, the gastric mucosa in *CDH1* mutation carriers is normal until a second "hit" inactivates the normal *CDH1* allele, which occurs frequently by promoter hypermethylation [6].

1.5.2 – Microsatellite instability

The emergence of microsatellite instability (MSI) is dependent on the deficiency or inactivation of one of the DNA mismatch repair (MMR) proteins – MLH1, MSH2, MSH6, or PMS2 – that render cells unable to repair DNA replication errors in short DNA sequence repeats, leading to the appearance of new alleles not present in the adjacent normal cells [104]. Noteworthy, the deficiency or inactivation of MMR proteins does not reside on a genetic basis since mutations are a rare event [72]. Promoter hypermethylation is frequently observed. Genetic instability at the level of microsatellites has been found in approximately 10% of sporadic GC [73], more commonly in the intestinal type [74].

1.5.3 – Selected acquired somatic alterations

Loss of expression of trefoil peptide 1 (TFF1) has been described in preneoplastic lesions and in approximately 50% of intestinal type GC [75]. However, loss of TFF1 expression does not stand on gene mutations, deriving instead from loss of heterozygosity (LOH) and promoter hypermethylation [76]. The biological significance of TFF1 loss was demonstrated in a *TFF1* knockout mouse model, in which mice displayed gastric hyperplastic lesions, and 30% of them developed multifocal intra-mucosal tumours [77]. These results strongly suggest TFF1 as a gastric tumour suppressor.

The *MET* encodes a transmembrane protein that acts as the receptor for hepatocyte growth factor (HGF), and has been found amplified in GC, more frequently in the diffuse than in intestinal type (39 % in diffuse versus 19% in intestinal type) [69, 78]. Another gene that has been reported to be amplified in GC is *HER2.* The human epidermal growth factor receptor 2

(HER2) is a tyrosine kinase with no known ligands but able to signal by establishing dimers with other receptor proteins. In addition to gene amplification, HER2 has also been shown to be overexpressed at the protein level in up to 20% of intestinal type GC, but only rarely in diffuse type [39, 69, 79].

The *TP53* gene encodes a nuclear protein that plays crucial roles in cell cycle control, DNA repair, and apoptosis [80]. *TP53* has consistently been found altered in GC, by LOH and mutations. In fact, *TP53* is the most frequently mutated gene in GC with a mutational frequency of up to 60%, regardless of histological type [12]. Interestingly, and possibly suggesting to play an initial role in gastric carcinogenesis is the detection of *TP53* mutations in preneoplastic lesions such as adenomas [81] and intestinal metaplasia [82]. Thus, *TP53* mutation seems not to be a GC specific event but instead an initial transformation-driver event. Also mutated in GC, one can find *KRAS*. In fact, the *KRAS* gene is frequently mutated in different cancers, and GC is no exception with approximately 5% of the intestinal type cases exhibiting *KRAS* mutations [83]. Curiously, and in clear contrast to colorectal cancer (CRC), *KRAS* mutations in GC are more frequently observed in MSI positive cases instead of MSS cases [84, 85].

The phosphatase and tensin homolog (*PTEN*) gene is one of the most frequently mutated or lost genes in cancer [86]. Due to promoter hypermethylation, PTEN expression is lost in up to 20% of GC cases, with no histological association [87]. Additionally, LOH has been referred as an underlying cause of PTEN loss of expression in GC [88].

RUNX3 is a member of the runt domain-containing family of transcription factors which regulates the *SMAD* gene family transcription and TGFβ signalling. RUNX3 is frequently inactivated in GC by protein mislocalization and promoter hypermethylation [89]. Further, RUNX3 knockout mice ($RUNX3^{-/-}$) showed elongated gastric glands with increased epithelial cell proliferation (hyperplasia) [90]. Additionally, $RUNX3^{-/-}$ mice showed loss of chief cells and development of SPEM, displaying also a higher susceptibility to GC following treatment with the chemical carcinogen N-methyl-N-nitrosourea (MNU). Those results gave functional support to the hypothesized role of RUNX3 as a gastric tumour suppressor [91].

2 – Inflammation and cancer

There is a tight connection between inflammation and cancer. However, the mechanistic of this complex relationship remains largely unknown. In this chapter, aspects of the relation between signalling mediators and cellular effectors involved in inflammation and cancer will be focused.

2.1 – Mechanisms of inflammation-driven cancer development

Inflammation is the natural response of an individual to relieve infection or tissue insult, in order to promote healing and tissue regeneration. However, in cases in which acute inflammation progresses into a condition of chronic persistence, one of the possible long-term consequences is cancer. The relation between cancer and inflammation was initially detected in the nineteenth century, when Rudolf Virchow observed the presence of inflammatory cells in tumour tissues and that tumours often arise at sites of chronic inflammation [92]. Nowadays, with the implementation of new and highly sensible detection techniques, it became evident that virtually every tumour contains inflammatory cells [93].

The inflammatory response is coordinated by several mediators, which are produced and secreted from inflammatory, epithelial, and mesenchymal cells. Of all the mediators released, cytokines are central players in the inflammatory process [94]. The secreted cytokines interact with receptors present on the cytoplasmic membrane of epithelial cells, modulate associated intracellular signalling cascades and culminate in the activation of transcription factors – such as nuclear factor-κB (NF-κB). The activation of NF-κB is responsible for the production of growth factors, the synthesis of cyclooxygenase (COX)-2, and increased amounts of reactive oxygen species (ROS) [95, 96]. In this scenario, the NF-κB-target gene COX2 will act as an inducer of cell growth and angiogenesis [97], whereas ROS have the ability to directly alter the normal function of different biomolecules [98].

In order to become a cancer, a normal cell has to undergo a series of irreversible, functionally relevant, and heritable molecular aberrations. Thus, inflammation must have the ability to induce DNA damage, leading to permanent alterations within the cell genome. [96]. In an almost ingenious manner, inflammation can enhance tumour formation by inducing the production of growth factors and cytokines that stimulate tissue-specific stem cell expansion. Acting in this manner, inflammation expands the pool of the ideal target cells for mutagenesis – undifferentiated, long-lived entities with the intrinsic ability to propagate their genome to the next generation [96].

It has been reported that ROS and reactive nitrogen intermediates (RNI) released by inflammatory cells are two major players in promoting DNA damage of epithelial cells. Additionally, Inflammatory cells may employ cytokines, such as IL1B or TNFα, to promote ROS formation in the surrounding epithelial cells [95, 96]. Independently of the mechanism behind, inactivating mutations in the tumour suppressor gene *TP53*, presumably caused by oxidative insults trough ROS, were detected in cancer cells and in inflamed, non-dysplastic colitis-

associated epithelial cells. This observation highly suggests that chronic inflammation is a precocious condition, able to induce genetic mutations [95, 99]. The same phenomenon was found in different chronic inflamed tissues, such as Barrett's oesophagus (BO) [100], and hepatitis C virus (HCV)-associated chronic hepatitis [101].

Inflammatory mediators are also able to inactivate or repress DNA repair pathways, e.g. the mismatch repair (MMR) system. Mutations or epigenetic silencing of MMR response genes are associated with the MSI phenotype, characterized by increased rates of DNA replication errors scattered throughout the genome [102]. Noteworthy, MSI can be detected in epithelial cells of inflamed non-dysplastic intestinal mucosa of patients with chronic ulcerative colitis (CUC), suggesting that inactivation of the MMR system is an early event in colon carcinogenesis [102, 103].

The connection between inflammation and cancer cannot be viewed as a one-way road, since there is evidence that DNA mutations in epithelial cells can lead to inflammation and thereby promote carcinogenesis. This interrelationship was uncovered following attempts to understand the reason why inflammatory cells and mediators are present in the microenvironment of virtually all cancers – even present in cancer types for which there is no evident underlying inflammatory condition [96, 104]. In a study using two transgenic mouse models in which mutated *KRAS* was expressed under the promoter of putative gastric stem cell markers (DCAMKL1 and K19), Okumura *et al.* demonstrated that mutated *KRAS* induced the expression of inflammatory mediators, such as IL1B and IL6. The authors also described that those mice developed preneoplastic lesions, starting from gastric atrophy, metaplasia, hyperplasia, and culminating in high-grade dysplasia [105]. Consequently, Mantovani *et al.* (2008) reported that the connection between inflammation and cancer can be described as consisting of two pathways: an extrinsic pathway, promoted by inflammatory conditions that increase cancer risk (e.g. *H. pylori* induced gastritis); and an intrinsic pathway, promoted by genetic mutations that stimulate the synthesis of inflammatory mediators that will nurture tumourigenesis (Figure 4) [104].

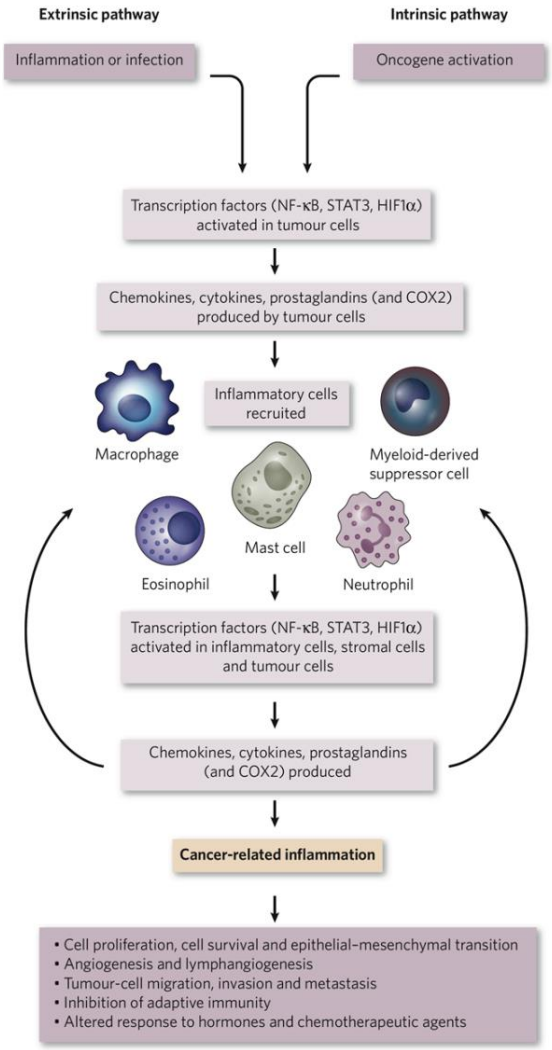


Figure 4. Inflammation and cancer associated pathways. Cancer and inflammation are connected by the intrinsic and the extrinsic pathways. The intrinsic pathway is originated by genetic aberrations that cause neoplasia. Transformed cells induce the production of inflammatory mediators, generating a tumor-inflammatory microenvironment for which there is no underlying inflammation. In the extrinsic pathway, inflammatory or infectious conditions increase the risk towards cancer. The two pathways converge into one point: the activation of transcription factors, which coordinate the production of inflammatory mediators. The inflammatory mediators recruit and activate more inflammatory cells, generating a cancer-related inflammatory microenvironment (adapted from Mantovani *et al.*, 2008 [142]).

Intracellularly, the two pathways converge, resulting in the activation of transcription factors that coordinate the production of inflammatory mediators, such as cytokines. Accordingly, transcription factors are considered the “masters and commanders” of the whole cancer-associated inflammatory process. The cytokines produced and released by cancer cells

activate the same transcription factors in inflammatory cells, stromal cells, and other tumour cells, ending with more inflammatory mediators being produced, generating a cancer-promoting inflammatory microenvironment [104].

Of the outmost importance, signalling pathways and transcription factors that are involved in progenitor/stem cell maintenance and renewal (e.g. STAT3) are targets of inflammation-induced expression [106], and are also frequently found up-regulated in cancer [107]. This observation is highly suggestive of a tight relation between inflammation and cancer, in which critical molecular effectors may play crucial functions in the inflammatory and carcinogenic processes.

2.2 – IL1B and cancer-related inflammation

IL1B is a potent pro-inflammatory cytokine that is not present in tissues under normal homeostatic conditions, but it is produced and secreted upon inflammatory signals [108]. When secreted at low doses, IL1B induces limited inflammatory responses, while at high quantities, it induces a strong and extensive inflammation that is followed by damage of the normal tissue architecture [109]. Remarkably, inflammatory responses are severely attenuated in IL1B knockout ($IL1B^{-/-}$) mice, confirming the crucial role of this cytokine in inflammation [110]. In addition to the destructive effects over tissue architecture, IL1B may be involved in the carcinogenesis process through the activation of infiltrating inflammatory cells or the target cells for transformation to produce ROS and RNI that can ultimately cause genetic aberrations [95].

The functional evidences about the relevance of IL1B on tumour formation were obtained from $IL1B^{-/-}$ mice. In one study, wild type (wt) and $IL1B^{-/-}$ mice were treated with the carcinogenic 3-methylcholantrene (3-MCA). After 110 days of treatment, 60–70% of wt mice developed fibrosarcomas, against only 5% of tumours in $IL1B^{-/-}$ mice [108]. Furthermore, IL1B was found to be involved in cancer cell invasiveness and metastization. Using an $IL1B^{-/-}$ mouse model as a cell recipient, Voronov *et al.* (2003) demonstrated that the local growth and invasion of inoculated B16 melanoma cells was inhibited in $IL1B^{-/-}$ mouse but not in wt animals [111]. Moreover, and contrarily to control mice that promptly developed lung metastases, $IL1B^{-/-}$ mice lacked the ability to form lung metastases after being inoculated with B16 melanoma cells [111].

IL1B is considered a pro-tumorigenic cytokine in different cancer models, conferring an aggressive phenotype to cancer cells. IL1B is expressed in 90% of invasive breast cancers, while in ductal in situ carcinomas (DISC) it is rarely expressed [112]. Moreover, elevated IL1B tumour content has been shown to be significantly associated with established aggressive

parameters (oestrogen receptor negativity and high tumour grade) [112, 113]. In the GI tract, IL1B is highly expressed in colorectal cancer (CRC) in comparison with normal colonic mucosa [114]. *In vitro* experiments performed on CRC cell lines indicate IL1B as a growth factor able to induce cell proliferation [115], inhibit chemically-associated apoptosis [116], and promote stemness and invasive properties [117].

Development of oesophageal adenocarcinoma (OAC) has been linked to chronic inflammation of the oesophagus [118]. The main risk factor for OAC is Barrett's oesophagus (BO), involving a progression into low-grade/high-grade dysplasia [119]. Using a transgenic mouse model with IL1B overexpression restricted to oesophagus, Quante *et al.* (2012) demonstrated that increased expression of this cytokine alone is sufficient for the stepwise formation of Barrett-like metaplasia, dysplasia and intra-mucosal OAC [120]. Noteworthy, the lesions observed in the transgenic IL1B mouse oesophagus share high degree of similarity at the histological and molecular levels with human BO and OAC [120].

2.2.1 – IL1B role on gastric cancer development

A robust amount of evidences supports the association between IL1B and GC development. The first evidences came from the observation that IL1B levels are strongly up-regulated in the gastric milieu of individuals infected with *H. pylori* [36, 38]. Additionally, IL1B was shown to be a potent repressor of the expression of the gastric tumour suppressor *TFF1* [121]. In one of the first studies to evaluate the biological roles of IL1B on gastric epithelial cells, Beales *et al*. (2002), reported that this cytokine exerts a significant pro-mitogenic effect [49]. In fact, the increase in cell proliferation was dependent on the activation of the extracellular signal-regulated kinase (ERK1/2) branch of the mitogen-activated protein kinase (MAPK) pathway [49]. Noteworthy, activation of the ERK1/2 signalling pathway was found to be increased in *H. pylori*-infected individuals [122] and in GC samples when compared with paired adjacent normal gastric mucosa [123-125]. To explore the *in vivo* impact of IL1B on *H. pylori*-mediated gastric epithelium behaviour, Shigematsu *et al.* (2013) infected $IL1B^{-/-}$ mice (BALB/C background) with the bacterium [126]. The authors observed a decrease in the proliferation rate and an increase in apoptosis levels of $IL1B^{-/-}$ gastric epithelium when compared with control wt gastric mucosa [126]. These results validated the previously described pro-mitogenic role of IL1B over the gastric epithelium [49].

A seminal work that supported and validated the role of IL1B in *H. pylori* induced gastric carcinogenesis came from a transgenic mouse model in which IL1B overproduction was

specifically targeted to the gastric epithelium [127]. With IL1B overexpression confined to the gastric mucosa, these transgenic mice developed, in a stepwise fashion that mimicked human gastric carcinogenesis, inflammation and hyperplasia, gastric atrophy, metaplasia, dysplasia, and GC. Relevantly, the formation of gastric lesions occurred even in the absence of *H. pylori* infection, which when introduced led to a reduction in time to lesions formation. Additionally, the pathological changes, including the progression towards GC, were refrained by administration of interleukin-1 receptor antagonist (IL1RA), proving beyond doubt that IL1B is the responsible for the pathological effects observed on the gastric epithelium [127].

2.3 – TGFβ and gastric cancer: from genetics to inflammation

Transforming growth factor (TGF)-β is a multifunctional cytokine with critical functions in many cellular responses such as cell growth, apoptosis, and differentiation [128]. In non-transformed epithelial cells, TGFβ inhibits proliferation through the activation of the cyclin-dependent kinase (CDK) inhibitors p15, p21, and p27 [129], and by repressing the expression of cell proliferation inducer c-Myc [130]. Studies on the TGFβ receptor complex and its downstream signalling mediators (SMADs) revealed TGFβ as an important tumour suppressor pathway [128] [131]. In fact, decreased or even loss of expression of *TGFBRI* or *TGBFRII* is observed in some GC cases [132]. Also, frameshift mutations in *TGFBRII* are a frequent event in gastric and colorectal cancers with MSI [133], and *SMAD4* inactivation by promoter methylation and loss of heterozygosity (LOH) was also detected in GC cases [134].

TGFβ is also a powerful immunosuppressive and anti-inflammatory mediator [135]. The evidence about TGFβ regulation of the tumour microenvironment emerged from the analysis of primary tumours and also from transgenic mouse models. Target deletion of *Smad4* from head and neck epithelia (HN-Smad4$^{-/-}$) promoted spontaneous formation of tumours in mice, characterized by increased inflammation and genomic instability [136]. Noteworthy, the levels of inflammatory mediators, such as IL1B, were found to be significantly increased in tumours derived from those HN-Smad4$^{-/-}$ transgenic mice [137]. In humans, the majority of gastrointestinal MSI cancers have inactivating mutations in *TGFBRII* [131] and are characterized by the presence of a strong lymphocytic infiltration [138]. The histological observation of inflammatory infiltrate in MSI tumours with mutated *TGFBRII* strengthens the anti-inflammatory role of TGFβ signalling pathway.

3 – Transcription factors as crucial effectors in normal and pathological conditions

The critical change in gene expression that follows processes such as cell proliferation, differentiation, expression of cell-specific genes, and response to inflammatory mediators is controlled mainly at the transcriptional level. Also in cancer, transcription factors are the final effectors of oncogenic signalling pathways [139]. In this chapter will be addressed the biological functions of CREB and C/EBP families of transcription factors in normal and pathological contexts.

3.1 – CREB transcription factor family

Extracellular stimuli elicit changes in gene expression in target cells through the activation of intracellular signalling kinase cascades that culminate in the phosphorylation and activation of critical transcription factors. The cyclic AMP (cAMP) responsive element-binding protein (CREB), with a molecular weight of approximately 43KDa [140], is one of the best characterized stimulus-induced transcription factors [141]. Originally, CREB was described to be activated in response to the second messenger cAMP [142]. Later, CREB was identified as a direct effector of other signalling pathways, activated by a diverse array of stimuli [143-145]. At the functional point of view, CREB mediates gene transcription by binding as a dimer to a conserved cAMP-responsive element (CRE) binding motif present on the promoter of target genes [146].

Phosphorylation of CREB at Ser133 plays a crucial role on the activation of CREB, inducing the transactivation of different target genes by promoting the recruitment and complex formation with the transcriptional co-activator CREB-binding protein (CBP) and its paralogue p300 [147, 148]. However, CREB-mediated transcription via a phosphorylation-independent mechanism also occurs, although in this case CREB co-activators are not CBP/p300 but are transducers of regulated CREB activity (TORCs) [149].

Shortly after the characterization of CREB, other two highly related gene products were described: activating transcription factor 1 (ATF1) [150] and cAMP-response element modulator (CREM) [151]. While CREB and ATF1 proteins are expressed in different cell types, CREM is expressed more specifically in neuroendocrine tissues. The primary protein structure of CREB family members uncovers a centrally positioned kinase-inducible domain (KID), inside of which the Serine 133 is located – and several potential phosphorylation sites [144, 152]. The KID region is flanked by hydrophobic glutamine-rich domains, Q1 and Q2, which function as

constitutive activators [153]. A basic leucine zipper (bZIP) dimerization domain and a nuclear localization signal are located in the protein carboxyl terminal (Figure 5) [144, 154].

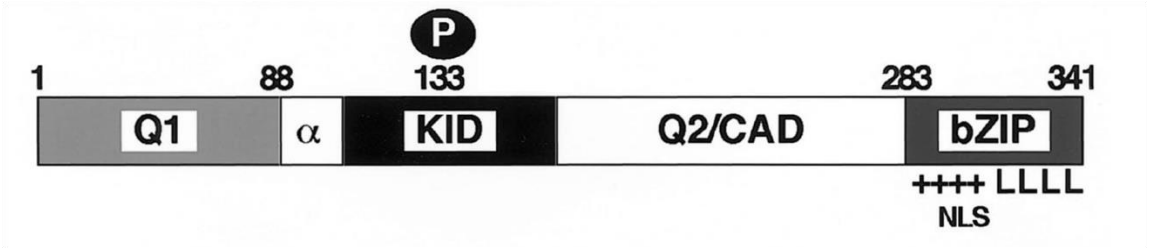


Figure 5. Functional domains of CREB protein. The scheme depicts the major domains of the protein. Numbers on top indicate the position of amino acid residues, (+) marks refer to the positively charged basic domain, and L refers to the leucine zipper domain. The position of the nuclear localization signal (NLS) is also shown as well as kinase-inducible domain (KID), and Q1/Q2 activation domains (adapted from Shaywitz & Greenberg, 1999 [154]).

The *CREM* gene can give rise to different transcripts by alternative splicing, encoding proteins with distinct activator or repressor properties [155]. Noteworthy, a truncated CREM isoform, known as inducible cAMP response element repressor (ICER), consistent with the lack of an activation domain, acts as a potent repressor of transcription mediated by CREB family members [156]. ICER avidly dimerizes with CREB or other family members and through a dominant negative inhibition mode of action, inhibits CREB-dependent transcriptional activity.

3.2 – CREB knockout and transgenic models

CREB null mice ($CREB^{-/-}$ mice) die immediately after birth from respiratory distress [157] due to a defective maturation of alveolar epithelium [158]. Furthermore, $CREB^{-/-}$ mice have an impaired T cell development, and a strong reduction in the corpus callosum [157]. Functionally relevant, CREM protein levels are up-regulated in $CREB^{-/-}$ mice possibly as a compensatory mechanism to re-establish the transcription of CREB-dependent target genes; however not sufficient to rescue the abnormal development of $CREB^{-/-}$ mice [158] On the other hand, CREM and ATF1 null mice do not exhibit any discernible phenotypic alterations, surviving to adulthood [159, 160]; however CREM males are sterile owing to enhanced apoptosis of post-meiotic germ cells.

Due to the absence of a CREB-specific phenotype in knockout mice due to a partial compensatory functional mechanism by other members of the CREB family [158, 161], a different approach to address CREB biology was achieved through the development of transgenic mouse models expressing dominant-negative forms of CREB. The development of

transgenic models, granted the identification of CREB has a crucial player in the control of survival and proliferation of different cell types [144]. As an example, expression of a dominant-negative transgene – described as A-CREB – in chondrocytes resulted in dwarfism due to a strong reduction in the cellular proliferation potential [161]. Further studies revealed that CREB is positively involved in the transcription of several cell-cycle mediators, such cyclin D1, cyclin A, and proliferating cell nuclear antigen (PCNA) [144, 162, 163]. A function for CREB in growth factor-dependent survival has been demonstrated in different cell models, in which the overexpression of a dominant-negative CREB protein induces cell death [164, 165]. Other arguments that point CREB as a pro-survival effector emerged from *in vitro* assays in which the expression of ICER – the endogenous antagonist of CREB – promoted apoptosis through inhibition of CREB-dependent transactivation of the anti-apoptotic gene *BCL2* [166, 167].

3.3 – CREB target genes

The differential transcriptional regulation of target genes by CREB is probably achieved by varying the sequence composition and spatial placement of the CRE-binding motif on gene promoters. Firstly described as an eight-base-pair palindrome, 5'-TGACGTCA-3' [146], the CRE-binding motif also occurs as a half-size motif (5'-CGTCA-3' or 5'-TGACG-3') [168, 169]. Furthermore, the occupancy of the CRE-binding motif by CREB seems to be possibly regulated by DNA methylation: the full-length CRE (5'-TGACGTCA-3') contains a central CpG dinucleotide, and methylation of the cytosine of this site inhibits, at least *in vitro,* CREB binding and subsequent gene transcription [170].

In the first approach to define the pool of CREB target genes, Mayr *et al.* (2001), compiled and described a total of 105 genes directly regulated by CREB [144]. However, because the predicted number of CRE-binding sites in the human genome largely exceeded that report, two genome-wide analyses to identify CREB target genes were performed [171, 172]. In one study, CREB was found to interact with more than 6300 promoter *loci* [171], and in the other CREB was found to occupy approximately 4000 promoters [172]. However, and despite the large number of CREB target genes described, Zhang *et al.* (2005) observed and reported that CREB activity may be targeted only to certain genes at the level of promoter occupancy, Ser133 phosphorylation, or recruitment of the transcriptional apparatus [172]. In fact, Cha-Molstad *et al.* (2004) observed that promoter occupancy by CREB is a dynamic process and varies from one cell type to another, thus showing that the ability of CREB to bind a particular CRE-binding motif represents a crucial component of gene regulation [173].

Niehof *et al.* (1997) reported that one hepatocyte specific CREB-target gene is the transcription factor CCAAT/enhancer-binding protein (C/EBP)-β [174]. Beyond hepatocytes, CREB acts as a critical activator of C/EBPβ expression also in preadipocytes [175]. In this cell model, when CREB is silenced, the other members of the family – ATF1 and CREM – are overexpressed, substituting CREB in the transactivation of *CEBPB* [176].

3.4 – CREB in inflammation

CREB was found to be an important player in the inflammatory process. For example in leukemia cell lines, CREB acts as a direct transcriptional regulator of *IL1B*. In the leukemic cell model, IL1B operates as an autocrine growth factor, where through a positive feedback mechanism that cytokine induces the transcription of it is own gene through the action of CREB [177]. In a different cellular context, in aminon-derived cells, CREB and C/EBPβ were found to be the transcriptional effectors necessary for IL1B expression [178]. Nevertheless, CREB acts not only as a direct transcriptional activator of inflammatory mediators but is also primed by inflammatory signals to regulate the expression of a specific set of inflammation-associated genes in different cellular contexts. In this regard, the increase in expression of MUC8 and MUC5AC – whose up-regulation is virtually always observed in airway inflammatory conditions [179] – in respiratory epithelial cells in response to inflammatory stimuli is mediated by the transcriptional activity of CREB [180, 181].

Kudo *et al.* (2007) reported the pattern and timing of expression and activation of different transcription factors in the gastric epithelium of Mongolian gerbils associated with *H. pylori* infection. CREB was found to be one of the up-regulated transcription factors in response to *H. pylori* infection, and was strongly correlated with gastric mucosa inflammation and ulceration [182]. Furthermore, *H. pylori*-induced gastritis was found to be significantly associated with COX2 expression [183]. This enzyme seems to contribute to the gastric carcinogenesis process, because it is frequently found overexpressed in preneoplastic lesions and GC, where it leads to the increase in the levels of ROS and RNI [184, 185]. Jüttner *et al.* (2003) provided a molecular pathway underlying *H. pylori*-dependent COX2 expression, in which CREB, after being activated by MAPK signalling, is responsible for COX2 expression [186].

3.5 – CREB in cancer

One aspect generally accepted in the cancer research field is that unrelated tumours can activate the same oncogenic pathway/s using alternative strategies to that particular end. This is true for CREB-dependent signalling because its activation, by direct or indirect mechanisms, is frequently observed in various tumour types [187].

The first evidence about CREB involvement in cancer emerged with the identification of the chromosomal translocation t(12;22) in clear cell sarcomas (CCS) that creates the fusion protein EWS-ATF1 [188]. More recently, the EWS-CREB fusion protein was also found in a gastrointestinal CCS [189]. Both in EWS-ATF1 and EWS-CREB fusion proteins, the KID domain of ATF1/CREB is substituted by the EWS activation domain, originating a new fusion-transcription factor that has a functional CRE-binding recognition domain and acts independently of phosphorylation events [190]. Remarkably, the presence of the CRE-binding domain has been shown to be mandatory for the cellular transformation and tumour cell survival [191].

3.5.1 - Leukemogenesis

The role of CREB in leukemogenesis has been supported by various studies in leukemia patients and leukemia-derived cell cultures [192-195]. Increased protein levels of CREB and phosphorylated CREB (pCREB) were found in bone marrow cells from patients with acute lymphoid leukemia (ALL) or with acute myeloid leukemia (AML) in comparison with control individuals [194, 195]. Furthermore, leukemia patients with increased CREB levels have a poor prognosis, characterized by a decrease in the time to relapse and a decrease in event-free survival [192, 193]. *In vitro* enforced overexpression and gene silencing assays in leukemia cell lines allowed to observe that CREB confers increased growth and survival advantages [193]. Furthermore, the *in vivo* silencing of CREB in an aggressive model of BCR/ABL-driven leukemia resulted in a significant increase in animal survival [196]. Taken together, these results indicate CREB as an important proto-oncogene in leukemogenesis.

Transplantation of bone marrow cells silenced for CREB expression in irradiated mice resulted in a smaller number of committed progenitor cells compared with control non-silenced cells. However, long-term engraftment revealed no major effects on mice, suggesting that CREB insufficiency is not essential for hematopoietic stem cell (HSC) activity [196]. Thereby, CREB seems to be a critical factor for the maintenance of leukemia cell biology but not essential for the normal function of HSCs. This aspect revealed the dependence of leukemia cells on CREB-dependent signalling, which can be used in future cancer-targeted therapies.

3.5.2 – Epithelial cancers

Similarly to leukemias, CREB expression has been reported as deregulated in cancers with an epithelial origin. Prostate cancer (PC) is the most frequent cancer type in men in developed countries [4]. At early disease stages, PC is responsive to androgenic regulation, but eventually progresses to a stage that is resistant to androgen deprivation and poorly responsive to the present available therapies [197]. Progression of PC to androgen resistance is associated with up-regulation of insulin-like growth factor-1 receptor (IGF1R) [198] and Genua *et al.* (2009) disclosed that CREB is responsible for IGF1R increased expression in PC [199]. Furthermore, the immunohistochemical analysis on normal prostate gland showed no detectable levels of pCREB, whereas positive pCREB staining was observed in all PC of the poorly-differentiated type and also associated bone metastasis [200]. This positive association between pCREB and metastasis suggests that this CREB-dependent signalling is involved in PC tumour progression and metastization [200].

In breast, CREB transcript level was significantly up-regulated in tumours compared with adjacent normal tissue [201]. Patients with higher transcript levels of CREB had a significantly shorter disease-free survival compared with patients with low levels of CREB mRNA [201]. Moreover, highly metastatic breast cancer cell lines express higher levels of CREB compared to non-metastatic cell lines. In highly metastatic cell lines, CREB expression is necessary for the transcriptional expression of genes *PTHrP*, *MMPs*, and *OPG*, which are closely involved in cancer metastasis and bone destruction [202]. Recently, a positive link between HER2-positive breast tumours and CREB expression was described, in which HER2 increases pCREB levels [203]. Based on *in vitro* and *in vivo* evidences, increased pCREB levels in HER2-positive breast tumours were found necessary for cell proliferation, survival and migration, as well as for tumour formation ability [203].

In never smoking patients with non-small-cell-lung cancer (NSCLC) CREB and pCREB were found significantly up-regulated in tumour tissue compared with adjacent normal tissues [204]. Moreover, in those NSCLC cases the authors reported an inverse association between the expression level of CREB and pCREB and disease free-survival [204].

3.6 – C/EBP transcription factor members

The first CCAAT/enhancer binding protein (C/EBP) member identified was reported to be able to interact with the CCAAT box motif present in the promoter of several genes [205]. Thereafter, the optimal C/EBP binding motif was identified as being RTTGCGYAAY, where R is A or G, and Y is C or T, though some additional variations are tolerated [206]. Additionally, five C/EBP members were identified, all characterized by possessing a conserved basic-leucine zipper (bZIP) type of DNA-binding and dimerization domain located at the protein C-terminus [207, 208]. To prevent possible nomenclature mistakes, Cao *et al.* (1991) proposed that each C/EBP member should be named with a Greek letter according to the chronological order of their discovery (C/EBPα – C/EBPζ) [209]. Four C/EBP-family members are intronless (C/EBPα, -β, -δ, and -γ), whereas C/EBPε and -ζ possess two and four exons, respectively (Figure 6) [208].

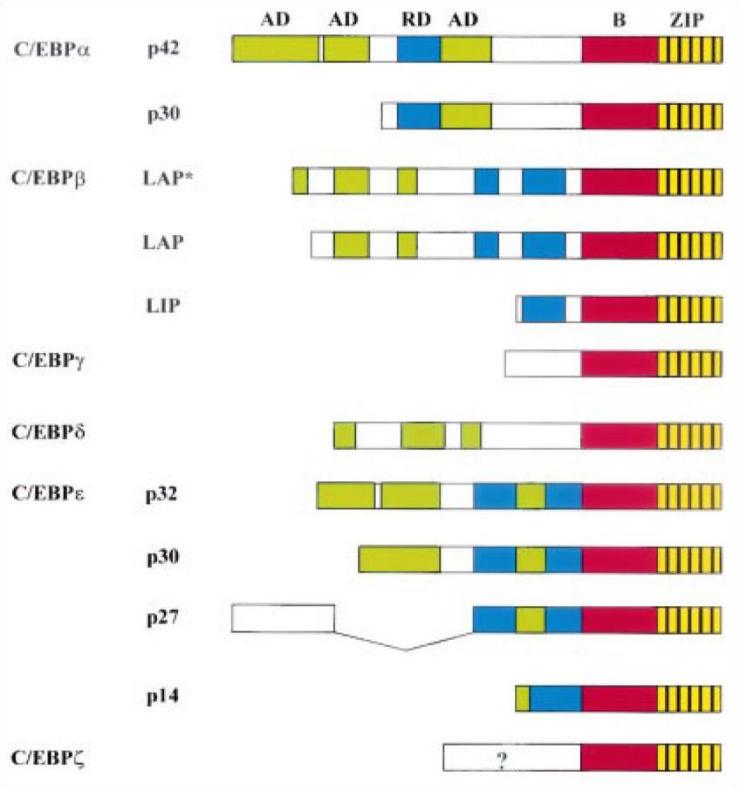


Figure 6. Representation of C/EBP family members. The leucine zipper is shown in yellow, with black vertical lines indicating the leucine residues; the basic region is in red. The position of the activation domains (AD) and negative regulatory domains (RD) are shown in green and blue respectively. ? indicates the N-terminus activation domain of C/EBPζ (adapted from Ramji & Foka, 2002 [208]).

In analogy to other transcription factors, C/EBP protein dimerization is mandatory for DNA binding [207, 210], and due to the highly conserved bZIP domain between all family members, C/EBP proteins are able to form heterodimers with different binding and transcriptional activities [208, 209, 211]. In opposition to the bZIP C-terminal portion of C/EBPs, the N-terminal part is quite divergent between members, with the exception of the activation domains that are important for the interaction with elements of the basal transcriptional apparatus [208, 212].

Notwithstanding the existence of six C/EBP members, the number of C/EBP proteins in a specific cell at a certain moment can be higher. The reason for this resides first, in the fact that C/EBPε can give rise to different peptides by alternative use of promoters and exon-alternative splicing [213], and second because C/EBPα and –β mRNA molecules can give rise to different peptides by a leaky ribosome scanning mechanism [214]. Through this mechanism, C/EBPα transcript can give rise to a full-length 42KDa and a truncated 30KDa isoforms, while C/EBPβ mRNA can originate three isoforms, 38KDa (LAP*), 35KDa (LAP), and 21KDa (LIP) [208, 214].

All C/EBP members exhibit a specific cell/tissue expression profile: C/EBPα and C/EBPβ are expressed in a wide range of cell types; C/EBPδ is expressed in adipose tissue, intestine, and lung [209, 211]; C/EBPγ and C/EBPζ are ubiquitously expressed [208, 215], and C/EBPε expression is largely confined to granulocytic cells [213].

Of the six C/EBP members, C/EBPα and C/EBPβ have been the most thoroughly studied in human and animal models, while research focusing the other members is still very scarce. Thus, from now on, in next sub-chapters it will be described the up-to-date data about the biological processes in which C/EBPα and C/EBPβ are involved.

3.6.1 – C/EBPs in cellular proliferation and differentiation

From the first observations about C/EBPα expression, reporting the strong protein levels in terminally differentiated cells that an anti-proliferative role was suggested for this transcription factor [216]. In fact, when C/EBPα expression is induced in *in vitro* assays it is able to efficiently inhibit cell proliferation [216, 217]. Mechanistically, the inhibition of cell proliferation triggered by C/EBPα can occur by the action of this protein on: a) the regulation, and activation of the CDK inhibitor p21 [218]; b) the inhibition of CDKs activity [219]; c) the repression of E2F-mediated transcriptional activity [220]; and interaction with the SWI/SNF complex [221, 222].

Mice silenced for C/EBPα (C/EBPα$^{-/-}$) die shortly after birth due to a limited production of liver enzymes necessary for glucose metabolism [223]. Nevertheless, cultured hepatocytes from C/EBPα$^{-/-}$ new-born mice display increased proliferative activity, with increased transcript levels of the proliferation activators Jun and MYC [224, 225]. Also, terminal differentiation of neutrophils [226] and the respiratory epithelium [227] are profoundly imbalanced in the absence of C/EBPα. During adipogenesis, C/EBPα expression is observed in the late stages of the process, when it is responsible for the transcription of genes specific of differentiated mature adipocytes [228]. Those observations supported the notion that a single transcription factor is able to regulate two essential features of differentiated cells: promote cell-specific gene expression and proliferation arrest.

In opposition to C/EBPα, C/EBPβ is able to play anti-proliferative or proliferative functions, depending on the cell type context. In keratinocytes, C/EBPβ seems to have anti-proliferative functions because enforced expression of C/EBPβ in mouse keratinocytes inhibited cell growth and induced phenotypical changes associated with a differentiated phenotype. Moreover, analysis of the epidermis of C/EBPβ$^{-/-}$ mice revealed epidermal hyperplasia and decreased expression of the differentiation markers keratin 1 (K1) and keratin 10 (K10) [229]. On the other hand, several reports indicate C/EBPβ as a promoter of cellular proliferation. As an example, C/EBPβ expression is promptly induced in hepatocytes after partial hepatectomy, where it is necessary for promoting liver regeneration [230]. Also, the mammary epithelial cells (MECs) from C/EBPβ$^{-/-}$ mice showed a decrease in the proliferative rate, ending in abnormal lobulo-alveolar morphogenesis [231, 232]. Enforced expression of C/EBPβ in human MEC resulted in hyper-proliferation that was accompanied by the acquisition of a partially transformed phenotype, characterized by an increase in the invasive ability [233]. Moreover, C/EBPβ was described as a critical transcription factor in mammary stem cells (MaSC), promoting the cellular outgrowth potential [234].

The preadipocyte differentiation model revealed the functional link between C/EBPβ and C/EBPα expressions. In this model, the differentiation from preadipocytes into adipocytes occurs through the serial induction of these two transcription factors. The expression of C/EBPβ (and also C/EBPδ) is rapidly induced in preadipocytes after the differentiation stimulus, diminishing its protein levels during the terminal phases of differentiation. In these last phases of adipocyte differentiation, C/EBPα expression is strongly induced, standing as the most highly expressed member of C/EBP-family. In fact, C/EBPα regulates the terminal adipocyte differentiation, turning on the battery of adipocyte-specific genes required for the full-functionality of mature adipocytes [235, 236] (Figure 7).

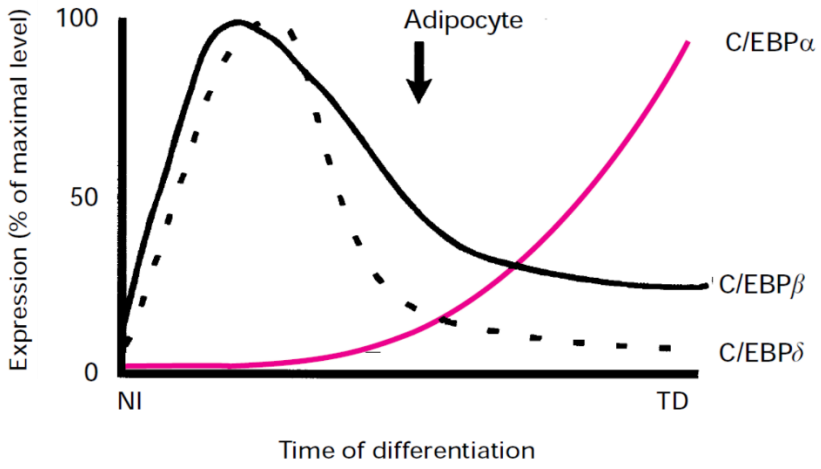


Figure 7. Temporal pattern of C/EBPs expression during preadipocyte differentiation. Abbreviations: NI, non-induced; TD, terminal differentiation. Arrow point to the approximate time when proliferation ends (adapted from Calkhoven & Ab, 1996 [236])

3.6.2 – C/EBPs and inflammation

Soon after the identification of C/EBPs that a functional link with inflammation was established based on the ability of inflammatory mediators to promote the activity or expression of members of this family of transcription factors. For example, C/EBPβ was first identified as an inducible protein in response to activation of IL6 signalling pathway [237]. In fact, IL6 stimulation was not only able to induce the expression of C/EBPβ but also was shown to decrease C/EBPα expression [208, 237, 238]. Additionally, subsequent studies allowed the identification of a broad range of inflammation-related genes activated by C/EBPs such as cytokines and their receptors, and elements of signal transduction pathways [208].

Work performed with mouse models allowed to discriminate relevant roles of C/EBPs in the inflammatory cells. Functionally relevant, C/EBPα and C/EBPβ are expressed in cells from the myeloid lineage [208, 209, 211]. C/EBPα$^{-/-}$ mice lack mature neutrophils due to defects in myeloid differentiation beyond the myeloblast stage, and C/EBPβ$^{-/-}$ mice lack mature or functional macrophages [208, 209, 211], [239]. In fact, macrophages from C/EBPβ$^{-/-}$ mice exhibit impaired expression of inflammatory mediators, such as TNFα [237, 238]. Additionally, C/EBPβ is also involved in epithelial cell response to inflammatory insults. In this regard, mice with an airway and alveolar epithelial-specific disruption of C/EBPβ (*Cebpb*$^{\Delta LE}$) displayed a

dramatic impairment of neutrophils and pro-inflammatory mediators to the lungs when compared with control mice [240].

TGFβ is a powerful immunosuppressive and anti-inflammatory cytokine [135] that is able to block the biological functions of IL6 [241]. The causative impairment in IL6 signalling activation occurs at the transcriptional level, where the SMADs complex operate as inhibitors of C/EBPβ-dependent transcription of acute-phase genes, such as haptoglobin (*HP*). The inhibitory effect of TGFβ over C/EBPβ was previously reported in rat intestinal epithelial cells, where TGFβ caused the attenuation of an inflammatory-dependent increase in expression of C/EBPβ and also the loss of binding of this transcription factor to *HP* promoter [242].

In the gastric epithelium it is known that *H. pylori* infection promotes COX2 expression through activation of toll-like receptors (TLR) [243]. Searching for a regulator of COX2 expression in GC, Regalo *et al.* (2006) identified C/EBPβ as promoter-activating transactivator of COX2 in GC cell lines [185].

3.6.3 – C/EBPs in cancer

From what has been exposed above, it is evident that C/EBPs play important roles in normal cell biology and in inflammation-associated processes. Nonetheless, C/EBPs were also found to play complex and important roles in cancer. Thus, in this sub-chapter, it will be described the involvement of C/EBPs in the carcinogenesis process.

3.6.3.1 – C/EBPα behaves as a tumour suppressor

The first evidences that suggested C/EBPα as a tumour suppressor were found in AML. In this blood cancer type, inactivating somatic mutations were found in approximately 10% of leukemic patients [244, 245]. Two-thirds of *CEBPA* mutations are bi-allelic: the combination of N- and C-terminal *CEBPA* mutations accelerates disease development and explains the clinical occurrence of this mutational configuration [246]. In addition to mutations, C/EBPα protein is also frequently found downregulated in AML as a direct consequence of *CEBPA* promoter hypermethylation [247]. Nevertheless, *CEBPA* mutations are not a common event in other malignancies, with only one report about *CEBPA* mutations in GC [248]. The mutation described, although possibly damaging is a very infrequent event (1 mutation in 142 GC cases) and lacks confirmation of the real pathological relevance.

To examine the role of C/EBPα in epithelial tumour development, Loomis *et al.* (2007) evaluated the function of C/EBPα in an epidermal-specific C/EBPα$^{-/-}$ mouse model. When C/EBPα$^{-/-}$ mice were subjected to chemically-induced carcinogenic insults, they displayed decreased tumour latency period and dramatic increases in tumour incidence and growth rate [222].

In lung adenocarcinoma, C/EBPα is frequently found downregulated in a large proportion of cases, and *in vitro* enforced expression led to a significant decrease in cell growth, emergence of morphological changes characteristic of differentiation, and apoptosis [249]. Tada *et al.* (2006) demonstrated that DNA hypermethylation and histone acetylation of the upstream *CEBPA* promoter were strongly associated with decreased or absent C/EBPα expression in lung cancers [250]. In addition to lung cancer, C/EBPα expression was also found downregulated in breast [251] and pancreatic cancers [252].

3.6.3.2 – C/EBPβ promotes tumourigenesis

The development of C/EBPβ$^{-/-}$ mice allowed the observation that this transcription factor is involved in the development of certain cancer types. One of the first studies suggesting this effect was performed to assess the tumorigenic potential of C/EBPβ during carcinogen-induced skin tumour formation. In this study, C/EBPβ$^{-/-}$ mice revealed to be totally refractory to tumour formation [229]. The reason behind the lack of tumour formation in C/EBPβ$^{-/-}$ mice resides in the critical role of C/EBPβ for keratinocyte survival though – a process triggered by activated RAS pathway [229]. Because keratinocytes of C/EBPβ$^{-/-}$ mice exhibit a dramatic increase in cellular apoptosis when submitted to carcinogens, this abnormal increase in cell death can be sufficient to confer total resistance to chemical-induced cancer formation [239]. The increase in apoptotic rates are possibly caused by an aberrant overexpression of p53 in the keratinocytes of C/EBPβ$^{-/-}$ mice, since C/EBPβ acts as negative regulator of p53 [253].

Breast cancer is perhaps the better characterized model for C/EBPβ tumourigenic role. In this cancer type, C/EBPβ was found expressed in 70% of cases [254]. Two independent studies revealed the importance of C/EBPβ on breast cancer formation and progression. In one of those studies, the overexpression of C/EBPβ isoform LIP in the mouse mammary gland was sufficient to generate hyperplastic lesions in 30-40% of the glands, and carcinomas in 9% of the glands [255]. In the other study, the enforced expression of C/EBPβ isoform LAP was able to transform a normal epithelial breast cell line (MCF10A), conferring it anchorage independent growth and acquisition of invasive properties [233]. Additionally, and from a mechanistic approach, C/EBPβ isoform LIP was described as being involved in breast cancer progression

by blocking the cell-growth inhibitory action of TGFβ signalling pathway [256]. Thereafter, various studies revealed C/EBPβ-target genes with important roles in breast cancer formation and metastization [257-261].

Noteworthy, a general oncogenic role for C/EBPβ was proposed by Lamb *et al.* (2003), who examined the gene expression profiles of different human tumours types characterized by increased levels of cyclin D1. The authors found that C/EBPβ overexpression was significantly correlated with cancer types marked by cyclin D1 up-regulation, indicating a possible functional link between C/EBPβ and cyclin D1 [262].

In respect to GC, C/EBPβ was shown to be overexpressed in tumour samples when compared to adjacent normal mucosa [263]. In independent studies, Regalo *et al.* (2006) and Milne *et al.* (2006) confirmed the up-regulation of C/EBPβ protein in gastric tumour samples, particularly in tumour with an intestinal or atypical histological differentiation [185, 264]. Moreover, C/EBPβ was found to be a direct transcriptional repressor of TFF1 expression [263], a well-established gastric tumour suppressor [77].

4 – Transcription factors as targets for cancer therapy

A selective number of transcription factors are overexpressed and/or overactive in most human cancers, making them putative relevant targets for the development of anticancer drugs. This rationale is supported by knowing that there are more oncogenic proteins and oncogenic signalling cascades upstream of these same transcription factors than are oncogenic transcription factors. Therefore, being transcription factors the terminal effectors of malignant gene expression patterns, they occupy a central role in all classic hallmarks of cancer [139, 265, 266]. Thus, effective anti-transcription factor drugs could be able to antagonize and inhibit the action of various upstream-activated oncogenic pathways [267].

Mechanistically, signalling cascade activation begins with the binding of extracellular proteins (ligands) to cell-membrane receptors that dimerize or oligomerize at the cell surface to start intracellular communication events. Cell-membrane receptors have been frequently found mutated or overexpressed in cancer. As examples, epidermal growth-factor receptor (EGFR) and platelet-derived growth-factor receptor (PDGFR) have shown to be overexpressed or mutated in different cancer types [268]. Then, specific cytoplasmic proteins will act as signal transducers, transmitting the information into the nucleus [267]. In this regard, intracellular tyrosine kinases activated by mutation are fairly common in cancer. As an example, *KRAS* is frequently found mutated in human cancers, including as previously mentioned GC [267].

Finally, the increase in expression and activity of transcription factors that are activated in a direct or indirect way by specific phosphorylation events may act as oncogenes, if involved in the transcriptional expression of genes with positive functions in cell proliferation and/or cell survival [139, 267].

Transcription factors with oncogenic roles in cancer can be classified into three main groups according to their functional behaviour [267]. The first group corresponds to the steroid receptors, and examples of expression in cancer are oestrogen receptors (OR) in breast and androgen receptors (AR) in prostate. Anti-oestrogen and anti-androgen drugs – such as tamoxifen and bicalutamide, respectively - have been in clinical use successfully for years [269, 270].

The second group of transcription factors is defined by resident nuclear proteins, which are activated by serine kinase signalling cascades [271]. Members of this second group comprises, among others, JUN, ATF-CREB-CREM family, and the C/EBP family of transcription factors [267, 271]. Regarding this second group, a battery of small-molecule inhibitors of CREB activity were recently described [272, 273]. These small-molecule inhibitors, designated KIX-KID interaction inhibitors, exert their effect by disrupting the interaction between CREB and its co-activators, namely CBP and p300. Notably, one of the KIX-KID inhibitors exhibited a strong *in vitro* effect in inhibiting the proliferation of breast cancer cells but not mammary normal epithelial cells, revealing the strong dependence of breast cancer cells for CREB-dependent transcriptional activity [273].

The third group of transcription factors is composed by “latent cytoplasmic factors”. The hallmark of this group is residence in the cytoplasm in an inactive form until they are activated by proteins that bind cell surface receptors [271]. As members of the latent cytoplasmic factors group one can found STATs, NF-κB, and β-catenin [267, 271]. The signal-transducer and activator of transcription (STAT)-3 acts as a key player in important oncogenic signalling pathways [107]. Constitutive STAT3 activation has been observed in various human cancers, usually associated with a poor clinical outcome [106, 274]. During years, numerous strategies were developed to inactivate STAT3. The first approach was the development of small-molecules that reportedly inhibited STAT3 activity. Unfortunately, those small-molecules demonstrated a general lack of specificity [275, 276]. Recently, a different approach to directly inhibit STAT3 activity was developed using double-stranded oligonucleotide decoys [277]. The STAT3 decoy exhibited selective STAT3-binding, resulting in *in vitro* inhibition of proliferation and survival of head and neck squamous cell carcinoma (HNSCC) cells [278]. A phase 0 clinical

trial was performed using double-stranded oligonucleotide decoys, confirming the efficacy of this approach in inhibiting STAT3 oncogenic signalling in HNSCC tumours [278].

NF-κB is a transcription factor that promotes the transcription of inflammatory cytokines [279, 280] and whose constitutive activation has been observed in various malignancies [281-284]. Noteworthy, a small molecule antagonist of NF-κB (DHMEQ) has been recently developed, and it acts on NF-κB-dependent signalling inhibition by directly interacting with the protein complex and blocking nuclear translocation [285].

Another protein with important transactivating properties is β-catenin. Although β-catenin do not bind DNA, it possess an amino-terminal domain that bind to transcriptional co-activators and to TCF/LEF DNA binding proteins [271]. Deregulated WNT signalling pathway is one of the most frequent and functionally relevant events in colorectal cancer. In this cancer model, inactivating mutations in the tumour suppressor *APC* result in the translocation of β-catenin to the cell nucleus, leading to the constitutive activation of WNT signalling [286]. A recently developed battery of small-molecules that lead β-catenin to degradation via proteasome pathway are showing promising results, because those small-molecules were able to selectively kill cancer cell lines with constitutive WNT signalling activation [287].

Noteworthy, in addition to the position that transcription factors occupy in oncogenic signalling pathways as the final cellular effectors, they can also act as the direct mutational targets after chromosomal or genetic aberrations. In fact, several genes involved in chromosome rearrangements are transcription factors – as examples, the EWS-ATF1 and EWS-CREB rearrangements observed in CCS, as previously mentioned [188, 189, 288]. Also, an increasing number of pathognomonic tumour-specific genetic and epigenetic events have been shown to directly inactivate tumour suppressor or activate oncogenic transcription factors [139].The increase in understanding about the role of transcription factors and allied networks in the carcinogenesis process provides a hope and a challenge for new innovative treatment strategies. Identification of the most appropriate transcriptional targets in distinct tumour types and efficient delivery methods are mandatory prerequisites for the development of effective pharmacological inhibitors of transcription factors.

RATIONALE AND AIMS

In clear contrast with other cancer models, there is a general lack of knowledge about the natural history of GC, especially the signalling pathways and molecular mechanisms that forge tumour formation through the disruption of the normal gastric homeostatic processes. The discovery and understanding of the molecular events underlying gastric carcinogenesis will ultimately inform about possible and more effective treatment strategies.

It is well recognized that chronic inflammation is a risk factor for the development of a wide range of cancers. In the gastric epithelium context, a robust set of evidences link *H. pylori*-associated chronic inflammation with the onset of GC. Moreover, the risk to develop GC further increases in individuals that have a genetic background responsible for a more intense inflammatory response. However, the signalling pathways modulated by inflammation and the mechanisms through which the inflammatory response drives the transformation of gastric epithelial cells remains to be elucidated. It is proposed that the link between enhanced chronic inflammation and GC depends on the long-term damaging effects of inflammatory mediators over the gastric mucosa. Coupled with mutagenic events, this could ultimately lead to increased risk of cell transformation and GC development.

In this regard, IL1B is particularly interesting because polymorphisms in its promoter region have been shown to be associated with increased risk of GC, possibly through the differential production of the cytokine. Also, IL1B was shown to act as a growth factor able to increase the proliferative rate of gastric epithelial cells and a transgenic mouse model with gastric-specific IL1B overproduction develops GC. Hence, inflammatory mediators, such as IL1B, are able to disrupt gastric homeostasis possibly through the modulation of signalling pathways. The signalling events converge in the activation of transcription factors that act as cellular effectors of inflammation-induced biological responses. Furthermore, inflammation-activated transcription factors are also found to play relevant biological roles in malignancies. This observation is highly suggestive of a tight relation between inflammation and cancer, in which major molecular effectors play crucial functions in the both inflammatory and carcinogenic processes.

Based on the exposed, **the main goal of this work was to increase our understanding of the signalling events and associated molecular mechanisms underlying inflammation-driven GC development**. To achieve our major goal, we focused on two specific aims:

- **Determine the expression patterns of inflammation-associated molecular effectors on normal gastric mucosa, preneoplastic and GC lesions.**

By pursuing this goal, we expected to observe and report for the first time the histological pattern of distribution of inflammation-associated transcription mediators over gastric epithelium. The comparison of the expression profile between normal gastric epithelium and different gastric lesions would function as an indication mark about the putative biological/pathological role of those transcriptional effectors.

- **Determine the inflammation-modulated signalling cascades responsible for the expression/activation status of molecular effectors and the underlying biological meaning through *in vitro* and *in vivo* approaches**

  Because the expression/activation status of inflammation-induced transcription effectors is the final step of a series of intracellular signalling events, we aimed to disclose the underlying inflammation-triggered signalling mechanisms. After that, through a series of *in vitro* and *in vivo* assays we attempted to translate our findings into a biological readout. We expected that the results obtained could help inform new strategies for prevention and treatment of GC, including the control of chronic inflammation and the identification of new therapy targets.

MATERIAL AND METHODS

Optimized and applied for article "C/EBP alpha expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis".

Tissue Material

Surgical specimens from 54 GC were resected and diagnosed at Hospital S. João/Faculty of Medicine, Porto, Portugal. Tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 3 mm were obtained from each block and used for routine staining with haematoxylin and eosin and immunohistochemistry. The procedures followed in this study were in accordance with the institutional ethical standards. All the samples enrolled in this study were delinked and unidentified from their donors.

Immunohistochemistry

Tissue sections were first treated with 10mmol/l citrate buffer (pH 6.0) for 40 min at 99ºC. Unspecific endogenous peroxidase activity was eliminated with a Hydrogen Peroxide Block solution (Labvision, UK) for 10 min. After washing, slides were incubated with monoclonal mouse antibody anti-C/EBPα 1:300 (Cell Signaling Technology, USA) or C/EBPβ (1:100, Santa Cruz Biotechnology, USA) 1 h at room temperature (RT). Sections were then washed and incubated with Dako Real Envision/HRP Rabbit/Mouse solution (DAKO, Denmark) for 30 min (RT). The slides were then developed for 10 min in Dako Real diaminobenzidine (DAB) (0.05%, DAKO) and sections counterstained with haematoxylin, dehydrated, and mounted. For immunofluorescence, after the primary C/EBPα antibody incubation, sections were incubated with a biotinylated secondary antibody and signal was obtained with Alexa Fluor (Molecular probes, Invitrogen, CA, USA) incubation. For double TFF1 and C/EBPα staining, two independent reactions were performed on the same slides. Sections were blocked for 15 min in 10% BSA with anti-mouse serum and incubated overnight in monoclonal antibody anti-C/EBPα at 1:100 (Cell Signaling Technology, USA). After washing, samples were incubated with anti-rabbit secondary antibody 1:200 (DAKO, DK) for 30 min and washed again. A final 1 h incubation with avidin-biotin-peroxidase 1:100 (DAKO, DK) was performed. Slides were then developed with DAB (DAKO, DK). After a washing step of 30 min in PBS at 60ºC, slides were again incubated overnight with monoclonal antibody anti-TFF1 1:100 (Zymed, USA) and developed with alkaline phosphatase (DAKO, DK) and Fast Red (Sigma-Aldrich, USA). Slides were reviewed by a pathologist, tumours were classified according to Lauren's classification,

and the sections were semi-quantitatively scored according to the intensity of staining when compared with the positive control: intense staining was classified as III; moderate intensity as II; and weak intensity or negativity as I. Cases were classified as 'downregulated' when >50% of the tumour cells were classified as I. All washing steps were performed in PBS buffer. Normal gastric mucosa was used as a positive control, and negative controls were performed by substitution of the primary antibody with immunoglobulins of the same class and concentration.

Cell Culture, Transfections, and Western Blotting

AGS and MKN28 cells were grown in RPMI medium with 10% FBS (GIBCO, Invitrogen, USA). AGS cells were grown until 60–80% confluence in six-well plates, and transfected using 3 μg of Plenti-C/EBPα expression vector with an appropriate TFX-50 (Promega, WI, USA) concentration and volume. For western blot analysis, cells were scrapped in PBS and lysed in RIPA buffer with protease and phosphatase inhibitors. A measure of 40 μg of total protein were loaded into acrylamide gels and separated by electrophoresis. The proteins were then transferred to Hybond membranes (Amersham Biosciences, UK). For dot blot, 20 μg of denatured protein extract were directly pipeted into Hybond membranes. After blocking, blots where incubated during 1 h with primary antibodies anti-C/EBPα 1:100 (Cell Signaling, USA), anti-P27 and anti-Cyclin D1 1:100 (Santa Cruz Biotechnology, USA), anti-tubulin 1:15000 (Sigma-Aldrich, USA), and in the case of the dot blot with anti-TFF1 1:100 (Zymed, USA) in PBS plus 5% non-fat dried milk and 0.5% tween-20. The blots were then washed three times in the same solution and incubated 45 min with an HRP-conjugated secondary antibody 1:1000 (Santa Cruz Biotechnology, USA) in PBS 0.5% tween-20. Blots were then washed three times in PBS 0.5% tween-20 and signal was detected with chemiluminescence using ECL (Amersham Biosciences, USA). For MAPK inhibition experiments, MKN28 cells were grown until 50–60% confluence and treated for 24 h with 10 μM SB239063 or PD98059 (Sigma-Aldrich, USA).

BrdU Incorporation Assay and Immunocytochemistry

AGS cells were harvested in 24-well plates with glass slides, and transfected using TFX50 (Invitrogen, USA) with empty vector and full-length C/EBPα expression vectors in OPTIMEM medium (GIBCO, USA). After 1 h, complete RPMI medium was added and cells were left growing for 48 h. MKN28 cells were grown in six-well plates with glass slides and

treated with MAPK inhibitors as described above. After incubating 1 h in 5-bromo-deoxyuridine (BrdU), cells in the glass slides were fixed in 4% paraformaldehyde, washed with PBS two times, and quenched by incubation with 2M HCl for 20 min. After washing, slides were incubated with anti-BrdU antibody 1:100 (DAKO, DK)) for 1h. For simple immunocytochemistry, MKN28 cells treated and untreated with MAPK inhibitors were blocked in PBS with 4% BSA and incubated in C/EBPα 1:100 (Cell Signaling, USA) antibody for 1 h. In procedures, cells were finally incubated with secondary anti-mouse FITC 1:100 (DAKO, DK)-conjugated antibody for 30 min. After washing, cells were mounted in Vectashield (Vector Laboratories, USA) with DAPI blue and scored for BRDU incorporation or C/EBPα expression on a fluorescence microscope.

Inhibition of C/EBPα by siRNA

MKN28 cells were grown until 50% confluence and pre-incubated in serum-free medium. The appropriate anti-C/EBPα target sequence (100 nM), and scrambled control siRNA (Qiagen, DE) were mixed with Metafectene (Biontex laboratories GmbH, Germany) in serum-free medium, incubated for 20 min and added to the cells. After overnight incubation, the medium was changed to complete RPMI and cells left to grow for 48 h, after which BRDU incorporation and protein expression analyses were performed.

Statistical Analysis

Comparison of GC cases regarding their clinicopathological features was performed using Fisher's and χ^2 test. Three independent measurements were performed for the BRDU incorporation experiments and results were compared by Student's t-test.

Optimized and applied for article "C/EBPβ/RUNX1t1 regulatory loop controls cell proliferation in gastric cancer".

Human gastric cancer samples and microarray data

Human tissue samples were derived from patients that had undergone resection for sporadic gastric adenocarcinoma at the Robert-Roessle Hospital (1995–2003). The selection of samples, the procedure for histological classification and staging, the second blinded evaluation by an independent pathologist including assessment of tumour content in the pieces that RNA was extracted from, and RNA extraction and microarray procedure has been described elsewhere [289].

Transgenic mice

C/EBPβ knockout (KO) animals were previously established in C57Bl/6 background [239]. C57Bl/6 RUNX3 KO mice were obtained from the group of Prof. Ito [90], and crossed with C/EBPβ KO mice. Due to the lethal phenotype of the single RUNX3 KO, C/EBPβ/RUNX3 heterozygote animals were bred and the phenotype analysed in the offspring at birth. Animals were bred and kept according to the institutional guidelines, and genotyped by PCR as previously described [90, 239].

C/EBPβ knockdown cells and in vivo tumorigenic assay

MKN45 and MKN74 cells were infected with lentivirus containing GFP-tagged control shRNA and shRNA against C/EBPβ. Efficiency of knockdown was assessed by Western Blot and proliferation was measured by BrdU incorporation assay. The effect of C/EBPβ expression on tumour formation was examined by subcutaneously implanting 3×10^6 cells of control MKN74/45 and ShRNA-mediated C/EBPβ-silenced MKN74/45 into 6-8-week-old male NIH(s) II-nu/nu nude mice, four mice per group. The animals were monitored weekly for tumour formation for 20 days after inoculation. Tumour sizes in two dimensions were measured with calipers, and volumes were calculated with the formula (a × b2) × 0.5, wherein "a" is the long axis and "b" is the short axis (in millimeters). Mice were maintained and sacrificed according to institutional guidelines, and at termination of the experiment tumours were excised, fixed, embedded and analysed by immunohistochemistry for Ki67 and C/EBPβ expression.

Co-immunoprecipitation

Flag-tagged RUNX1t1 was expressed in MKN28 and MKN45 cell lines. Cells were harvested and lysed in buffer containing 50 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 0.2% NP-40, 5 mM MgCl2, 50 µM, ZiCl2 and protease inhibitor cocktail (Roche, CH). Protein lysates were incubated at 4 °C with Protein A sepharose beads (Sigma, USA) for 1h. Beads were then washed 4 times in lysis buffer and examined by Western Blot analysis.

Immunohistochemistry

Stomachs were obtained from three month-old C/EBPβ knockout (KO) mice, and new-born C/EBPβ$^{-/-}$, RUNX3$^{-/-}$ and compound C/EBPβ/RUNX3 KO animals. Stomachs were longitudinally excised, formalin-fixed and embedded in paraffin. Gastric cancer tissue microarrays were obtained as described elsewhere [290]. Serial sections were obtained, deparaffinised and stained with Haematoxylin and Eosin, examined by a pathologist, and measured. An additional group of sections were treated with 10 mM citrate buffer and stained with 1:100 anti-Ki67 (DAKO, DK), 1:500 anti-C/EBPβ, 1:50 anti-TFF1 (Santa Cruz Biotechnology, USA), or 1:500 anti-RUNX1t1 (Sigma, USA) antibody. After washing with PBS with 0.02% Tween and incubation with horseradish peroxidase-bound secondary antibody (GE Healthcare, USA) development was performed using DAB.

BrdU proliferation assay

Cells with stable C/EBPβ knockdown were sorted and plated to 40% confluence. Cells were also transfected with RUNX1t1 and analysed for BrdU incorporation after 48 h. Briefly, cells were incubated with 1 M BrdU for 20 minutes, trypsinized and harvested in ice-cold PBS. Cells were then fixed, permeabilized, and stained with fluorescent anti-BrdU antibody according to the APC-BrdU flow kit protocol (BD Biosciences, USA). Dead cells were stained with 7-AAD and BrdU positivity was then assessed by flow cytometry.

Total RNA extraction, cDNA synthesis and quantitative real-time PCR

For RNA extraction from mouse tissue, stomach sections were frozen in liquid nitrogen after excision, and finely grinded in a mortar. For RNA extraction from gastric cancer cells, these were harvested in ice-cold PBS and pelleted at 2000 rpm. Lysis buffer was then added to the obtained powder or to the pellet which was then vigorously resuspended using a 3ml syringe. RNA was extracted using a universal RNA extraction kit (Roboklon, DE). RNA was quantified, cDNA synthesized by standard methods and SYBR green quantitative real-time PCR performed. Primers used are listed as follows:

Primer name	Sequence (5' to 3')
Human_CEBPB_Fw	GACAAGCACAGCGACGAGTA
Human_CEBPB_Rv	AGCTGCTCCACCTTCTTCTG
Human_FOG2_Fw	TGGGGACACACAGTCAGAGA
Human_FOG2_Rv	CCTCAGAGATGGCCTTCGTA
Mouse_Fog2_Fw	TGGGATGGACCAGGAGAG
Mouse_Fog2_Rv	GACGAGCTCTTCACCCTCTG
Human_SPARCL1_Fw	AGAGCACCAAGAGGCCAAG
Human_SPARCL1_Rv	CTCTCATCCGTAGAGGAAACTGA
Mouse_Sparcl1_Fw	TCCTGCTTGTACGGACTTTG
Mouse_Sparcl1_Rv	TTCCTTCAAGGTGATGTGCTT
Human_RUNX1t1_Fw	CCCTCGCTAGACGTGAACTC
Human_RUNX1t1_Rv	TGCTGTTTGGTAAAGCATCG
Mouse_Runx1t1_Fw	AGTTCGCACCCTTGT
Mouse_Runx1t1_Rv	TTCGTGCTGAGCGAG
Mouse_Ki67_Fw	CCACACTGTGTCGTCGTTTG
Mouse_Ki67_Rv	CCGTGCGCTTATCCATTCA
Mouse_Pcna_Fw	CGAAGCACCAAATCAAGAGA

Mouse_Pcna_Rv	CGGCATATACGTGCAAATTC
Mouse_Muc6_Fw	CCTCTGCTGCGACTGTCTAA
Mouse_Muc6_Rv	TGGGAGTGGGAAGATAATGG
Mouse_Muc5ac_Fw	CTGTGGAGCATGGGGAAAT
Mouse_Muc5ac_Rv	GAACCACAGACCTGCTCCAC
Mouse_Cyclin A1_Fw	GCTACCTTCCAGAAGCTGAAGT
Mouse_Cyclin A1_Rv	CAGGGTCTCTGTGCGAAGTT
Mouse_Cyclin E1_Fw	GCAGCGAGCAGGAGACAGA
Mouse_Cyclin E1_Rv	GCTGCTTCCACACCACTGTCTT
Mouse_Cyclin D3_Fw	TGCCAAAACGCCCCAGTAC
Mouse_Cyclin D3_Rv	CGGGATGCCCGAAGGA
Mouse_p15_Fw	AGATCCCAACGCCCTGAAC
Mouse_p15_Rv	CCCATCATCATGACCTGGATT
Mouse_Bcl2_Fw	ATGTGTGTGGAGAGCGTCAACC
Mouse_Bcl2_Rv	TGAGCAGAGTCTTCAGAGACAGCC
Mouse_Birc5_Fw	CTGATTTGGCCCAGTGTTTT
Mouse_Birc5_Rv	GCCACAAAACCAAAGAGAGG

Plasmids

For the construction of C/EBPβ isoform expression vectors, LAP*, LAP and LIP were cloned from human cDNA by PCR, following digestion with restriction enzymes, ligation into pcDNA3-flagged plasmid and ampicillin selection. TFF1-luciferase reporter plasmid was similarly cloned from human cDNA into a pGL3-basic plasmid. RUNX1t1 expression plasmid (pCMV-3xFlag-ETO) was obtained from (ADDGENE, UK) (ref: #12507). For the construction of C/EBPβ knockdown vectors, shRNA (5'-gccgcgacaaggccaagatgc-3') was inserted into a pLVTH-M lentivral vector.

Tissue culture, transfection, and luciferase assays

MKN28, MKN45 and MKN74 cell lines were grown in RPMI medium (GIBCO, USA). For transfection, cells were trypsinized, seeded, and grown to 50-60% confluence. C/EBPβ isoform plasmids and/or RUNX1t1 plasmid were re-suspended in serum-free medium with transIT (Mirus Bio, USA) transfection reagent and added to the cells. Protein and RNA were extracted after 48 h and analysed by Western Blot and real-time PCR.

RUNX1t1 promoter methylation analysis

Methylation analysis of the RUNX1t1 promoter was determined by methylation-specific PCR (MSP), as previously described [291]. MSP method distinguishes unmethylated from methylated alleles in a gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracil. Subsequently, PCR using primers specific to either methylated or unmethylated DNA was performed. Genomic DNA (350ng) was bisulfite-treated and purified with EZ DNA Methylation Kit Gold (Zymo Research, USA). The primer sequences of RUNX1t1, for methylated and unmethylated reactions were as previously described [291]. A quantity of 100 ng of bisulfite-modified DNA was used in each PCR. Amplification was carried out for 36 cycles (30 s at 95°C, 30 s at 56°C, and 30 s at 72°C). Control PCRs lacking genomic DNA were performed for each set of reactions. Amplified products were separated by electrophoresis in a 2.5% agarose gel.

RUNX1t1 gene mutational screening

Molecular analysis was performed on DNA extracted from a cohort of 26 tumour samples. DNA samples were extracted in Hospital São João/ Faculty of Medicine of University of Porto and belong to the Tumour Bank of that institution. All primers were newly designed using Primer 3 software. Each primer pair was designed to flank each coding exon of *RUNX1T1* gene. We amplified 20 ng of DNA in a 20 µl reaction that included 1× Master Mix Solution (Qiagen, DE), and primers at 0.4 mM. PCR was performed with an initial incubation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58ºC for 45 s, and extension at 72°C for 60 s, and with a final extension for 10 min. We used 5 µl of PCR product to run an electrophoresis in 2% agarose gel to check the DNA amplification. Next, we purified the remaining PCR product by adding an enzyme mixture of exonuclease I and alkaline

phosphatase (Fermentas, USA). After that, PCR fragments were directly sequenced on an ABI 3100 capillary sequencer (Applied Biosystems, USA) with BigDye terminator mix. The primers used for mutational screening are as follows:

Primer name	Sequence (5' to 3')
RUNX1T1_exon1_Fw	TGGGTATGATACTTCAGACTGGTT
RUNX1T1_exon1_Rv	TCTGGTACGTAAGTAAATTGCAAAA
RUNX1T1_exon2_Fw	CCACTTGAAAAACTGAGGTGCT
RUNX1T1_exon2_Rv	CCCTTGATTTTTCATTTGCAG
RUNX1T1_exon3_Fw	CTTGGTGGCAATTTCCTCAT
RUNX1T1_exon3_Rv	CTGAGTCTCCCACCCACACT
RUNX1T1_exon4_Fw	TTTGTGTTTTTACTATTTACCAACAGG
RUNX1T1_exon4_Rv	AAATCAAAGAGCCCCTAAATG
RUNX1T1_exon5_Fw	CAACAGGACAGAAGAAAACTTTCAG
RUNX1T1_exon5_Rv	ATGCCACAGGTATGGGAAAA
RUNX1T1_exon6_Fw	CTGAACTGTGCTGGTTTCTGA
RUNX1T1_exon6_Rv	CCCAATCCCGTAAGAAGTGA
RUNX1T1_exon7_Fw	TTACATCGAGTTTGCCCACA
RUNX1T1_exon7_Rv	CCTCACTCCAGTTGTTTTCCA
RUNX1T1_exon8_Fw	TGTGATGATTTATATGCTCTTCCCTA
RUNX1T1_exon8_Rv	CAGCATAAGAAATATGTGTTTTCGAG
RUNX1T1_exon9_Fw	AGGAGAATGGGCATTGCTTA
RUNX1T1_exon9_Rv	ACTGCACACAGCTGCCAGA
RUNX1T1_exon10_Fw	TTCGGCTAACTGAGAGGTGTT
RUNX1T1_exon10_Rv	GCACTCTAATGAATGAAAACTATCTTG
RUNX1T1_exon11_Fw	TGCCTAACATATTTGTCAGACTATTG
RUNX1T1_exon11_Rv	TCGCGTTGGTTGTGTTGT

Electrophoretic Mobility Shift Assay (EMSA)

MKN28 and MKN45 cells were transfected with increasing amounts of RUNX1T1. Nuclear extracts were prepared from transfected cells, quantified, and incubated with previously radioactively labelled (α-32P dCTPs) nucleotides, containing an optimized C/EBPβ binding sequence. Protein/Labelled-DNA complexes were then run in a 15% acrylamide gel in non-denaturing conditions and binding affinity assessed by intensity of radioactive signal. Anti-C/EBPβ antibody was added to the protein/labelled-DNA complex as a control, and a supershift was observable, confirming that it was C/EBPβ what bond to DNA. Competition with non-labelled C/EBPβ binding sequence, confirmed the specificity of the observed signal.

Bioinformatic microarray data analysis and statistical analysis

The raw data files (.text files for murine Agilent Technologies® arrays and excel files for human Affymetrix GeneChips) were imported into GeneSpring GX 12.1 software (Agilent Technologies, USA) as two separate species-specific experiments. Pre-processing (background correction, normalization and probe summarization) was performed according to the RMA algorithm followed by baseline transformation to the median of all samples (in one experiment). Quality control was done by assessment of inter-array correlation analysis calculating the correlation coefficient of each array to every other one. The human arrays yielded correlation coefficients between 0.829 and 0.972, with an arithmetic mean of 0.917 and the murine arrays between 0.991 and 0.924 with a mean of 0.9. In the murine array experiment, only probes owning “detected” flags in at least 3 arrays (34,150 probes) were used for further analyses. Genes whose expression between groups of samples was significantly different were identified by Welch-test with $p \leq 0.01$ being the significance cut-off. The fold change (FC) of expression between groups was calculated as the fold difference between group means. Gene annotation information was obtained from GeneSpring GX software (state of 08/2012). For hierarchical clustering, ‘Euclidean distance’ and ‘complete linkage’ were used as distance metric and linkage algorithm. The migration of genes between the murine and human microarray experiment was performed using the Orthology Search Tool of bioDBnet at http://biodbnet.abcc.ncifcrf.gov/.

Optimized and applied for article "Interleukin-1B signalling leads to increased cell survival of gastric carcinoma cells through a CREB-C/EBPβ-associated mechanism"

Tissue material

Surgical specimens from 66 GCs were resected and diagnosed at Hospital S.João/Faculty of Medicine of University of Porto, Portugal. Tissue fragments were first fixed in 10% formaldehyde followed by paraffin embedding. Tumour-representative areas of each GC were selected to create a tissue microarray (TMA) block. Briefly, sections of 0,5 mm of diameter were extracted from each one of the 66 paraffin embedded GCs and inserted into a new paraffin block. Serial sections of 3 μm were obtained from the TMA block and used for routine staining with hematoxilin and eosin and immunohistochemistry. The procedures followed in this study were in accordance with the institutional ethical standards. All the samples enrolled in this study were unidentified.

Immunohistochemistry and immunofluorescence

Tissue sections from formalin fixed paraffin-embedded (FFPE) tissues were first deparaffinised, hydrated, and then treated with 1x citrate buffer (pH 6.0) (Thermo Scientific, CA, USA) for 45 minutes at 100°C. All the following steps were performed at room temperature (RT). Unspecific endogenous peroxidase activity was eliminated with 3% hydrogen peroxidase in methanol for 15 minutes. To reduce nonspecific background staining, slides were blocked with Ultra V Block (Thermo Scientific, USA) for 10 minutes. Slides were rinsed in PBS-0.1% Tween20 and incubated for 1h with antibody anti-CREB [E306] (Abcam, UK) diluted 1:1000, overnight (ON) with anti-C/EBPβ (Abcam, UK) diluted 1:1000 in UltraAB Diluent (Thermo Scientific, USA). Slides were then incubated with Dako Real EnVision HRP Rabbit/Mouse solution (Dako, DK) for 30 minutes. Slides were washed, developed for 1-3 minutes with 2% Dako REAL™ DAB+ Chromogen solution (Dako, DK), counterstained with haematoxylin, dehydrated, and mounted with mounting medium (Thermo Scientific, USA). All washing steps were performed in PBS-0.1% Tween20 buffer. Normal gastric mucosa was used as control, and negative controls were obtained by substitution of the primary antibody with immunoglobulins of the same class and concentration. Slides were reviewed by a pathologist,

and the percentage of positive cells was semi-quantitatively scored as: 0, corresponding to positivity in <5% of the tumour cells; 1, corresponding to positivity in >5% and <50% of the tumour cells; 2, corresponding to positivity in >50% and <75% of the tumour cells; and 3, corresponding to positivity in >75% of the tumour cells.

For immunofluorescence, FFPE tissue from normal stomach was deparaffinised, hydrated, and antigen retrieval was performed with 1x citrate buffer (pH 6,0). After unspecific protein blocking with Ultra V Block (Thermo Scientific, USA) for 10 minutes, slides were incubated with rabbit antibody anti-CREB [E306] (Abcam, UK) diluted 1:1000 for 1h, followed by mouse antibody anti-C/EBPβ (Abcam, UK) diluted 1:1000 ON. After washing twice with PBS-Tween 0,021% for 10 minutes, slides were incubated with a mixture of two secondary antibodies raised in different species (with Texas Red-conjugated against rabbit and FITC-conjugated against mouse) for 45 min at room temperature and protected from light. To counterstain cell nucleus, slides were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, USA). Finally, slides were visualized and images captured (Apotome acquired) under a fluorescence microscope (Zeiss, DE).

Cell culture, chemical treatments, and transfections

AGS and GP202 cell lines were maintained in RPMI medium (GIBCO, USA), supplemented with 10% foetal bovine serum (FBS) (PAA, AUS), and 100 IU/mL penicillin and 100 µg/mL streptomycin (GIBCO, USA), in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. Cells were grown until 60-80% confluence in 6-well plates, and treated with 10 ng/mL of IL1B (Sigma-Aldrich, USA) and incubated for 24 h.

For ERK1/2 inhibition, cells were treated with 25 µM U0126 (Cayman, USA) alone and together with IL1B for 24 h. As U0126 was diluted in DMSO, appropriate DMSO volume was added to the cells as the control condition. For CBP-CREB interaction inhibition cells were treated with 25 µM specific inhibitor (Merck-Millipore, DE) or vehicle DMSO (Sigma, USA) for 24 h and 48 h. All experiments were performed in complete RPMI cell culture medium.

For silencing experiments, AGS cells grown until 60-80% confluence in 6-well plates were transfected with 1.0 µg of anti-CREB shRNA expression vector or 1.0 µg scrambled shRNA (Origene, USA) using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's specifications. At 48 h post-transfection, cells were selected by adding 1.0 µg/mL of puromycin (Sigma-Aldrich, USA) to culture medium. Individual puromycin-resistant

colonies were isolated after 2 weeks of selection and expanded in the presence of puromycin (1.0 µg/mL). For transient silencing of CREB, AGS cells were transfected either with 100 nM of siRNA against CREB (Qiagen, DE) or with 100 nM of siRNA control, using Lipofectamine 2000 (Invitrogen, USA) as a vehicle. In parallel, C/EBPβ silencing was performed by transfecting AGS cells with 150 nM of siRNA against *CEBPB* or siRNA control (Qiagen, DE). The protein downregulation after gene silencing was evaluated by western blotting following 72 h of culture.

Western blotting

Cells were washed with 1x PBS (pH7.4) and lysed in NP-40 buffer supplemented with phosphatase (Sigma, USA) and protease inhibitors (Roche, DE). After Bradford protein quantification, 40 µg of total protein were loaded into 12.5% acrylamide gels, separated by SDS-PAGE under denaturing conditions and electro-transferred to Hybond ECL Nitrocellulose Membrane (GE Healthcare, UK). After blocking, membranes were incubated for 1.5 h with primary antibodies anti-ERK1/2 #9102 (Cell Signaling, USA) diluted 1:1000, anti-pERK1/2 # 9106 (Cell Signaling, USA) diluted 1:1000, anti-CREB [E306] (Abcam, UK) diluted 1:500, anti-pCREB [E113] (Abcam, UK) diluted 1:1000, anti-C/EBPβ [H-7] (Santa Cruz Biotechnology, USA) diluted 1:500, anti-cyclin D1 (Santa Cruz Biotechnology, USA) diluted 1:500, and anti-α-tubulin (Sigma-Aldrich, USA) diluted 1:10000 in PBS-0.5% Tween20 plus 5% non-fat dried milk or 4% BSA (bovine serum albumin). The blots were then washed with PBS-0.5% Tween20 and incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:10000 in PBS-0.5% Tween20 plus 0.5% non-fat dried milk. Chemiluminescent bands were visualized using Western Blot ECL (GE Healthcare, UK).

BrdU incorporation assay

Previously to AGS, GP202, and MKN28 cells addition to the six-well plates, three glass slides (10 mm x 10 mm) were positioned far apart in each well of the six-well plate. Cells were allowed to reach 60-80% confluence and 1x BrdU was added to culture medium for 1 h. Cells were then washed with 1x PBS and fixed in freshly prepared 4% (v/v) paraformaldehyde at room temperature for 30 minutes. The glass slides were removed from the six-well plate, transferred to individual wells in a 12-well plate, and washed with 1x PBS. To denature the DNA and permeabilize cells, hydrochloric acid (HCl) 2 M was added to each slide during 20

minutes following by washings with PBS-0.5% Tween 20 plus 0,05% BSA. Cells in glass slides were incubated for 1 h with mouse primary antibody against BrdU (Dako, DK) diluted 1:10, washed two times with PBS-0.5% Tween 20 plus 0.05% BSA, and incubated for 30 minutes with anti-mouse secondary antibody marked with Alexa Fluor 594 (Invitrogen, CA, USA) diluted 1:100. Glass slides were rinsed in PBS-0.5% Tween 20 plus 0.05% BSA two times, mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, USA), and finally visualized under fluorescent microscopy. For each experiment, BrdU technique was performed in triplicates. In each assay, at least 1000 cells were counted and BrdU incorporation expressed as the rate between DAPI and BrdU positive cells.

Promoter analysis and chromatin immunoprecipitation (ChIP)

The nucleotide sequence of human *CEBPB* promoter was obtained from the UCSC Genome Browser (http://genome.ucsc.edu/). The putative CRE-binding sites present on *CEBPB* promoter were identified using the Genomatix MatInspector software (http://www.genomatix.de/solutions/genomatix-genome-analyzer.html).

ChIP assay was performed using a Magna ChIP G Kit (Millipore, USA), according to the manufacturer's protocol instructions. Briefly, $1x10^7$ of AGS cells were cross-linked with 1% formaldehyde during 10 minutes at RT, and reaction was stopped by 1x Glycine solution for 5 minutes. Cells were rinsed with 1xPBS, lysed in order to isolate nuclei, and then sonicated in Nuclear Lysis to shear the chromatin to sizes of 200-500 base pairs (bp). Then, 50 µl of the supernatant was immunoprecipitated by adding 2 µg of rabbit antibody anti-CREB (Abcam, UK) or 2 µg of control rabbit polyclonal anti-IgG antibody (Abcam, UK), and the mixture was placed on a rotator at 4°C ON in the presence of magnetic G beads. DNA–protein cross-links were reversed by heating samples at 62°C for 2 h in a shaking platform. To elute DNA, a series of wash steps followed by elution (50 µl) were performed in spin columns. Precipitated DNA was analyzed by PCR using the following conditions: 95 °C for 15 min, 35 times (95 °C for 1 min, 58º - 60ºC for 1 min, 72 °C for 1 min). The reactions were carried out with HotStarTaq DNA Polymerase (Qiagen, DE) as described by the manufacturer, using 2 µl of DNA template. The PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR primer pairs flanking CREB-binding sites and for control region (CR) were designed using Primer 3 software.

Primer name	Sequence (5’ to 3’)
CEBPB_BS1_Fw	AGGCTCTGTTCTAGGCACCA
CEBPB_BS1_Rv	CTCAGGTCTCAGCCCAAAAG
CEBPB_BS2_Fw	GATGAGGGCATTTCATTTGG
CEBPB_BS2_Rv	CCATGAAGGGTGTCGCTACT
CEBPB_BS3_Fw	GTCCTCCCGGGGGTCTCG
CEBPB_BS3_Rv	CTCCTGAGCCCGGTTATTTA
Primer_Unspec_Fw	GCAACCCACGTGTAACTGTC
Primer_Unspec_Rv	CCCAAAAGGCTTTGTAACCA

RNA isolation, cDNA synthesis, and quantification of CEBPB mRNA transcript

Total RNA was isolated from AGS cells grown in 6-well culture plates, using the RNeasy Mini Kit (Qiagen, De). RNA concentrations were measured using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, USA). Reverse transcription was performed using SuperScript II Reverse Transcriptase KIT (Invitrogen, USA) with 1000 µg of total RNA in a 20 µl volume reaction, after treatment with DNaseI (Invitrogen, USA).

To determine the relative amount of the *CEBPB* transcript, we performed quantitative target amplification, using cDNA as template, with SYBR Green PCR Kit (Qiagen, DE), according to the manufacturer protocol. As an internal control, we quantified the expression levels of Beta-actin transcript.

Primer name	Sequence (5’ to 3’)
CEBPB_Fw	AAGATGCGCAACCTGGAG
CEBPB_Rv	CGCGAGCTCAGCACCCTG
Beta-actin_Fw	GGCATCGTGATGGACTCCG
Beta-actin_Rv	GCTGGAAGGTGGACAGCGA

Analysis of In vivo tumour growth by chicken embryo in vivo tumourigenesis assay

The chicken embryo chorioallantoic membrane (CAM) model was used to evalua*te the* growth capability of AGS cells, transfected with scrambled or shCREB RNA (n=16). Briefly, fertilized chick (Gallus gallus) eggs obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5-2 ml of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. Cells, re-suspended in 10 µl of complete medium, were placed on top of E10 growing CAM and $2x10^6$ cells from each cell line (Scrambled VS shCREB) were placed into a 3 mm nylon ring under sterile conditions, per embryo. The eggs were re-sealed and returned to the incubator for an additional 5 days. After removing the ring, the CAM was excised from the embryos, photographed *ex ovo* under a stereoscope, at 20x magnification (Olympus, SZX16 coupled with a DP71 camera). The area of CAM tumours was determined using the Cell A (Olympus, JP) program.

Statistical analysis

The clinicopathological features of GC cases were compared using the χ^2-test. Independent triplicate measurements were performed for the BRDU incorporation, and transcript quantification by real time-PCR. When two conditions were compared, Student's t-test was used, whereas ANOVA was employed when the comparison involved more than two conditions. For tumour area comparison, the paired *t*-test was used. In order to accurately access putative differences in tumour areas between the two cell lines, only eggs bearing two tumours with areas ≥ $1mm^2$ (n=16) were considered, independently of the cell group. Values of $p<0.05$ were considered statistically significant (*); $p<0.01$ (**) and $p<0.001$ (***).

RESULTS

Article: C/EBP alpha expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis

Gonçalo Regalo, Carlos Resende, Xiaogang Wen, Bárbara Gomes, Cecília Durães, Raquel Seruca, Fátima Carneiro and José C Machado. *Laboratory Investigation*, (2010) **90,** 1132–1139

Introduction

Gastric carcinoma (GC) is still one of the most common cancers worldwide, despite its decreasing incidence in the developed countries. The continued inflammation of the gastric epithelium by chronic *Helicobacter pylori* infection is a major contributor to carcinogenesis, most likely by promoting disruption of the balance between proliferation and differentiation in the regenerating inflamed mucosa. Although this process has been well characterized phenotypically, the main molecular players in gastric neoplastic transformation are largely unknown [292].

Proteins of the CCAAT/enhancer-binding protein (C/EBP) family are important transcription factors that link gene expression to proliferation/differentiation control [293]. We have recently shown that C/EBPβ is overexpressed in preneoplastic lesions and GC [185]. Most notably, C/EBPβ overexpression is associated with loss of TFF1, an established differentiation marker, and a putative gastric tumour suppressor [121].

Members of the C/EBP family are known to heterodimerize among themselves, giving rise to different functional transcriptional complexes. Moreover, they often act with a high degree of coordination [208]. This is well demonstrated in adipogenesis, where sequential expression of different C/EBP members underlies the process of differentiation from preadipocytes to fully mature adipocytes [294]. After the differentiation stimulus is given, C/EBPβ is expressed in immature preadipocytes and primes cells to differentiate by inducing C/EBPα expression [295]. Once active, C/EBPα drastically reduces cell proliferation, and promotes the expression of peroxisome proliferator-activated receptor γ (PPARγ) [296]. In this and other models, C/EBPα is a crucial effector of lineage commitment and terminal differentiation programs. The disruption of these programs has been shown to be oncogenic in several cellular contexts. For instance, C/EBPα is a consensual tumour suppressor in acute myeloid leukemia (AML) where deleterious mutations have been described in a proportion of cases [244]. C/EBPα may also have a role in other cancer models [297] such as lung cancer, where it was found downregulated by methylation [250]. However, the expression pattern and

functional relevance of C/EBPα in normal stomach and in GC has never been described. In this study, we characterized the expression of C/EBPα in the normal gastric mucosa and in GC. Furthermore, we investigated the effects of expressing C/EBPα in GC cells, and aimed at clarifying the link between pathways of C/EBPα modulation and gastric carcinogenesis.

Results

Immunohistochemical analysis of C/EBPα expression

In the normal mucosa of the stomach, C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity and mostly localized in the mucous surface epithelium (Figure 1a). This expression pattern contrasts with that of C/EBPβ whose expression is concentrated to the neck zone (Figure 1b). This expression pattern was confirmed using immunofluorescence, where C/EBPα staining was again stronger in the foveolar and surface epithelium, with fewer positive cells observed in the neck zone (Figure 1c). As described earlier, infiltrating inflammatory cells were also found to express C/EBPα. To confirm that C/EBPα expression does correlate with the differentiation status of the gastric epithelium, we performed double staining with TFF1, a well-established gastric differentiation marker. A clear overlap was observed between TFF1 and C/EBPα in the surface epithelium (Figure 1d).

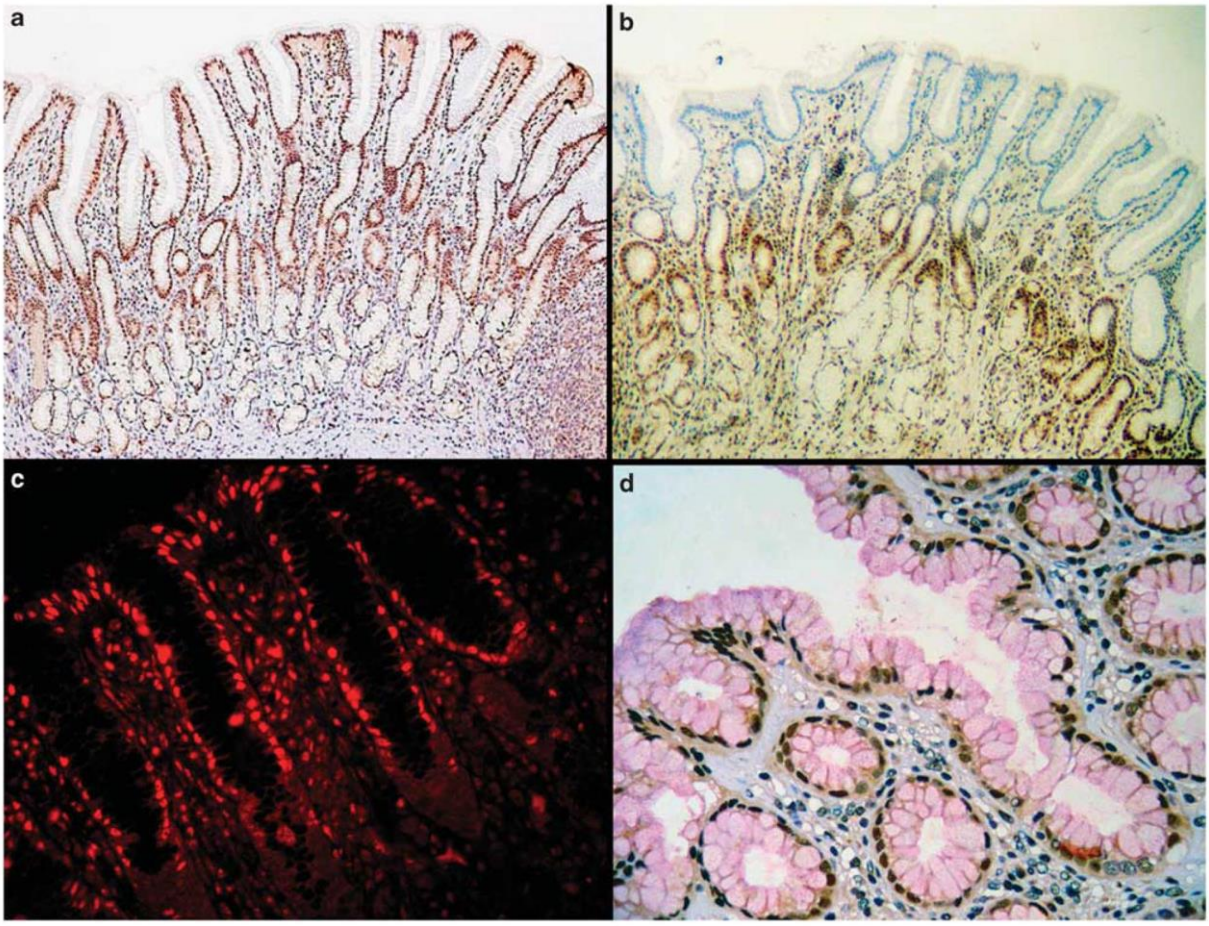


Figure 1. C/EBPs expression in normal gastric mucosa. (a) C/EBPα immunostaining in non-neoplastic mucosa, showing strong expression in the superficial epithelium. (b) C/EBPβ expression in normal gastric mucosa of the antrum, showing strong localization in the neck zone. (c) C/EBPα immunofluorescence, showing expression in differentiated gastric foveolae, and few positive cells toward the neck zone. (d) C/EBPα (brown) and TFF1 (red) double staining, showing co-expression of the two proteins in gastric foveolae.

Similarly to what was observed in the normal gastric mucosa, in GC C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity (Figure 2a). In GC, C/EBPα was considered downregulated in 30% of the tumours (Figures 2b–d). No statistical significant relationships were found between C/EBPα expression and any clinicopathological features of the cases (Table 1).

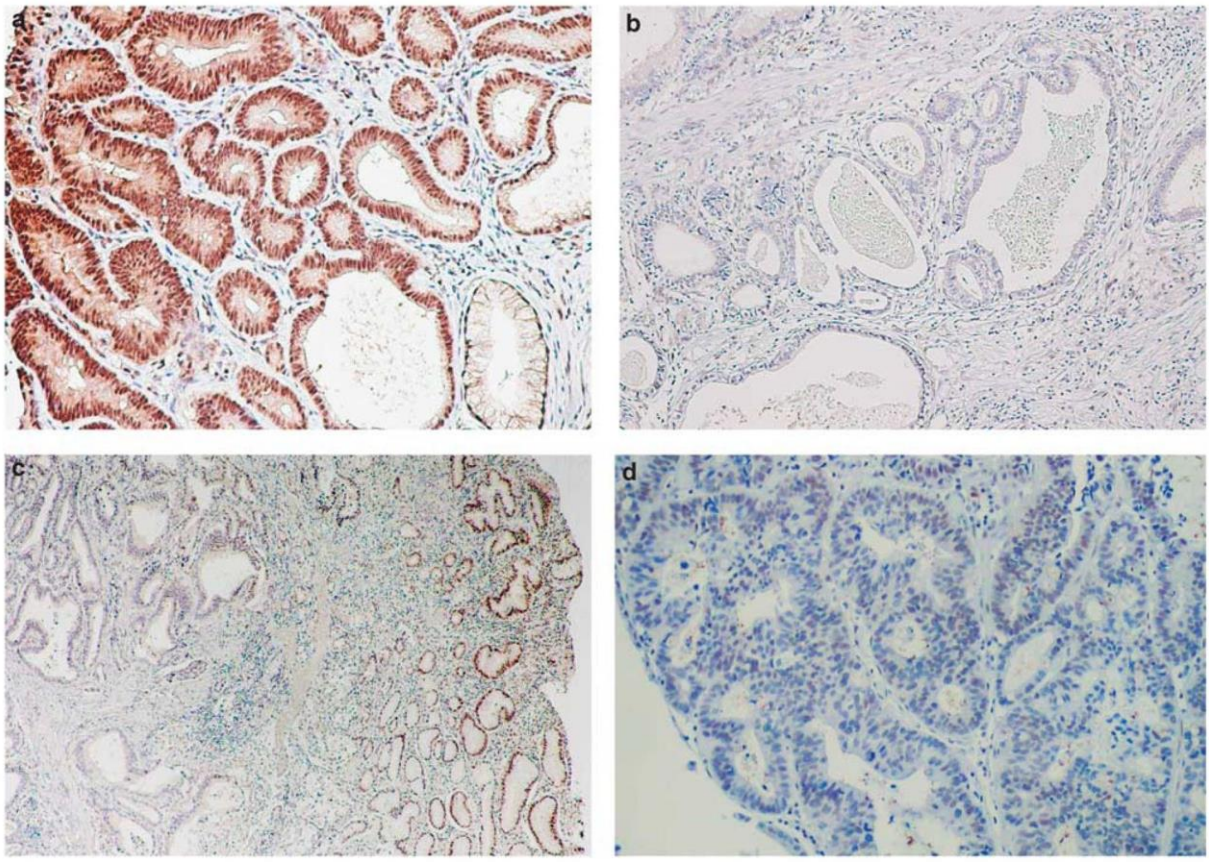


Figure 2. C/EBPα staining in intestinal-type GC. (a) C/EBP positive tumour. (b) Tumour showing complete loss of C/EBPα expression. (c) GC displaying downregulation of C/EBPα expression (positive cells to the right are located in non-neoplastic gastric epithelium). (d) GC negative for C/EBPα expression.

Table 1. Relationship between the clinicopathological features of GC and C/EBPα expression scoring

		C/EBPα downregulation		
	No. of cases (%)	Yes	No	*P*-value
Age (years)				
≤40	2 (4)	1 (50)	1 (50)	NS
40–65	18 (36)	2 (11.1)	16 (88.9)	NS
≥65	30 (60)	12 (40)	18 (60)	NS
Gender				
Male	30 (60)	11 (36.7)	19 (63.3)	NS
Female	20 (40)	4 (20)	16 (80)	NS
Histological type				
Intestinal	27 (54)	11 (40.7)	16 (59.3)	NS
Diffuse	16 (32)	1 (6.3)	15 (93.7)	NS
Atypical	7 (14)	3 (42.9)	4 (57.1)	NS
Depth of invasion				
T1	2 (4)	0 (0)	2 (100)	NS
T2	26 (52)	10 (38.5)	16 (61.5)	NS
≥T3	22 (44)	5 (22.7)	17 (77.3)	NS
Vascular invasion				
Absent	15 (30)	5 (33.3)	10 (66.7)	NS
Present	35 (70)	10 (28.6)	25 (71.4)	NS
Metastasis				
Absent	12 (24)	6 (50)	6 (50)	NS
Present	38 (76)	9 (23.7)	29 (76.3)	NS
Total	50 (100)	15 (30)	35 (70)	

NS: non-significant. Cases are classified according to the intensity and percentage of positive cells. Cases classified as downregulated present >50% of tumour cells classified as I.

Effect of C/EBPα expression on cell proliferation and differentiation

To assess the effect of C/EBPα on the proliferation status of GC cells, we transfected the C/EBPα-negative GC cell line AGS with an expression vector for the full-length *CEBPA* gene and measured the incorporation of BRDU after 48 h. We observed that re-expression of C/EBPα on AGS cells led to a 15% reduction ($P = 0.001$) in cell proliferation in comparison with the control (Figure 3a). Conversely, inhibition of C/EBPα by siRNA in the MKN28 cell line led to an increase ($P < 0.001$) in cell proliferation in comparison with the control (Figure 3b).

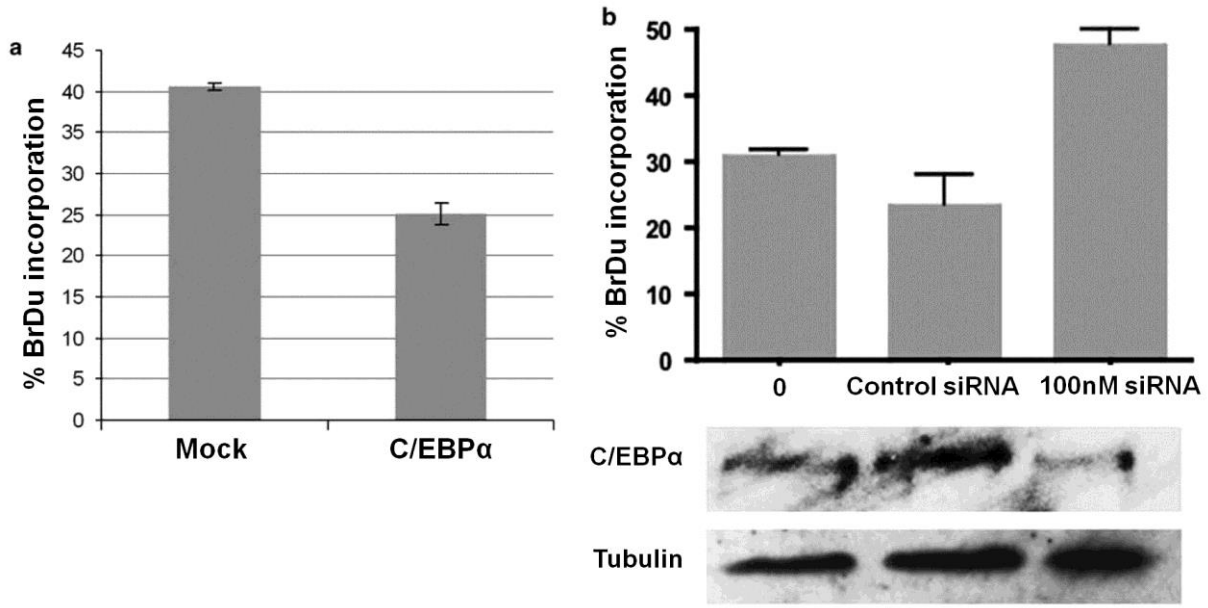


Figure 3. BrdU incorporation assay in GC cells. (a) Decreased proliferation rates of C/EBPα -transfected AGS cells in comparison with the control ($P = 0.001$). (b) C/EBPα inhibition by siRNA leads to increased BRDU incorporation in MKN28 cells. In all, 1000 cells were counted and BrdU incorporation expressed as the rate between DAPI and BrdU positive cells. The y axis represents the % of BrdU positive cells. Error bars represent s.d. Tubulin was used as protein-loading control.

To confirm this inhibitory effect of C/EBPα on proliferation, we analysed by western blotting the expression of two cell-cycle proteins typically associated with the control of gastric epithelial cell division. We observed decreased expression of cyclin D1, a cell-cycle inducer, and increased expression of p27, a cyclin-dependent kinase inhibitor (Figures 4a and c). Both these changes are consistent with an inhibitory effect on proliferation. The results on the effect of C/EBPα on proliferation, together with its expression pattern in the normal gastric mucosa,

suggested C/EBPα to have a role on proliferation arrest and on the differentiation of gastric epithelial cells. That being the case, increased expression of TFF1 would be expected in the presence of higher levels of C/EBPα. In accordance with this hypothesis, after transfection of AGS cells with the C/EBPα expression vector, we observed an increase in the expression of TFF1 (Figures 4b and c).

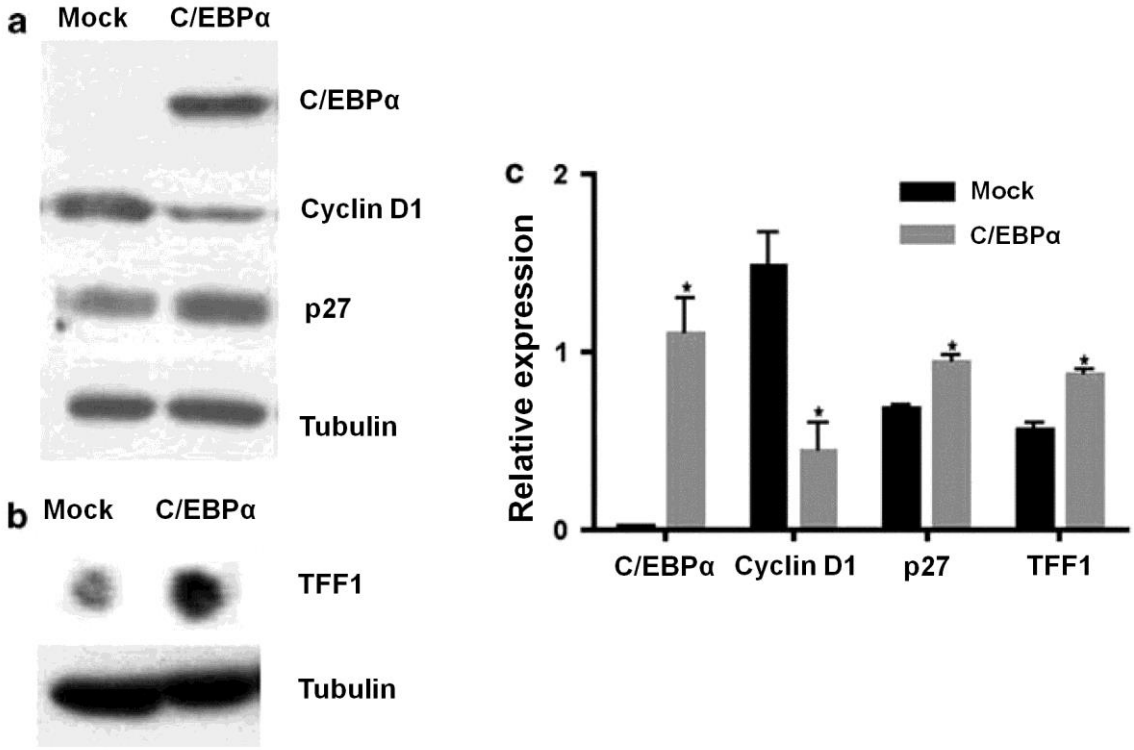


Figure 4. Effect of C/EBPα expression on AGS cells. (a) Western blot for cell-cycle proteins, showing increased p27 and decreased cyclin D1 expression after transfection with C/EBPα. (b) Dot blot showing increased TFF1 expression in C/EBPα-transfected cells. (c) Expression of C/EBPα, cyclin D1, p27, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between mock- and C/EBPα-transfected cells ($P < 0.05$).

Effect of MAPK inhibitors on the expression of C/EBPα and cell proliferation

The Ras/MAPK signalling pathway is one of the most consistently altered in human cancers. In GC, the Ras/MAPK pathway is constitutively activated through mutation of several of its receptors and signal-transducing members [84]. To explore the possibility of C/EBPα regulation by the Ras/MAPK pathway in GC, we treated MKN28 cells, which express C/EBPα,

with specific p38 (SB239063) and ERK1/2 (PD98059) inhibitors. Treatment with inhibitors led to a marked increase in C/EBPα expression and nuclear localization as detected by immunocytochemistry (Figure 5).

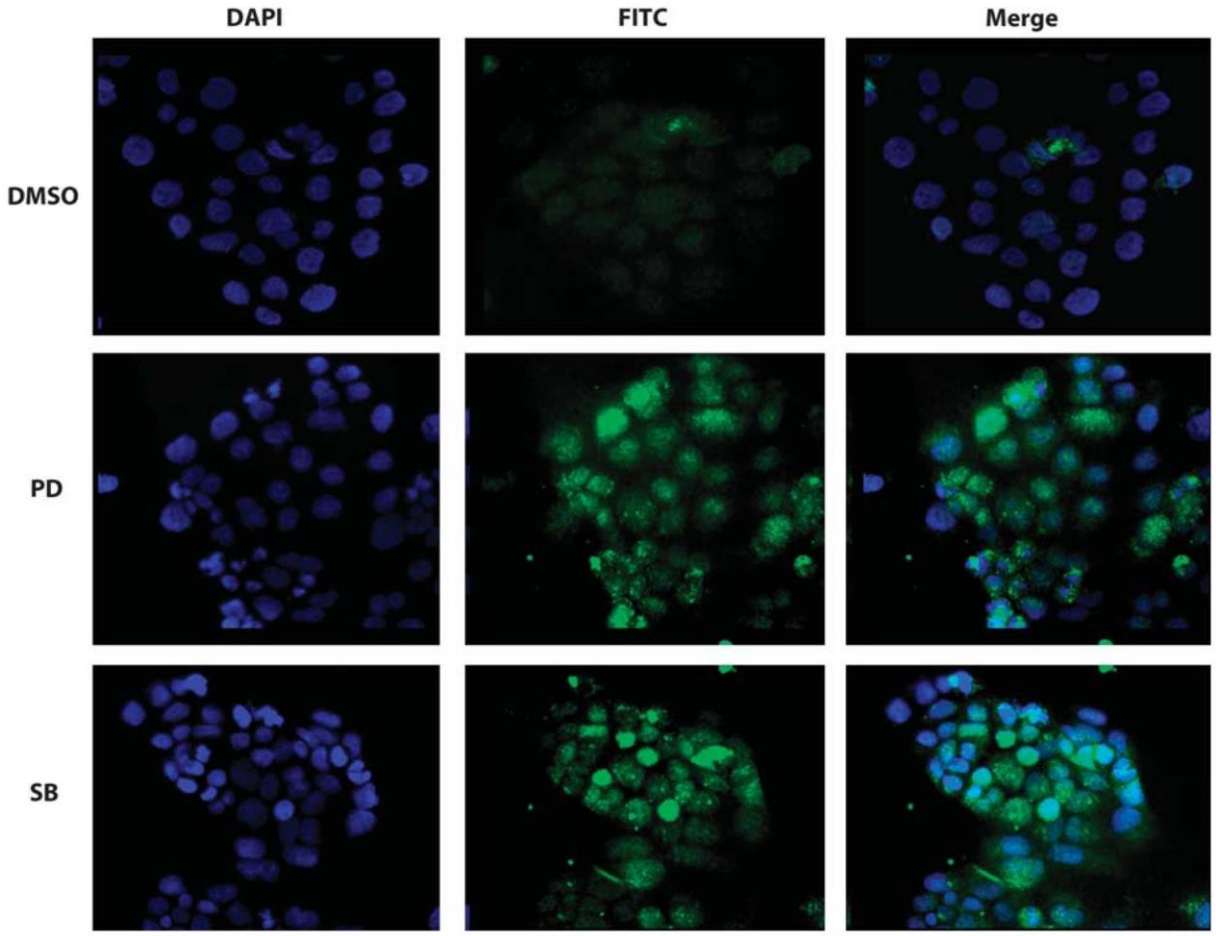


Figure 5. Treatment of MKN28 cells with p38 (SB) and ERK1/2 (PD) inhibitors leads to an increase in C/EBPα expression with nuclear localization. C/EBPα is stained green with FITC and nuclei are stained blue with DAPI for contrast.

This increase in C/EBPα expression was further confirmed by western blotting, and shown to be accompanied by an increase in TFF1 expression (Figures 6a and c). Concomitantly, we observed a decrease in cell proliferation by BrdU incorporation (Figure 6b) in cells treated with p38 inhibitor (P = 0.009) and in cells treated with ERK1/2 inhibitor (P = 0.003). This decrease in proliferation was accompanied by a decrease in Cyclin D1 expression (Figure 6c).

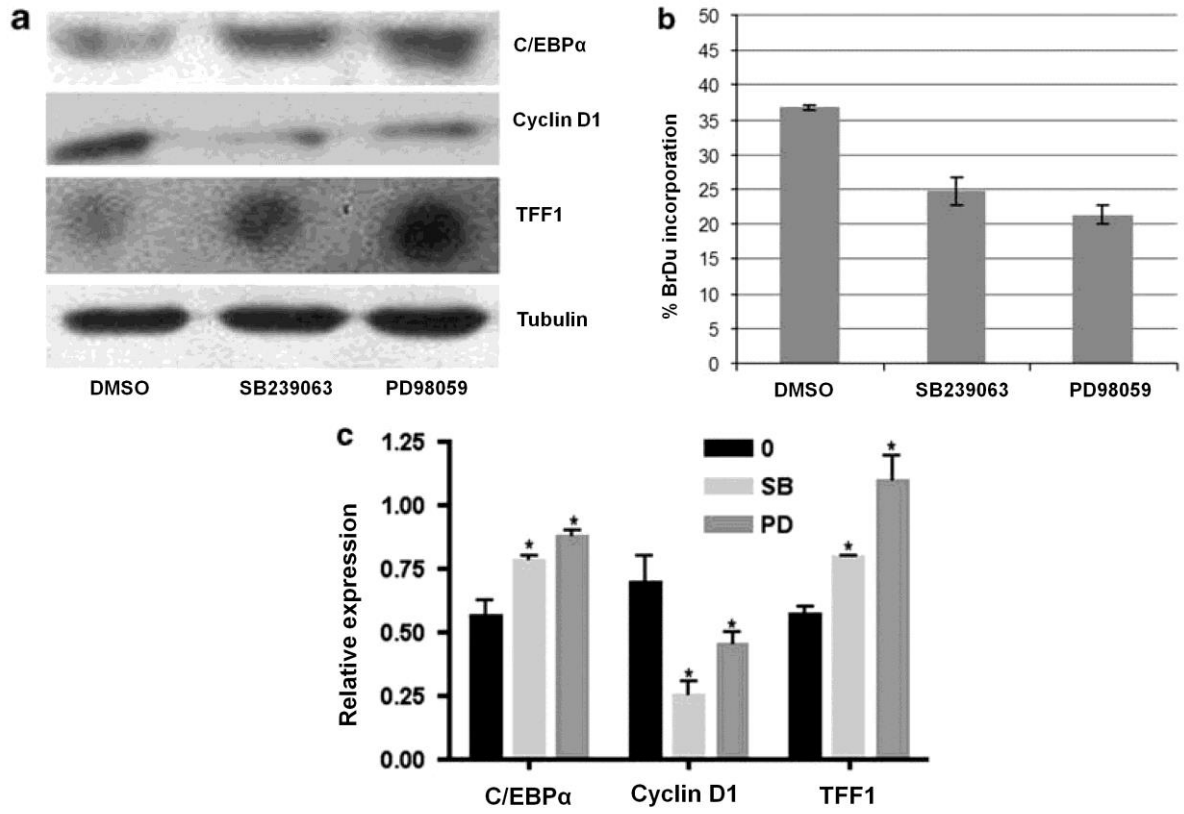


Figure 6. Effects of the treatment of MKN28 cells with a p38 (SB) and an ERK1/2 (PD) inhibitor in cellular proliferation and differentiation. (a) Western blot showing that treatment of MKN28 cells with SB and PD leads to an increase in C/EBPα and TFF1 expression and to a decrease in cyclin D1 levels. (b) Decrease of cell proliferation by BrdU incorporation assay of MKN28 cells treated with SB (P = 0.009) and PD (P = 0.003) inhibitors. (c) Expression of C/EBPα, cyclin D1, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between treated and non-treated cells (P < 0.05).

Discussion

We have shown that C/EBPα is expressed in the differentiated epithelial compartment of the superficial gastric mucosa. This expression pattern mirrors that described for C/EBPβ, which is expressed in the proliferative neck zone of the normal gastric mucosa. We have previously argued that C/EBPβ may have a role in maintaining a balance between proliferation and differentiation in the normal gastric mucosa [185]. In the proposed model, C/EBPβ would have a pro-proliferative activity in gastric epithelial stem-like cells. The presence of C/EBPα in differentiated cells, together with its ability to reduce cell proliferation and to up-regulate the gastric differentiation marker TFF1, suggest that C/EBPβ and C/EBPα may have

complementary roles in maintaining a balance between proliferation and differentiation in the normal gastric mucosa. By analogy to the model of adipogenesis, one feels tempted to speculate that C/EBPβ is expressed in gastric epithelial stem-like cells and may prime gastric epithelial cells to differentiate by inducing C/EBPα expression. Once active, C/EBPα would reduce cell proliferation, and promote the expression of gastric differentiation markers such as TFF1. C/EBPα was first described as a tumour suppressor gene in AML. In normal hematopoiesis, C/EBPα is essential to define cell lineages through interaction with other transcription factors. C/EBPα disruption by mutation leaves bone marrow cells in an undifferentiated, hyper-proliferative state being this event causal for a percentage of leukemias [298]. Downregulation of C/EBPα was additionally found in several epithelial tumour types, namely lung, breast, and skin cancers [250, 251, 299, 300]. In all these examples, a role for impaired C/EBPα function in tumourigenesis was strengthened by the observation that C/EBPα re-expression is able to inhibit tumourigenesis in vivo and in vitro [251, 300]. In our study, we observed downregulation of C/EBPα in about 30% of GC cases. In an earlier study, we have described a frameshift mutation of C/EBPα in a GC. This mutation was deleterious and absent from adjacent non-neoplastic tissue [248]. These results in the GC model are in keeping with the before described role of C/EBPα in tumourigenesis, whereby loss of C/EBPα would be associated to loss of differentiation and sustained proliferation of tumour cells. On top of C/EBPα loss of expression, we have shown earlier that C/EBPβ is overexpressed in cells retaining a proliferative phenotype such as those seen in dysplastic and cancer lesions. C/EBPβ is able to counteract, either by heterodimerization or repression of expression, the differentiating activity of C/EBPα. Altogether, either aberrant overexpression of C/EBPβ or loss of expression of C/EBPα are present in the majority of GC cases. Hence, these results suggest that changes in expression/function of C/EBPα and C/EBPβ may be pieces of the same puzzle rather than independent events in gastric carcinogenesis. This possibility, together with other putative mechanisms of post-translational or protein–protein interaction, would help explaining why expression of C/EBPα is still seen in about 70% of GC cases. In other cancer models, loss of C/EBPα has been linked with oncogenic Ras activation. In GC, activating RAS mutations do occur in a subset of microsatellite unstable (MSI) tumours. By using specific inhibitors for p38 and ERK1/2, downstream effectors of Ras signalling, we were able to show that inhibition of C/EBPα expression was dependent on the activation of this pathway. Moreover, inhibition of p38 and ERK1/2 increased TFF1 expression and strongly reduced MKN28 cell proliferation and cyclin D1 levels, in a set of alterations most likely linked with the observed increase in C/EBPα expression.

In summary, we show that in normal gastric mucosa, C/EBPα is expressed mainly in the differentiated foveolar epithelium where it co-localizes with TFF1. We show that C/EBPα is downregulated in a considerable percentage of GC. We additionally show that C/EBPα re-expression in a C/EBPα -negative cell line leads to a reduction in proliferation that is accompanied by an increase in p27 and reduction of cyclin D1 levels. In parallel, we show an increase in the expression of TFF1 in C/EBPα -transfected cells. Finally, we show that treatment of a C/EBPα expressing cell line with MAPK inhibitors leads to increased C/EBPα and TFF1 expression, and a concomitant reduction on cell proliferation and cyclin D1 expression. Overall, these results substantiate the role of the C/EBP transcription factor family in homeostasis of the gastric epithelium and in the process of gastric carcinogenesis.

Article: C/EBPβ/RUNX1t1 regulatory loop controls cell proliferation in gastric cancer.

Goncalo Regalo, Susann Förster, Carlos Resende, Bianca Bauer, Barbara Fleige, Wolfgang Kemmner, Peter M Schlag, Kosei Ito, Suk-Chul Bae, Thomas F. Meyer, Yoshiaki Ito, José C Machado, Achim Leutz (Submitted to JCI)

Introduction

The transcription factor C/EBPβ has been suggested to play a pro-oncogenic role in cancer, leukemia, and lymphoma through shielding from apoptosis and promotion of cell proliferation in conjunction with cyclin D1 [229, 233, 255, 293, 301-306]. In intestinal type gastric cancer (GC), C/EBPβ is highly expressed and associated with enhanced cyclooxygenase-2 (COX2) expression and loss of the mucous-associated protein TFF1 [185, 263] [75, 307, 308]. Mice that overexpress COX2 or are deficient for TFF1 develop gastric tumours, underscoring the importance of these proteins and a potential involvement of C/EBPβ in gastric carcinogenesis [77, 309].

Diffuse type GC is associated with loss of the adhesion protein E-Cadherin, however, despite the predominance and histological coherence of the intestinal type of GC, characterized by expansive growth and maintenance of a glandular structure, no central common molecular pathway has been convincingly shown as aberrantly regulated [53, 310]. Here, we examined the homeostatic functions of C/EBPβ in the murine stomach. Our results show that C/EBPβ controls the balance between proliferation and differentiation in the murine stomach. Cross-species analysis of gene expression between mouse C/EBPβ KO stomachs and human GC led to the identification of a subgroup of intestinal-type tumours that showed a strong C/EBPβ-regulation signature. Within this signature, RUNX1t1 was identified as a potential tumour suppressor. It interacts with C/EBPβ and causes release from DNA, counteracting the pro-proliferative properties of C/EBPβ. The RUNX1t1 promoter was also hypermethylated in a large fraction of human GC cases and ectopic expression of RUNX1t1 reduced proliferation in GC cell lines. Our data suggest C/EBPβ activation and RUNX1t1 silencing as important events in the gastric carcinogenesis process.

Results

C/EBPβ knockdown reduces the tumorigenic potential of gastric cancer cells

Enhanced C/EBPβ expression mainly in the human intestinal type GC subtype has been recently reported [185, 263] and was confirmed by real-time PCR, as shown in Figure 1A. The functional importance of high C/EBPβ expression in GC was examined by stable knockdown in human GC cell lines using a viral-based GFP-tagged short hairpin RNA. C/EBPβ-isoform knockdown efficiency in two cell lines approximated 70%, as confirmed by protein immunoblotting (Figure 1B). Proliferation of a cell line derived from intestinal-type GC (MKN74) and a cell line derived from diffuse GC (MKN45) were examined by BrdU incorporation and, as shown in Figure 1C, proliferation of both cell lines was reduced after C/EBPβ knockdown. The tumorigenic potential of cell lines, before and after C/EBPβ knockdown, was compared by xenotransplantation in immune-compromised mice, as shown in Figure 1D. Equal numbers of freshly sorted control and knockdown MKN74 or MKN45 cells were injected. Twenty days post-injection, C/EBPβ knockdown cells formed markedly smaller tumours than parental cells, with less weight and volume (Figure 1D). The difference was more pronounced in MKN74 intestinal type-derived tumours than in the diffuse MKN45 cell line. Ki67 staining showed reduction of cell proliferation in tumours originating from C/EBPβ knockdown cells in comparison to controls (Figure 1C). Interestingly, proliferation in tumours was accompanied by re-expression of C/EBPβ and, in tissue culture knockdown cells required frequent sorting to prevent overgrowth of cells that regained C/EBPβ expression, suggesting selection for C/EBPβ expression. These results show that C/EBPβ plays an important role in GC cell proliferation.

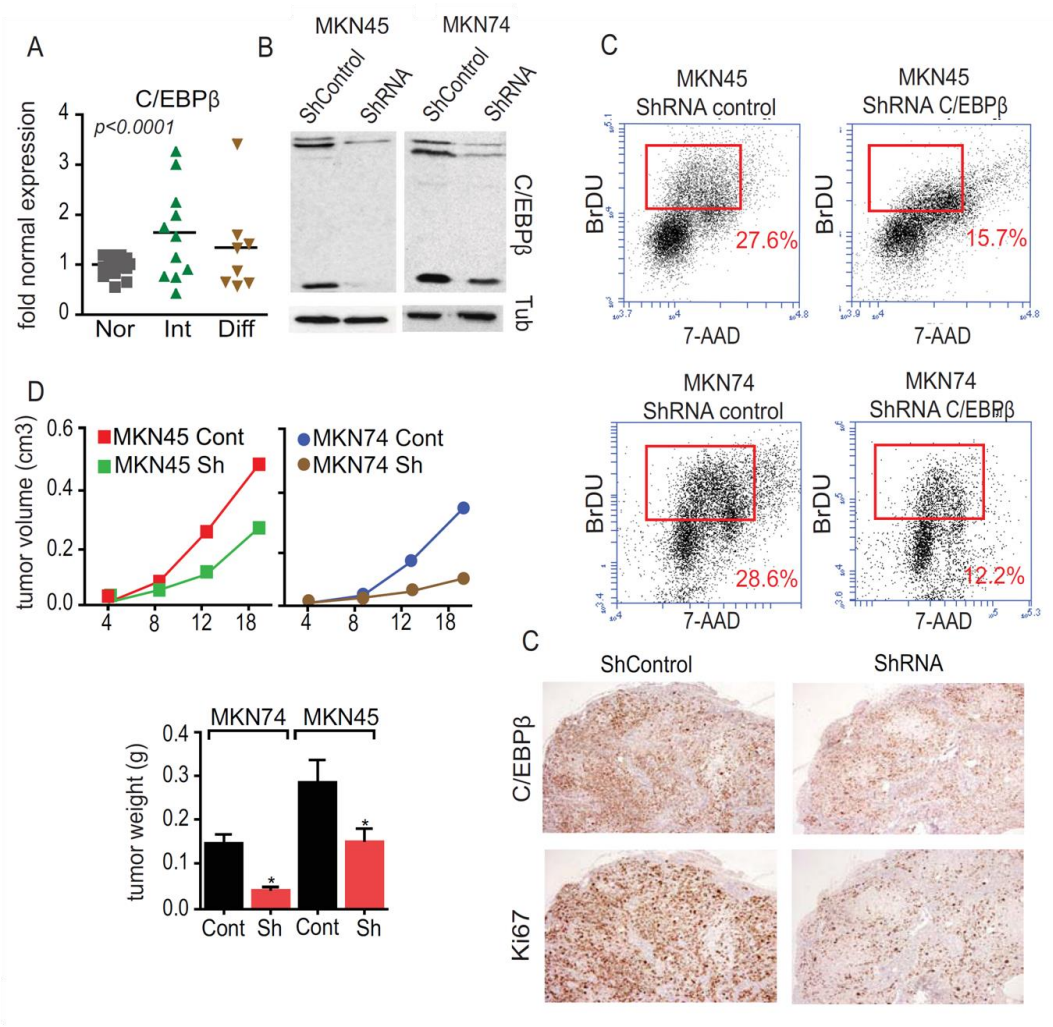


Figure 1. C/EBPβ controls gastric cancer cell proliferation. A) RNA expression of C/EBPβ in intestinal and diffuse gastric cancer cases as determined by real-time PCR. Tumour versus normal ratios were established for each case. Values above 1 entail up-regulation, whereas expression below 1 refers to downregulation (p value refers to normal vs Intestinal comparison). B) Stable knockdown of C/EBPβ in gastric cell lines evaluated by protein blotting (left panel MKN28, right panel MKN45). C) Cell proliferation was determined by BrdU analysis. Cells were labelled with BrdU and incorporation was determined by flow cytometry (FACS) and plotted against 7- AAD-positive cells, as a measure of DNA content. FACS plots show a reduced percentage of BrdU incorporation in gastric cells with C/EBPβ KO. S-phase percentages are highlighted in the FACS plots. D) Gastric cell lines with stable C/EBPβ KO were injected into nude mice and tumour volume and weight was assessed at different time points. Tumours originated from C/EBPβ KO cells were smaller than tumours in the controls. E) Ki67 staining revealed reduction of proliferation in the KO-derived tumours.

C/EBPβ knockout mice display imbalanced differentiation/proliferation of the gastric mucosa

Histological analysis of C/EBPβ expression in the murine stomach revealed restriction to the proliferative zone and overlap with Ki67 staining (Figure 2A), concordantly to observations in human stomach tissue [185]. Analysis of nullizygous C/EBPβ (C/EBPβ$^{-/-}$) stomachs revealed a significant reduction in the thickness of the gastric epithelium and diminished numbers of Ki67-positive cells, as compared to the wild type (WT), particularly in the posterior antral section of the stomach, although no histological abnormalities were observed (Figure 2B and 2C).

To gain further insight into the causes of reduced mucosa thickness, expression of cell cycle-related genes and apoptosis rates were examined. As shown in Figure 2C, reduction of Ki67 and of proliferating cell nuclear antigen (PCNA) in the KO mucosa was evident by quantitative PCR (qPCR) in accordance to histological observations. Additionally, reduced expression of Cyclin A1, Cyclin D3 and Cyclin E1, and increased expression of the CDK inhibitor p15 was found. Apoptosis rate of the C/EBPβ KO mucosa, as revealed by TUNEL assay, showed enhanced cell death in C/EBPβ KO mice compared to WT (supplementary Figure 1A) and qPCR expression analysis showed decreased expression of BCL2 and BIRC5 (survivin) (supplementary figure 1B).

C/EBPβ has previously been reported to repress the gastric differentiation marker and tumour suppressor TFF1 [121, 263]. Similarly to human gastric mucosa, expression of TFF1 was excluded from proliferating cells of the neck zone in murine WT gastric epithelium and expression of C/EBPβ and TFF1 were mutually exclusive (Figure 2D, upper panel). qPCR confirmed increased expression of TFF1 in C/EBPβ KO mucosa, similar to the differentiation genes MUC6 and MUC5AC (Figure 2D, lower panel). Taken together, these data confirmed a repressive role of C/EBPβ on gastric differentiation genes in proliferating cells [329, 359] and regulation of apoptosis in the normal gastric mucosa.

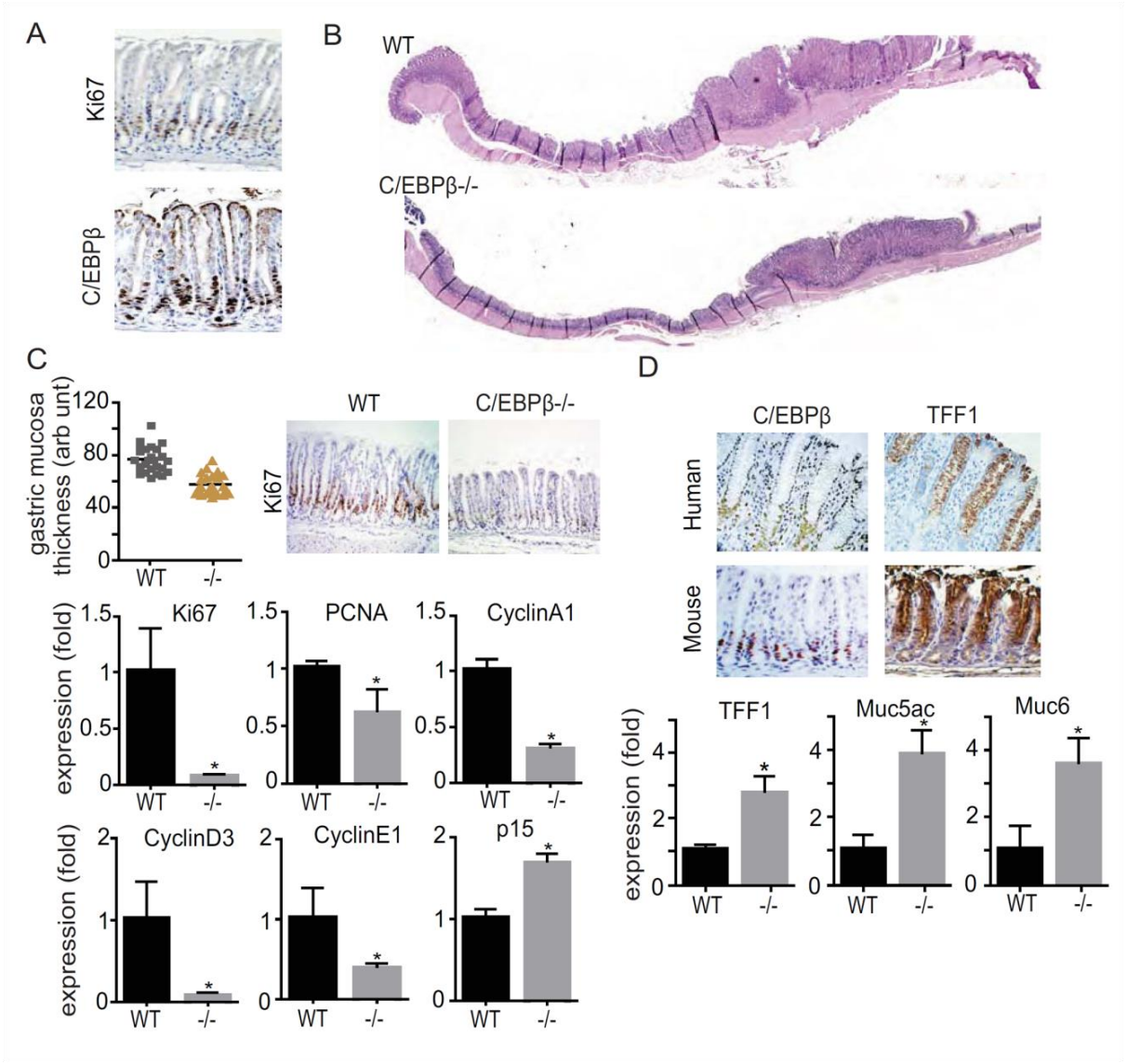
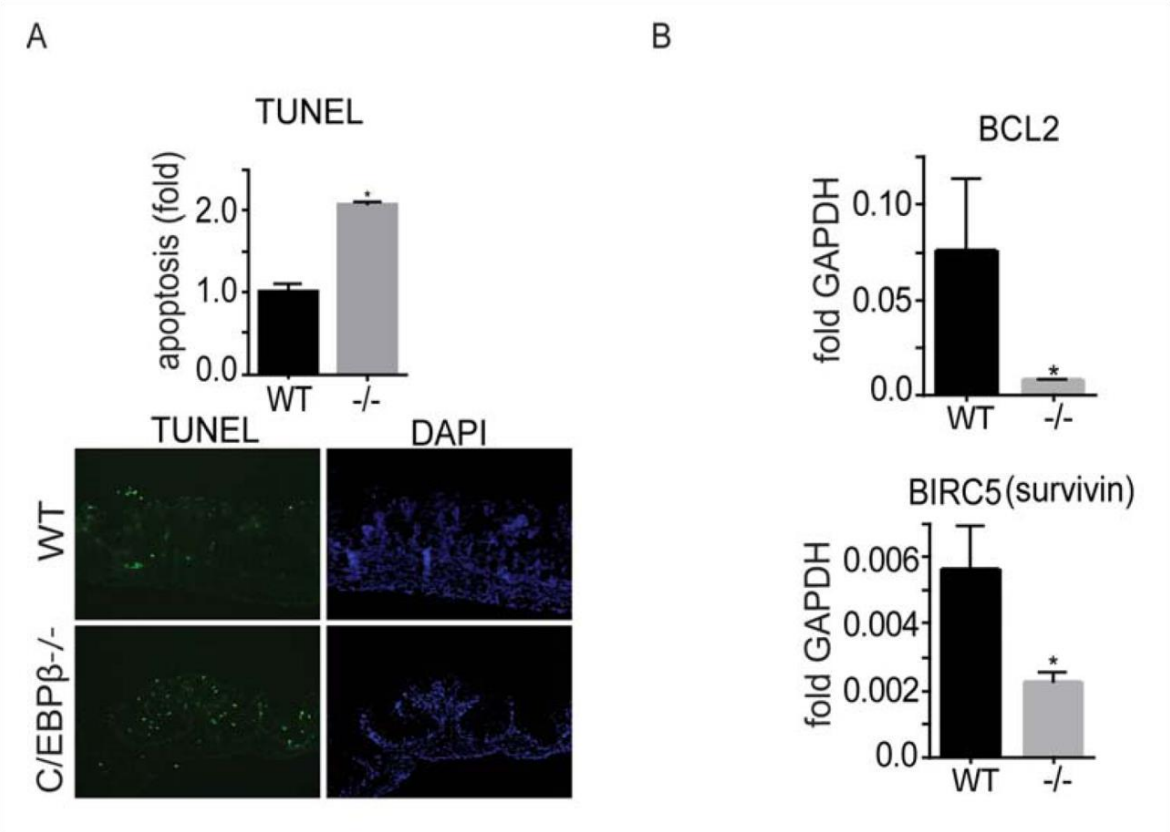


Figure 2. Analysis of the gastric phenotype of the C/EBPβ knockout (KO) mouse. A) Immunohistochemical analysis shows overlap of nuclear C/EBPβ expression and Ki67 expression in the proliferative zone in normal mouse mucosa. B) HE staining of a longitudinal section of wild type (WT) and C/EBPβ KO mucosa, showing reduction of the thickness of the antral KO gastric mucosa. C) Quantification of the C/EBPβ KO mice and WT antral gastric mucosa thickness (in arbitrary units). Adjacent immunohistochemical panel depicts the reduction of Ki67-positive cells in the C/EBPβ KO mucosa. Lower panels show qPCR evaluation of Ki67, PCNA, Cyclin A1, D3, E1 and p15 in the gastric mucosa of WT and C/EBPβ KO mouse stomach (5 animals/group, 3 months old). Values are presented as fold of WT expression, and asterisks refer to p-value of 0.05 or inferior. D) Mutually exclusive expression of TFF1 and C/EBPβ in the normal human (upper panel) and mouse (lower panel) stomach epithelium; C/EBPβ is expressed in proliferative cells of the neck zone and TFF1 in differentiated mucous epithelium. Increased expression of mRNA of differentiation proteins TFF1, MUC5AC, and MUC6 in the C/EBPβ KO mouse gastric mucosa as measured by qPCR.



Supplementary figure 1 Apoptosis analysis of the C/EBPβ KO mouse. A) TUNEL assay showing increased number of apoptotic TUNEL positive cells (upper panels FITC positive) in the C/EBPβ KO mucosa when compared to the WT stomach. Upper graph shows quantification ($p<0.05$). B) qPCR analysis of RNA levels shows reduced levels of anti-apoptotic proteins BCL2 and BIRC5/survivin in the gastric mucosa of the C/EBPβ KO mice.

Cross-species gene expression profiling reveals a subset of intestinal-type gastric tumours with a C/EBPβ-regulation signature

The similarities between human and murine gastric C/EBPβ biology raised the question to what extent the homeostatic and oncogenic C/EBPβ-dependent proliferation share common molecular mechanisms. We therefore compared gene expression profiles derived from C/EBPβ KO mice with previously analysed human GC samples [289].

Differentially expressed genes between the C/EBPβ KO (n=5) and WT (n=4) mice were identified by Welch-test. Significance in differential expression was accepted at $p \leq 0.01$ and a meaningful difference in expression at fold change (FC) larger than 1.5. These cut-off criteria yielded 171 unique annotated and 25 unique non-annotated transcripts (233 probes) as up-regulated in the C/EBPβ KO and 79 unique annotated transcripts and 12 unique non-annotated

ones (135 probes) as downregulated (supplementary Table 1 and 2 show the 15 most significantly regulated genes).

p-value	FC	Gene symbol	Gene name
1.37E-06	2.1	Crim1	cysteine rich transmembrane BMP regulator 1 (chordin like)
1.17E-05	35.3	Krtap3-2	keratin associated protein 3-2
1.33E-05	5.6	Cwh43	cell wall biogenesis 43 C-terminal homolog (S. cerevisiae)
3.74E-05	1.6	Tnip1	TNFAIP3 interacting protein 1, transcript variant 1
6.92E-05	2	Cidec	cell death-inducing DFFA-like effector c
7.80E-05	2	Pik3ip1	phosphoinositide-3-kinase interacting protein 1
9.68E-05	1.9	Unc5b	unc-5 homolog B (C. elegans)
1.21E-04	2.1	Sys1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)
1.64E-04	2.3	E130012A19Rik	RIKEN cDNA E130012A19 gene
1.80E-04	2	Gm8221	predicted gene 8221, non-coding RNA
2.03E-04	2.1	Ly86	lymphocyte antigen 86 (Ly86)
2.06E-04	2.7	Muc13	mucin 13, epithelial transmembrane
2.59E-04	2.8	Adck3	aarF domain containing kinase 3, nuclear gene encoding mitochondrial protein, transcript variant 1
2.67E-04	3.5	Gkn2	gastrokine 2
2.83E-04	1.5	Procr	protein C receptor, endothelial

Supplemental table 1. List of the 15 genes with higher up-regulation score in the C/EBPβ KO stomach.

p-value	FC	Gene symbol	Gene name
2.37E-07	112.5	Cpsf3l	cleavage and polyadenylation specific factor 3-like
5.87E-07	20.5	Gltpd1	glycolipid transfer protein domain containing 1
8.00E-07	17.4	BC021614	cDNA sequence BC021614
1.45E-06	44.2	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
6.13E-05	2.2	Pusl1	pseudouridylate synthase-like 1
7.01E-05	3.6	Sftpd	surfactant associated protein D
7.37E-05	2.8	Tdh	L-threonine dehydrogenase
1.24E-04	3	Nppa	natriuretic peptide type A
1.30E-04	3.4		CYT3_MOUSE (P35175) Stefin 3, partial (93%)
1.46E-04	17.4	Defb3	defensin beta 3
1.47E-04	4.7	Slc5a5	solute carrier family 5 (sodium iodide symporter), member 5
2.31E-04	1.8	Fam132a	family with sequence similarity 132, member A (Fam132a),
2.56E-04	1.9	Nr1h3	nuclear receptor subfamily 1, group H, member 3, transcript variant 1,
2.71E-04	1.6	Larp1	La ribonucleoprotein domain family, member 1
2.73E-04	2.2	Flrt1	fibronectin leucine rich transmembrane protein 1

Supplemental Table 2. List of the 15 genes with higher downregulation score in the C/EBPβ KO mucosa

Next, the combined list of up- and downregulated genes (FC>1.5, p≤0.01) derived from the C/EBPβ KO mouse profiling data was used to cluster the human GC microarray samples. The resulting gene expression heatmap suggested that the majority of genes did not show any overt deregulation in human cancers (whitish spots in heatmap). However, a group of genes showed explicitly strong regulation (indicated by dark bluish and reddish spots in the heatmap) across the cancer samples (Supplementary Figure 2, regulated gene cluster, indicated by box). Genes contained in this subset were then used to re-cluster the human cancer samples. The resultant cancer sample dendrogram and expression heatmap (Figure 3) revealed a group of cancer samples (Figure 3, black box) that exhibit downregulation of the majority of these genes. The group consisted of 16 of the original 59 (≈27%) samples and contained primarily cancers of the intestinal histological type. Importantly, genes downregulated in this particular cancer

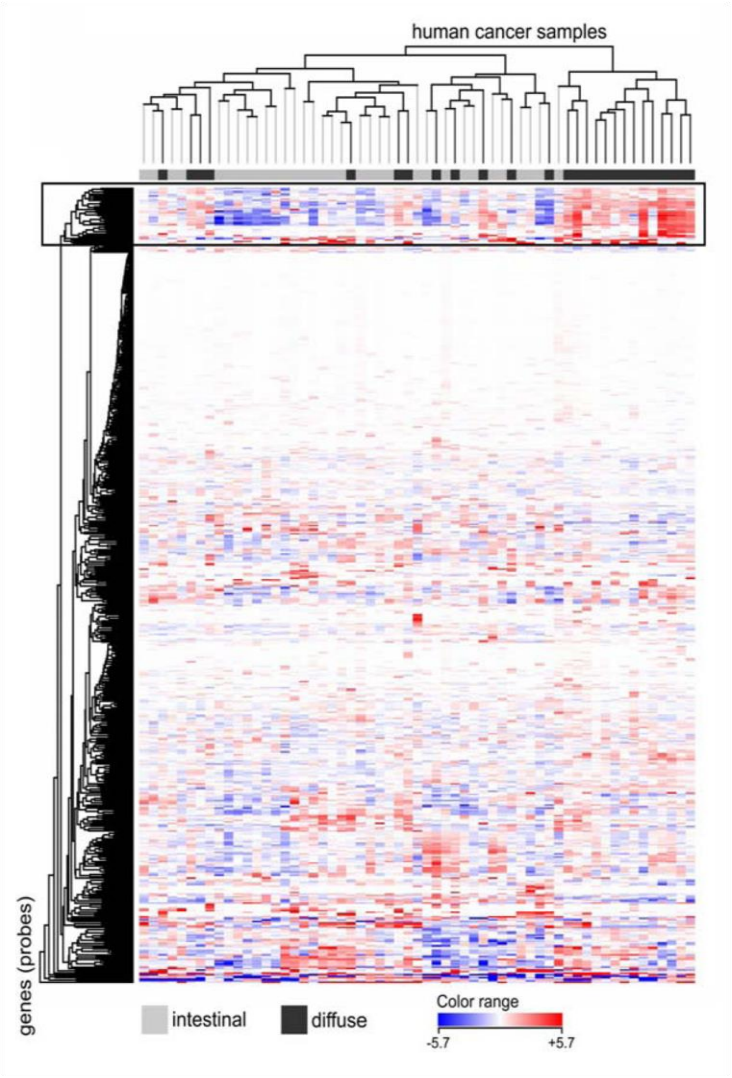
subgroup are mostly up-regulated in the C/EBPβ KO gastric mucosa (changes ranging from 1.5 to 2.3 fold; Table 1) identifying them as C/EBPβ repressed genes.

Gene symbol	Gene name	p (int. vs. diff.)	Regulated in intestinal GC	FC	p (C/EBPβKO vs. WT)	Regulated in C/EBPβ KO	FC
COL4A6	collagen, type IV, alpha 6	0.026065	down	2.2	0.003434	up	1.8
COX7A1	cytochrome c oxidase, subunit VIIa 1	1.34E-07	down	2.6	0.008941	up	2.2
CPE	carboxypeptidase E	8.69E-04	down	2	8.96E-04	up	1.6
GLI1	GLI-Kruppel family member GLI1	3.39E-05	down	1.9	0.001579	up	1.6
HLF	hepatic leukemia factor	9.51E-04	down	2.3	0.007635	up	1.6
MAMDC2	MAM domain containing 2	1.44E-06	down	6.2	0.007404	up	2.3
PDZRN4	PDZ domain containing RING finger 4	0.005077	down	2.9	0.007338	up	2
PTCHD1	patched domain containing 1	0.020572	down	2.1	0.00189	up	1.5
PTGER3	prostaglandin E receptor 3 (subtype EP3)	1.89E-07	down	3.7	0.004076	up	1.6
RAI2	retinoic acid induced 2	6.97E-05	down	2.6	0.008717	up	1.6
RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	1.32E-08	down	5.2	0.00741	up	1.5
SPARCL1	SPARC-like 1	9.97E-10	down	2.7	0.007544	up	1.5
ZFMP2 / FOG2	zinc finger protein, multitype 2	9.81E-08	down	4.4	0.001298	up	1.6

Table 1. Genes from the C/EBPβ clustered intestinal type genes, showing their regulation in the glandular tumours and C/EBPβ KO stomachs. The genes downregulated in intestinal type tumours are exclusively up regulated in C/EBPβ KO.

To validate the results obtained by microarray comparison, we selected three C/EBPβ repressed genes, FOG2, SPARCL1, and RUNX1t1, and analysed their expression by qPCR. Examination of the expression of these genes in WT and C/EBPβ KO stomach confirmed up-regulation in the gastric mucosa of C/EBPβ KO mice (5 animals/group; supplementary figure 3B). It was also important to examine the expression of FOG2, SPARCL1, and RUNX1t1 in normal human gastric mucosa as no normal tissue samples were available for the initial human

GC microarray analysis [289]. As shown in supplementary Figure 3A, expression of all three genes was downregulated in intestinal type GC, however, a subset of diffuse type tumours overexpressed RUNX1t1, compliant with the different aetiology of these tumours.



Supplementary figure 2. Heat map originated by the comparison of mouse microarray data displaying differences between C/EBPβ KO and WT mouse stomach, and a set of human gastric cancer samples. The majority of murine genes were not regulated in human gastric cancer (whitish spots in the map). However, one cluster of genes showed explicitly strong regulation (highlighted). The members of this strongly regulated gene cluster showed downregulation in the intestinal tumours compared to the diffuse-type ones with differences ranging between 1.8 and 6.2 with and overall classification correctness for histological type of 78%.

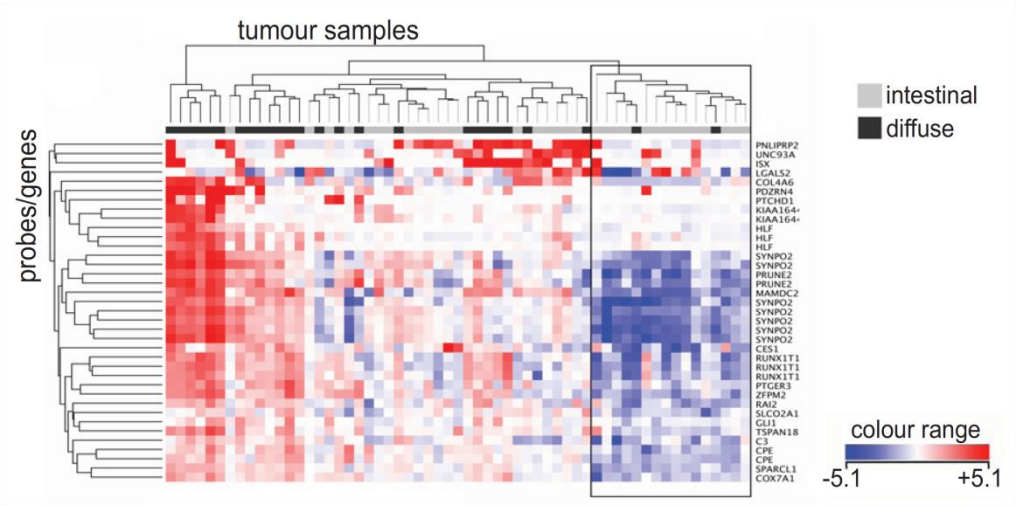
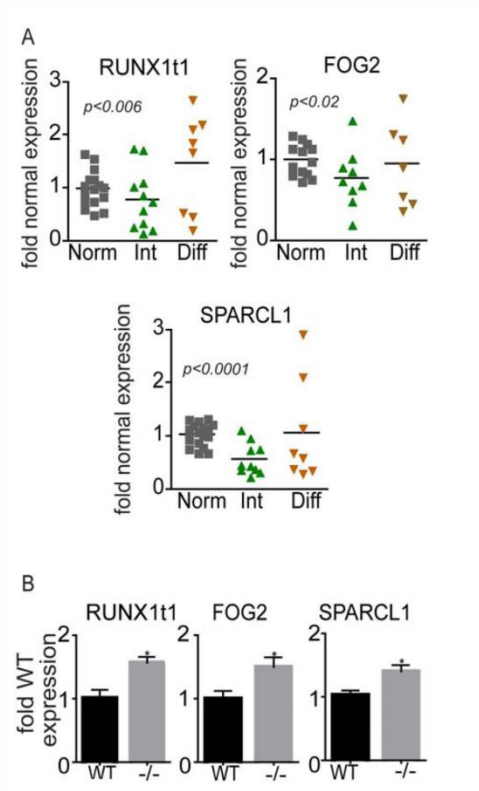


Figure 3. Cross-species comparison of gene expression. Two-way hierarchical clustering was performed using a strongly-regulated gene cluster (shown in Supplementary Figure 2) from microarray-derived murine genes that differed between C/EBPβ KO and WT stomach (p≤0.01, FC≥1.5) and human gastric cancer samples. Depicted are the resultant gene and sample dendrograms and the corresponding expression intensity heat map. The black box indicates a tumour cluster in which most of the genes show downregulation (bluish spots). This tumour group consisted of 16 of the original 59 (≈27%) samples and contained primarily cancers of the intestinal histological type.



Supplemental Figure 3. Confirmation of the microarray expression analysis results. A) RNA expression analysis of FOG2, SPARCL1 and RUNX1t1 in intestinal and diffuse gastric cancer cases as determined by real-time PCR. Tumour vs. normal ratios were established for each case. Values above 1 entail up-regulation, whereas expression bellow 1 refers to downregulation. Visible is the decreased expression of the gene set in intestinal type gastric cancer cases in comparison to normal. Significance displayed in graphic refers to Normal Vs Intestinal-type comparison. B) Expression evaluation of FOG2, SPARCL1 and RUNX1t1 expression in wild-type (WT) and C/EBPβ KO stomach (5 animals/group) by qPCR, showing upregulation in the C/EBPβ KO.

C/EBPβ expression is mandatory for the hyperplastic phenotype in the RUNX3 KO mice stomach

The RUNX3 KO mouse is an established model of early GC initiation and hyper-proliferation [90], although the mechanism underlying the RUNX3-deficient neoplastic phenotype remains under debate [311, 312]. As shown in Figure 4A, Ki67 staining confirmed increased proliferation of the epithelial stomach layer of new-born RUNX3-null mice and E-Cadherin staining confirmed the epithelial nature of the proliferating cells. C/EBPβ is highly expressed in the hyper-proliferative gastric mucosa of RUNX3 KO mice and staining of serial sections showed co-localization of C/EBPβ and Ki67 in the proliferative mucosa of the RUNX3 KO (Figure 4A).

C/EBPβ null animals were crossed with RUNX3 KO mice to evaluate the functional contribution of C/EBPβ in the neoplastic RUNX3 KO stomach tissue. Analysis of the stomach tissue of single RUNX3 KO and the compound C/EBPβ/RUNX3 KO showed that stomach wall thickness was reduced to WT levels in double KO animals. Ki67 staining confirmed an almost complete reversion of the hyper-proliferative phenotype in RUNX3 KO by removal of C/EBPβ (Figure 4A and 4B) that was accompanied by a substantial increase in the number of apoptotic cells (supplementary Figure 4A and 4B). We conclude that expression of C/EBPβ is mandatory for the neoplastic gastric phenotype of RUNX3 deficient mice.

To understand to what extent the role of C/EBPβ in the RUNX3 KO gastric phenotype reflects the gene regulation identified in the microarray analysis, we compared FOG2, SPARCL1, and RUNX1t1 in RUNX3 KO and in the compound C/EBPβ/RUNX3 KO. In the hyper-proliferative RUNX3 KO mucosa only RUNX1t1 displayed reduced expression, whereas FOG2 and SPARCL1 remained within the range of WT mucosa. Importantly, RUNX1t1 expression was partially rescued in the compound KOs, as shown in Figure 4C. These data suggested opposing functions and regulation of RUNX1t1 and C/EBPβ in proliferation control. Indeed, transfecting C/EBPβ isoforms (LAP*, LAP and LIP) into MKN28 and MKN45 cell lines led to the repression of RUNX1t1 expression (Figure 4D).

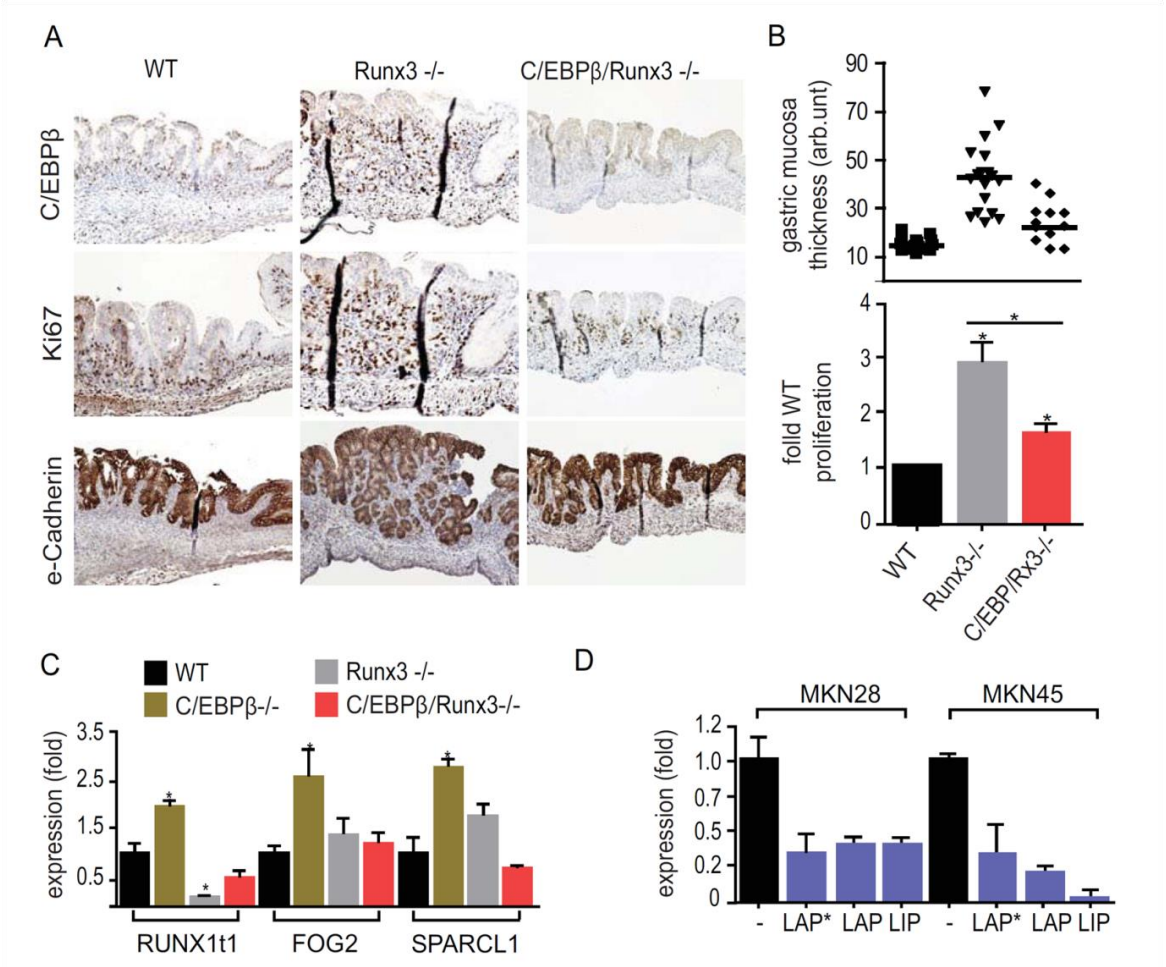
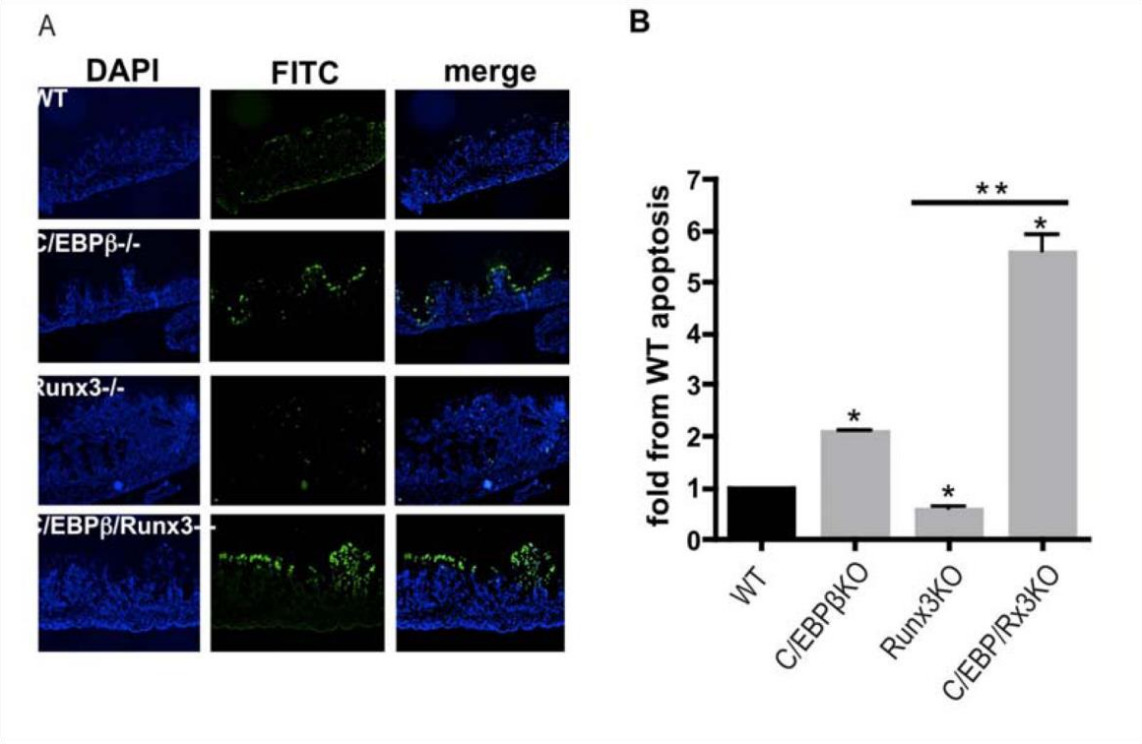


Figure 4. C/EBPβ in the RUNX3 knockout (KO) mouse. A) Immuno-staining shows increased C/EBPβ and Ki67 expression in the RUNX3 KO mouse stomach and reversion in the C/EBPβ/RUNX3 double KO. E-cadherin staining shows that hyper-proliferation is confined to the epithelial compartment. B) Quantification of the mucosal thickness and Ki67 expression ($p<0.05$) in the wild type (WT) and RUNX3 KO and reversal of the mucosal thickness and hyper-proliferative phenotype in the compound C/EBPβ/RUNX3 double KO. C) qPCR analysis of RUNX1t1, FOG2, and SPARCL1 in RUNX3 KO and C/EBPβ/RUNX3 KO stomachs shows that only RUNX1t1 is downregulated in the hyper-proliferative mucosa of the RUNX3 KO ($p<0.005$) and reverted to almost WT levels in the compound C/EBPβ/RUNX3 KO stomach. D) Transfection of C/EBPβ isoforms LAP*, LAP, and LIP into gastric cell lines MKN28 and MKN45 repressed RUNX1t1 expression as measured by quantitative PCR.



Supplemental figure 4. A) Apoptosis in the gastric mucosa of the single KO and compound C/EBPβ/Runx3 double KO was analyzed by TUNEL assay, being observable an increase in the cell death rate in the compound C/EBPβ/Runx3 KO when compared with the other mutants. B) shows quantification of TUNEL-positive cells ($p<0.05$).

RUNX1t1 plays a tumour suppressive role in human gastric cancer and modulates C/EBPβ activity

Expression of RUNX1t1 protein was evaluated by tissue microarray immunohistochemistry on 64 human GC samples. Nuclear staining was classified as strong, moderate, weak or absent, referencing to the expression of RUNX1t1 in the normal mucosa (classified as moderate). From the analysed tumours, 25 out of 64 (38%) showed weak or absent RUNX1t1 protein staining (Figure 5A). To further assess whether C/EBPβ is responsible for downregulation of RUNX1t1 in GC, we selected tumour-RNAs showing reduced levels of RUNX1t1 (supplementary Figure 3A). The majority of cases (7 out of 10, Figure 5B), however, failed to show a convincing inverse correlation between low RUNX1t1 and high C/EBPβ expression, suggesting alternative means of RUNX1t1 downregulation in GC. Sequencing of RUNX1t1 from 26 GC patients failed to disclose mutations that would explain loss of RUNX1t1 protein (data not shown), however, analysis of the *RUNX1t1* promoter by methylation-specific

PCR revealed hypermethylation in 10 out of 20 GC DNA samples (Figure 5C). Next, we examined the functional consequences of RUNX1t1 downregulation in GC cells. As shown in Figure 5D, overexpression of RUNX1t1 in MKN28 and MKN45 cell lines led to decreased cell proliferation, as determined by BrdU incorporation. These data suggest that RUNX1t1 inhibits proliferation and is frequently downregulated in GC.

RUNX1t1 has previously been reported to interact with C/EBPβ, to inhibit its DNA binding, and to block its pro-proliferative functions during the clonal expansion phase in adipogenic differentiation [313]. Ectopic expression of flag-tagged RUNX1t1 in MKN28 and MKN45 cell lines and subsequent immunoprecipitation showed that RUNX1t1 interacts with all endogenous C/EBPβ isoforms in cell lines (Figure 5E). Electrophoretic mobility shift assay (EMSA) led to a dose dependent decrease of C/EBPβ binding to its DNA consensus sequence in cell lines, although RUNX1t1 did not significantly alter nuclear C/EBPβ expression (Figure 5F). These results suggest that the tumour-suppressive function of RUNX1t1 is mechanistically connected to the suppression of pro-oncogenic C/EBPβ functions.

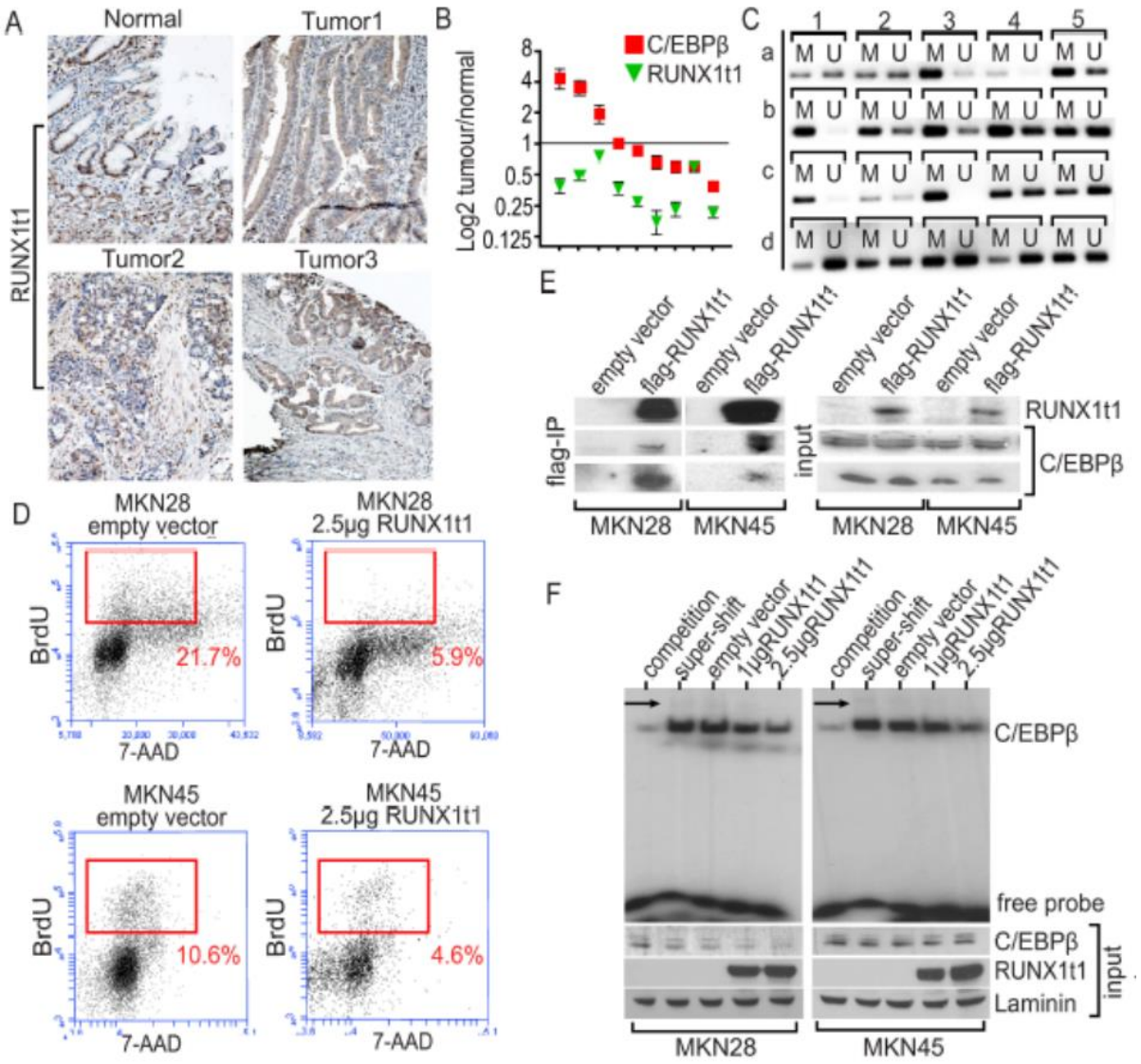


Figure 5. RUNX1t1 and gastric cancer. A) RUNX1t1 expression was evaluated by immunohistochemistry in 64 human gastric cancer samples, and staining was classified by comparison to the expression in the normal mucosa (left panel). 38% of the cases showed reduced expression of RUNX1t1 (right panel). B) In 10 gastric tumours with reduced RUNX1t1 RNA levels were examined for C/EBPβ expression by qPCR. Only 3 out of 10 cases showed higher C/EBPβ expression as compared to WT. C) The methylation status of the RUNX1t1 promoter was evaluated by methylation-specific PCR. Bisulfite treatment of tumour DNA converts unmethylated but not methylated cytosines to uracil, and subsequent methylation-specific PCR detects either methylated (M) or unmethylated (U) DNA. 50% of the analysed human gastric cancer cases (rows a-b, columns 1-5) present RUNX1t1 promoter hypermethylation. D) Ectopic expression of RUNX1t1 in MKN28 and MKN45 gastric cancer cell lines reduces gastric cancer cell proliferation as measured by BrdU incorporation assay. S-phase percentages are indicated in the FACS plots. E) Immunoprecipitation of flag-tagged RUNX1t1 co-precipitates C/EBPβ. Visible in the input Western Blot is also that RUNX1t1 does not affect C/EBPβ expression. F) Electrophoretic mobility shift assay (EMSA) using a radiolabeled C/EBPβ DNA probe and nuclear extracts from MKN28 and MKN45 cells. Transfection of RUNX1t1 reduces the binding of C/EBPβ to DNA in cell lines in a dose dependent manner. Arrow indicates the super-shift.

Discussion

GC is one of the leading causes of cancer-related death in the developing world [314] and our data suggest a function of C/EBPβ particularly in the intestinal type of GC. Comparison of gene expression profiles from C/EBPβ KO mice and human GC samples provided insight in C/EBPβ-related molecular mechanisms. Absence of C/EBPβ from the murine stomach shifts the balance from epithelial proliferation towards differentiation and apoptosis.

Data presented here suggest that the function of C/EBPβ in GC is embedded in the homeostatic regulation of the gastric mucosa. Deregulation of pathways that sustain C/EBPβ functions such as inflammatory signals may favour uncontrolled proliferation and repression of differentiation genes such as TFF1 that ultimately unbalances the physiological homeostasis of the gastric epithelium and promotes tumour development [77].

C/EBPβ is mandatory for the hyper-proliferative phenotype of the RUNX3 KO mice and for the tumorigenic potential of GC cell lines. Expression profiling data of human GC samples and comparison with C/EBPβ KO mouse-derived expression data identified a subset of tumours owning a C/EBPβ-regulated signature. These tumours mostly belong to the intestinal type and may define a novel subtype. One of the genes characterizing this tumour cluster, RUNX1t1, has previously been connected to gastrointestinal abnormalities [315] and to suppression of C/EBPβ functions [313] and was consistently downregulated in the murine RUNX3 KO tumour model. RUNX1t1 is also a candidate tumour suppressor in ovarian cancer [291] and loss of RUNX1t1 expression has been associated with metastasis in pancreatic cancer [316]. Downregulation of RUNX1t1 during homeostasis and initially in intestinal type GC may occur through C/EBPβ. However, analysis of DNA methylation showed that the RUNX1t1 promoter was frequently methylated in human GC samples. RUNX1t1 promoter hypermethylation has also been observed in ovarian cancer [291] and suggests alternative routes of RUNX1t1 gene silencing in gastric carcinogenesis.

RUNX1t1, also known as MTG8 or ETO, is the recurrent t(8;21) translocation partner of the AML-ETO (RUNX1/MTG8) fusion protein. AML-ETO accounts for 15% of acute myeloid leukemia and 40% of M2-type leukemia, probably by interference with the differentiation inducing functions of C/EBPα and PU.1 [317, 318]. Few reports have focused on RUNX1t1 independently of the AML-ETO context, yet suggest involvement of RUNX1t1 in several co-repressor complexes [318]. Our results support the notion of RUNX1t1 as a suppressor of GC development and suggest a regulatory loop between C/EBPβ and RUNX1t1 in homeostasis and disruption in cancer. High expression of C/EBPβ leads to reduction of RUNX1t1 expression and high RUNX1t1 expression leads to the inhibition of C/EBPβ functions. Antagonism between

both proteins was reported in the adipogenic clonal expansion phase, which requires balanced expression of C/EBPβ and RUNX1t1 to prevent premature induction of C/EBPα and terminal fat cell differentiation [313]. The connection between C/EBPβ and RUNX1t1 may also be relevant in hematopoietic malignancies involving the AML-ETO translocation. It has recently been shown that RUNX1 and C/EBPβ bind to all hematopoietic genes in embryonic stem cells that undergo hematopoietic differentiation [319]. It thus seems plausible that the fusion of RUNX1 and RUNX1t1 in the t(8;21) AML-ETO translocation may counteract distinct functions of C/EBPβ in earmarking lineage commitment and differentiation genes and thus contribute to oncogenic conversion.

It is proposed that most neoplasms arise from a single cell of origin, and tumour progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines. Tumour cell populations are genetically more unstable than normal cells, perhaps from activation of specific gene loci in the neoplasm, continued presence of carcinogen, impact of inflammatory mediators, or even nutritional deficiencies within the tumour. Hence, each patient's cancer may require individual specific therapy, and even this may be thwarted by emergence of a genetically variant subline resistant to the treatment. More research should be directed toward understanding and controlling the evolutionary process in tumours before it reaches the late stage usually seen in clinical cancer.

Article: Interleukin-1B signalling leads to increased cell survival of gastric carcinoma cells through a CREB-C/EBPβ-associated mechanism

Carlos Resende, Gonçalo Regalo, Cecília Durães, Marta Teixeira Pinto, Wen Xiaogang, Ceu Figueiredo, Fátima Carneiro, José C. Machado (accepted for publication on Gastric Cancer)

INTRODUCTION

Gastric carcinoma (GC) is the second cancer-related cause of death in the world. Although its incidence is decreasing in developed countries, it remains a health burden in the developing world. The main risk factor for the onset of GC is life-time infection with *Helicobacter pylori* (*H. pylori*), a stomach colonizing bacterium [314]. Infection with *H. pylori* leads to chronic gastritis that may progress to gastric atrophy, intestinal metaplasia, dysplasia and finally GC [1, 14].

The risk of developing GC depends both on environmental factors and host-related factors [6, 39]. In this model, gene polymorphisms that increase the production of pro-inflammatory mediators, lead to enhanced chronic inflammatory response to *H. pylori* infection and to increased risk of progression towards GC [31, 39]. There are numerous studies demonstrating that polymorphisms in genes such as *IL1B, IL1RN, TNFA* and *IFNGR1* are associated with risk of developing GC [32, 40, 47, 50, 320]. Moreover, these polymorphisms have been shown to be associated with increased gene expression, both *in vitro* and *in vivo* [44]. Perhaps the most striking evidence favouring this model comes from a transgenic mouse model showing that overexpression of the *IL1B* gene in gastric mucosa leads to increased risk of developing gastric disease, including dysplasia and GC, even in the absence of *H. pylori* infection [127].

According to the prevailing model, the link between enhanced chronic inflammation and GC depends essentially on the "destructive" effects of inflammation over the gastric epithelium, resulting in atrophy of the gastric mucosa and increased cell turnover and mucosal repair [49, 321]. However, it is well demonstrated that inflammatory mediators, and other growth factors secreted by inflammatory cells, can act directly on other cell types, such as epithelial cells. Therefore, in addition to the mucosal destruction and repair effect, enhanced chronic inflammation could also play a role in gastric carcinogenesis by providing gastric epithelial cells with a survival stimulus through the secretion of growth factors [322, 323]. Coupled with

mutagenic events, this could ultimately lead to increased risk of cell transformation and GC development.

In this regard, IL1B is particularly interesting since polymorphisms in its promoter region, have been shown to be associated with increased risk of GC [32, 40, 47, 48]. IL1B is a powerful pro-inflammatory cytokine that activates different transcriptions factors [324], some of which are also activated by *H*. *pylori* infection [182, 325]. One of the IL1B-activated transcription factors is CCAAT/enhancer-binding protein beta (C/EBPβ) [326]. We previously reported that C/EBPβ is overexpressed in pre-malignant lesions and in GC, suggesting that this protein might be relevant for gastric carcinogenesis by inducing the expression of COX-2 [185]. Furthermore, C/EBPβ expression in GC was significantly associated with loss of expression of the putative gastric tumour-suppressor TFF1 [263, 327].

Another important IL1B-activated transcription factor is cAMP response element-binding protein (CREB), which has been described as a major player in inflammation [324, 328]. In non-small cell lung cancer, IL1B induces the activation of CREB through ERK1/2 signalling, resulting in the expression of a set of pro-angiogenic cytokines that are crucial factors for tumour progression [329]. Furthermore, CREB was recently described to play an important pro-oncogenic role in both cancer development and progression, being found overexpressed in several cancer types [193, 201, 204, 330]. It has been demonstrated, both in hepatocytes [174] and in preadipocytes [176], that CREB is able to regulate the transcription of the *CEBPB* gene by directly interacting with its promoter.

The main objective of this study was to determine whether chronic inflammation-associated IL1B signalling, as seen in the context of *H*. *pylori* infection, could be linked to gastric carcinogenesis by modulating the behaviour of gastric epithelial cells. We addressed this objective by showing that CREB and C/EBPβ transcription factors can be activated by IL1B signalling in the GC context. We also demonstrate that CREB acts upstream of C/EBPβ in GC cell lines. Finally, we show *in vitro* and *in vivo* that this signalling mechanism promotes GC cell survival.

RESULTS

IL1B increases C/EBPβ and CREB expression in an ERK1/2-dependent manner

To evaluate the effect of IL1B over the expression and activation status of C/EBPβ and CREB, we incubated GC cell lines AGS and GP202 with IL1B for 24 hours. Both in AGS and in GP202 cells, the incubation with IL1B led to an increase in expression of all isoforms (LAP*, LAP, and LIP) of C/EBPβ (Fig. 1). Regarding CREB, we observed an increase in both expression and phosphorylation levels in both cell lines (Fig. 1).

Since ERK1/2 has been previously implicated in the regulation of C/EBPβ and CREB, we investigated whether it could mediate the effect of IL1B over those two transcription factors. Incubation of AGS and GP202 cells with the ERK1/2 inhibitor U0126 led to a decrease in expression of C/EBPβ and CREB and to a decrease in phosphorylation levels of CREB (Fig. 1). The level of ERK1/2 phosphorylation in both cell lines was measured as a control for the efficacy of inhibition with U0126 (Fig. 1). These results demonstrate that IL1B is able to regulate the expression/activation status of both C/EBPβ and CREB in GC cells.

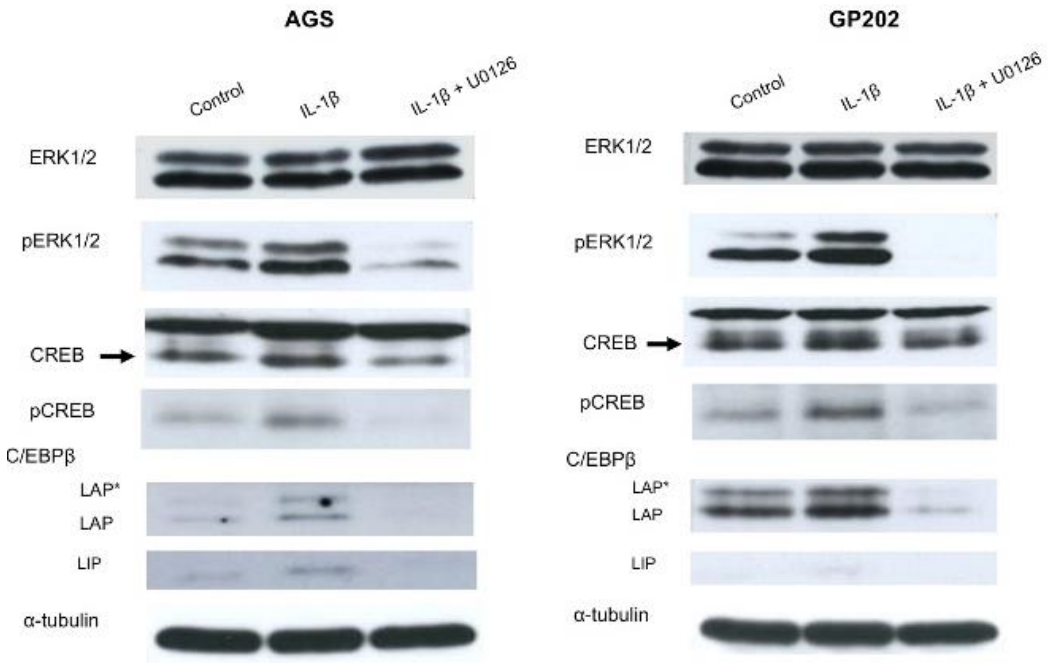


Figure 1: IL1B stimulation and ERK1/2 inhibition effects on CREB, pCREB, and C/EBPβ protein levels. Both AGS and GP202 cells, treated with 10 ng/mL of IL1B for 24h, exhibited an increase in activated ERK1/2 (pERK1/2). In parallel, the expression levels of CREB, pCREB, and C/EBPβ were also increased. The ERK1/2 chemical inhibitor U0126 (25 μM) reverted the effect of IL1B on CREB, pCREB, and C/EBPβ protein levels.

CREB is a transcriptional regulator of C/EBPβ in GC cells

Knowing that CREB is a transcriptional regulator of the *CEBPB* gene in other cell types, we decided to investigate whether the same regulatory mechanism could be at work in GC cells. Since both AGS and GP202 cells yielded the same results and the IL1B-stimulatory effect was more pronounced in AGS cells, we decided to perform the next set of experiments only in the AGS cells. Using both small interfering RNA (siRNA) and short-hairpin RNA (shRNA), we found that knocking-down CREB results in downregulation of C/EBPβ expression (Fig. 2a). Conversely, silencing of C/EBPβ by siRNA had no impact on CREB expression (Fig. 2a). These results show that CREB acts upstream of C/EBPβ in this regulatory mechanism.

To verify if CREB acts directly on the *CEBPB* gene, we analysed the *CEBPB* promoter (2663 base pairs) in order to find putative cAMP response element (CRE)-binding motifs. We employed the nucleotide position numbering as previously described [174]. The analysis revealed the presence of three CRE-binding sites, ranging from nucleotides -2174 to -2171 (BS1), from -959 to -956 (BS2), and from -66 to -63 (BS3) (Fig. 2b). Using chromatin immunoprecipitation (ChIP) we observed that CREB binds all three CRE-binding sites on the *CEBPB* promoter (Fig. 2c).

These results were validated by showing that 24h chemical inhibition of the interaction between CREB and its co-activator CREB binding protein (CBP), led to a reduced binding of CREB to the CRE-binding sites on the *CEBPB* gene promoter (Fig. 3a). To confirm that the decrease in CREB binding to the *CEBPB* promoter actually leads to downregulation of transcription and protein synthesis, we evaluated the relative amount of C/EBPβ mRNA and protein after 48 hours of treatment with the CBP-CREB interaction inhibitor. This experiment resulted in a significant reduction of both C/EBPβ transcript levels (Fig. 3b) and C/EBPβ protein levels (Fig. 3c).

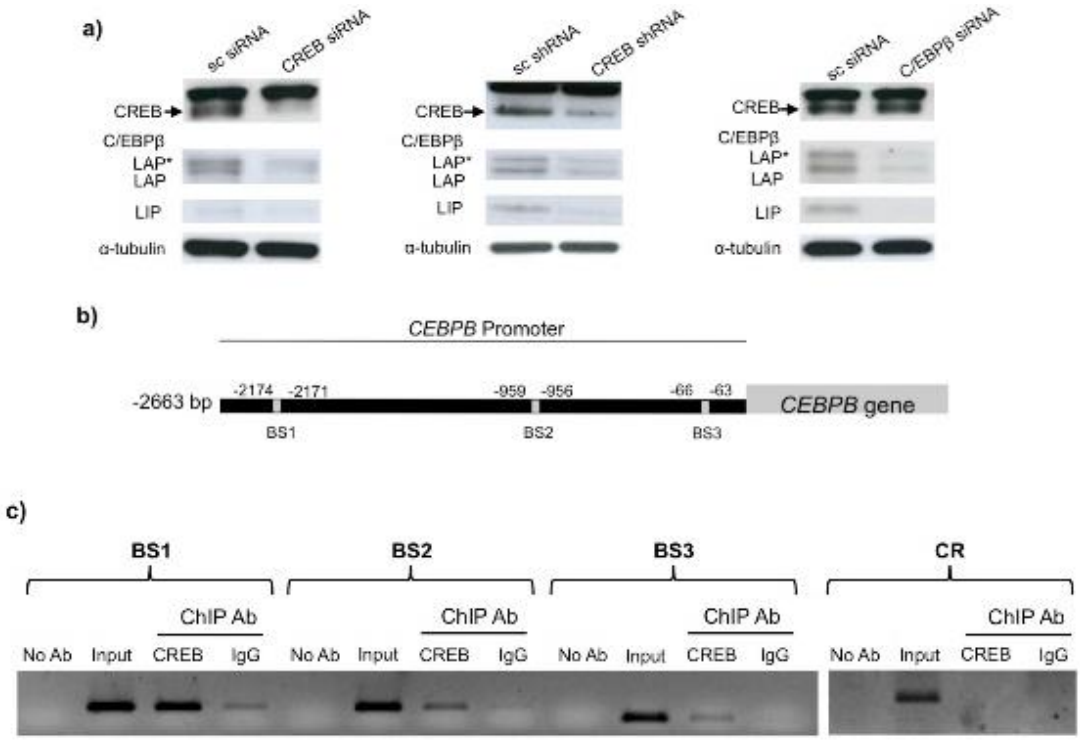


Figure 2. Effect of CREB downregulation on C/EBPβ protein levels. a) CREB silencing (CREB-specific bands are indicated by arrows; the upper bands on the CREB blots represent unspecific binding of the CREB antibody) was followed by a downregulation of C/EBPβ protein levels, whereas the silencing of C/EBPβ had no impact on CREB protein; b) schematic representation of the *CEBPB* promoter (2663 bp) with identification of the three CRE-binding sites (BS1, BS2, and BS3) and a control region (CR) located at the 3' end of *CEBPB*; c) CREB interacts with all three CRE-binding motifs present on the *CEBPB* promoter. No Ab: no antibody used; Input: 1/100 of the sheared initial chromatin; CREB: chromatin immunoprecipitated using an anti-CREB antibody; IgG: chromatin immunoprecipitated with an unspecific antibody of the same family of the anti-CREB antibody.

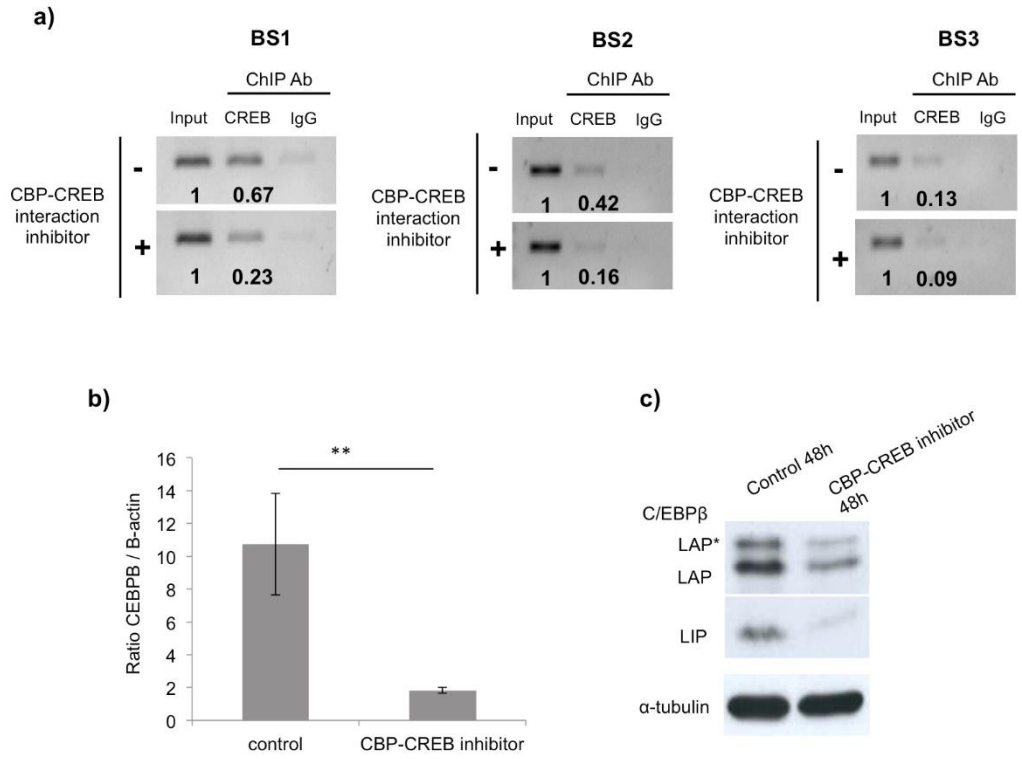


Figure 3. Effects of CBP-CREB interaction inhibition on CREB activity over C/EBPβ. a) ChIP performed on AGS cells after treatment with CBP-CREB interaction inhibitor (+) revealed a decrease in the amount of CREB linked to the *CEBPB* promoter comparatively to untreated cells (-); b) AGS cells treated with CREB-CBP interaction inhibitor (25 μM) for 48h showed a significant decrease in C/EBPβ transcript levels d) and in protein expression. Real-time PCR results represent the mean ± S.D. of three independent experiments. *: significant ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

CREB and C/EBPβ proteins are co-expressed in normal gastric mucosa and in GC

To determine whether there is an association between the expression of CREB and C/EBPβ, we analysed the immunohistochemical (IHC) expression of these two proteins in a series of 66 cases of GC. In normal gastric mucosa we found that both C/EBPβ and CREB are expressed in the nucleus of epithelial cells in a glandular region within which is located the proliferative isthmus/neck zone (Fig. 4a and 4b). To confirm that CREB and C/EBPβ are expressed in the same cells, we performed double immunofluorescence in a tissue fragment of normal gastric mucosa. (Fig. 4g – 4j). In GC, we observed that CREB and C/EBPβ are expressed in the cell nucleus of 93% and 73% of the cases, respectively. The comparison of the IHC results, shows that there is a statistically significant association (P=0.04) between CREB and C/EBPβ expression in our series of GC. We also observed that GC cases with a higher CREB expression score were significantly associated with intestinal and mixed

histological subtypes (*P*=0.003) (Table 1). No associations were detected between the expression of CREB and other clinicopathological characteristics of the tumours.

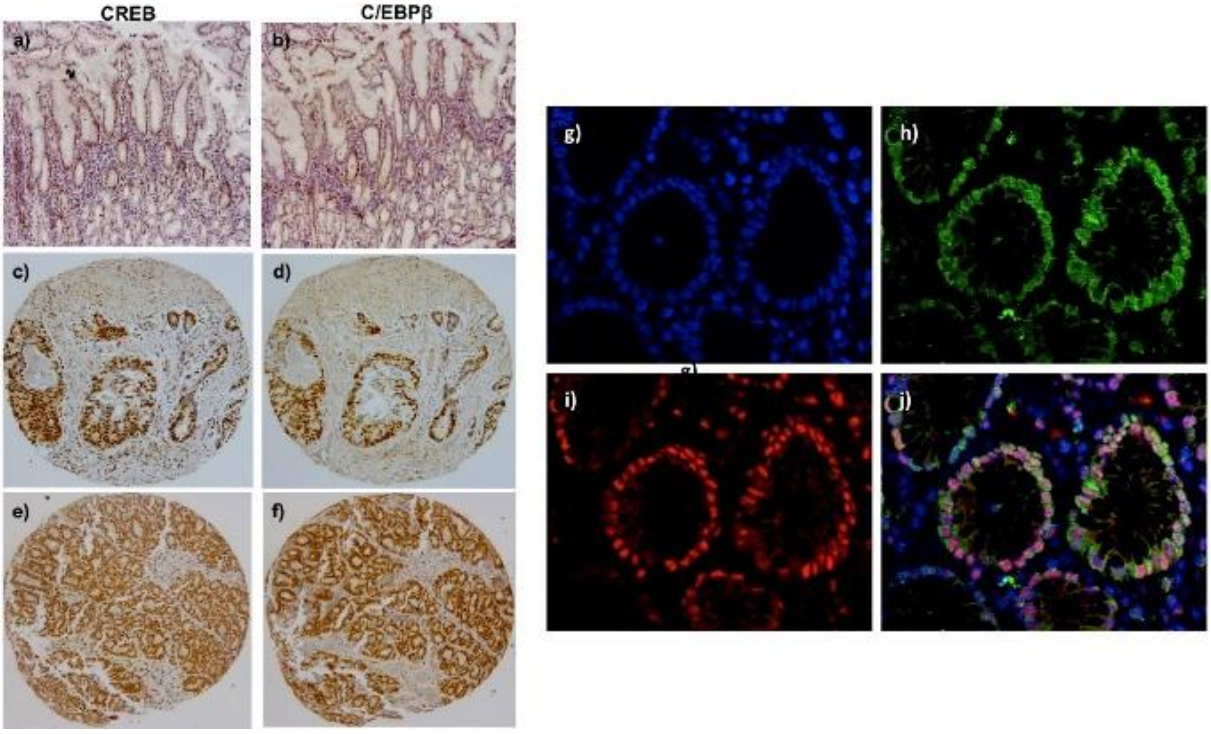


Figure 4. Immunohistochemical expression of CREB and C/EBPβ in normal gastric mucosa and in GC cases. a) CREB is expressed in epithelial cells located in the neck/isthmus region of normal gastric glands; b) C/EBPβ is also expressed in neck/isthmus normal epithelial cells; c) and e) examples of CREB-positive GC cases (scoring 3); d) and f) C/EBPβ is overexpressed in the same GC cases that are positive for CREB. Magnification 100x; g-j) double immunofluorescence staining for CREB and C/EBPβ in normal gastric mucosa, showing co-expression of the two proteins in gastric epithelial cells; g) DAPI nuclear staining; h) C/EBPβ staining; i) CREB staining; j) merged image for DAPI, C/EBPβ, and CREB staining. Magnification 400x.

		Nº of cases (%)	CREB score (%)				*p* value
			0	1	2	3	
C/EBPβ scoring							
	0	18 (27.3)	4 (22.2)	3 (16.7)	5 (27.8)	6 (33.3)	0.04
	1	23 (34.8)	0 (0)	4 (17.4)	9 (39.1)	10 (43.5)	
	2	12 (18.2)	0 (0)	1 (8.3)	4 (33.3)	7 (58.3)	
	3	13 (19.7)	0 (0)	0 (0)	3 (23.1)	10 (76.9)	
Histological type							
	Intestinal	25 (37.9)	1 (4.0)	2 (8.0)	7 (28)	15 (60.0)	0.003
	Diffuse	11 (16.7)	0 (0)	3 (27.3)	7 (63.6)	1 (9.1)	
	Mixed	20 (30.3)	0 (0)	3 (15.0)	4 (20.0)	13 (65.0)	
	Unclassified	10 (15.1)	3 (30.0)	0 (0)	3 (30.0)	4 (40.0)	
Venous invasion							
	Present	48 (72.7)	3 (6.2)	6 (12.5)	13 (27.1)	26 (54.2)	NS
	Absent	18 (27.3)	1 (5.6)	2 (11.1)	8 (44.4)	7 (38.9)	
Perineural invasion							
	Present	42 (63.6)	3 (7.1)	6 (14.3)	12 (28.6)	21 (50.0)	NS
	Absent	24 (36.4)	1 (4.2)	2 (8.3)	9 (37.5)	12 (50.0)	
Tumour extent							
	T1	2 (3.1)	0 (0)	0 (0)	2 (100.0)	0 (0)	NS
	T2	32 (48.5)	1 (3.1)	4 (12.5)	11 (34.4)	16 (50.0)	
	T3	23 (34.8)	2 (8.7)	3 (13.0)	4 (17.4)	14 (60.9)	
	T4	9 (13.6)	1 (11.1)	1 (11.1)	3 (33.3)	4 (44.4)	
Lymph node invasion							
	N0	8 (12.1)	0 (0)	0 (0)	5 (62.5)	3 (37.5)	NS
	N1	23 (34.8)	2 (8.7)	1 (4.3)	7 (30.4)	13 (56.5)	
	N2	21 (31.8)	2 (9.5)	4 (19.0)	5 (23.8)	10 (47.6)	
	N3	14 (21.2)	0 (0)	3 (21.4)	4 (28.6)	7 (50.0)	

Scoring: 0 – IHC positivity in <5% of tumour cells: 1 – IHC positivity in 6% to 50% of tumour cells; 2 – IHC positivity in 51% to 75% of tumour cells; 3 – IHC positivity in >75% of tumour cells.

Table 1. Relationship between the clinicopathological features of gastric cancer and CREB expression scoring.

CREB modulates IL1B-induced proliferation of GC cells

To determine whether IL1B is able to increase cell survival of GC cells we measured BrdU incorporation and performed TUNEL assays in the AGS GC cell line after incubation of cells with IL1B. In parallel, we determined whether any of the effects of IL1B is mediated by CREB. Our control experiments shows that CREB expression is downregulated by the shRNA used (Fig. 5a). Figure 5b shows that IL1B is able to significantly increase cellular proliferation and that this effect can be reverted by downregulating CREB levels. IL1B had no significant effect on the rate of apoptosis (data not shown).

To explore the role of CREB in GC cell proliferation we evaluated the expression of the cell-cycle regulator cyclin D1. After performing a dose-response experiment to determine the effect of the CBP-CREB interaction inhibitor over cell proliferation we selected a concentration of 25uM. In the AGS cell line, CREB inhibition had a significant inhibitory effect over cell proliferation (Fig. 5c). The effect was more pronounced after 48h of treatment. Moreover, this

effect could also be confirmed by measuring the expression level of cyclin D1 after CREB inhibition both at 24 and 48-h of treatment (Fig. 5f). These results were confirmed in the GP202 GC cell line (Figs. 5d and 5g) and in the intestinal-type GC cell line MKN28 (Figs. 5e and 5h). Overall, these results further support that CREB plays an important role in GC cell survival, both in diffuse and intestinal histological types, by regulating proliferation of GC cells.

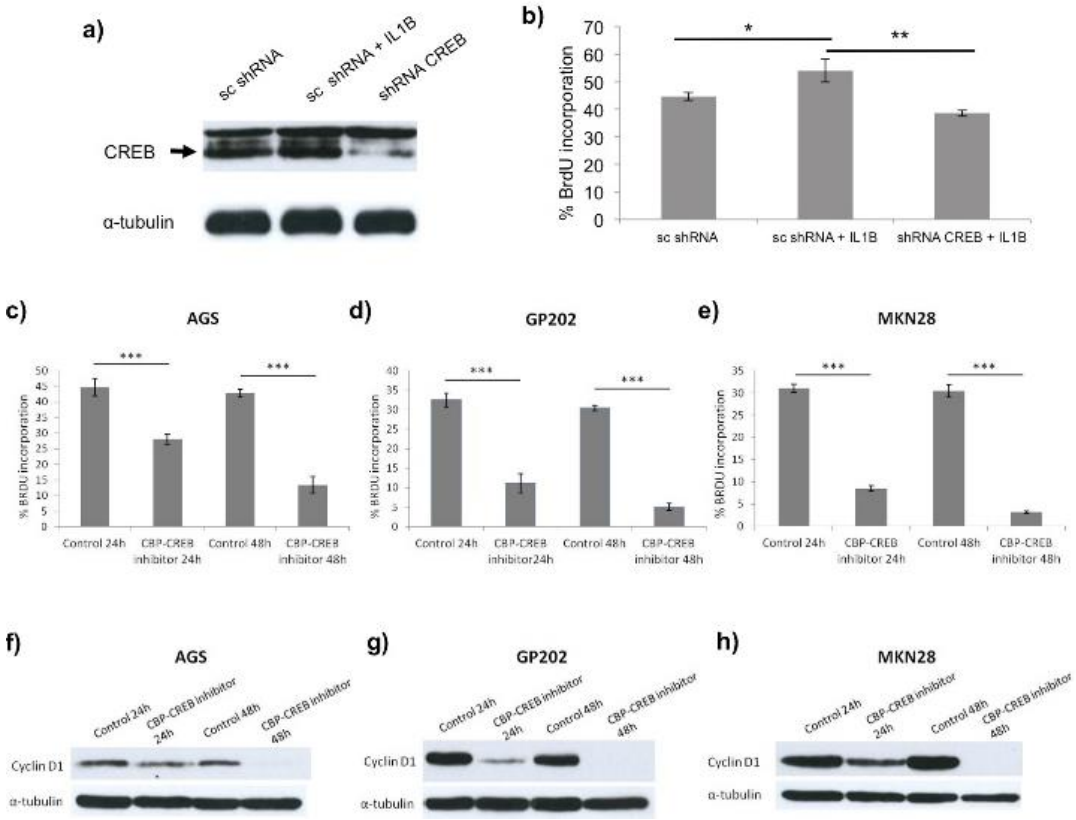


Figure 5. CREB modulates both IL1B-induced and basal cell proliferation. a) Downregulation of CREB expression using shRNA; b) AGS cells expressing normal levels of CREB (transfected with scrambled shRNA) showed an increase in cell proliferation after 24h of treatment with 10 ng/mL IL1B, while CREB downregulation (transfected with anti-CREB shRNA) was responsible for a significant decrease in IL1B-induced cell proliferation. sc shRNA (control scrambled shRNA); shRNA CREB (anti-CREB shRNA); c) AGS, d) GP202, and e) MKN28 cells were treated for 24 and 48h to assess the time-dependent impact of treatment over GC cell proliferation; the protein levels of cyclin D1 were checked in f) AGS, g) GP202, and h) MKN28 cells after 24 and 48h of CBP-CREB interaction inhibitor treatment. BrdU results represent the mean ± S.D. of three independent experiments. *($p<0.05$), **($p<0.01$) and ***($p<0.001$).

CREB inhibition decreases GC cell growth *in vivo*

To evaluate the effect of CREB on tumour growth, we used the chicken embryo chorioallantoic membrane (CAM) model. The CAM effectively supports the growth of inoculated human cancer cells due to the chick immunodeficiency during early developmental stages. Before CAM inoculation, we confirmed the knockdown of CREB protein on AGS cells transfected with anti-CREB shRNA (figure 6a). To avoid inter-animal differences in the results, scrambled shRNA and anti-CREB shRNA transfected AGS cells were inoculated in distinct areas of the CAM of the same egg and allowed to proliferate for 6 days. At this end point, the tumour area was quantified. As can be seen in figure 6b, inhibition of CREB led to reduced growth of the inoculated cells. Figure 6c shows that on average the tumour growth area was significantly smaller in cells with CREB inhibition. These results demonstrate that CREB-mediated signalling is important for GC cell growth in vivo.

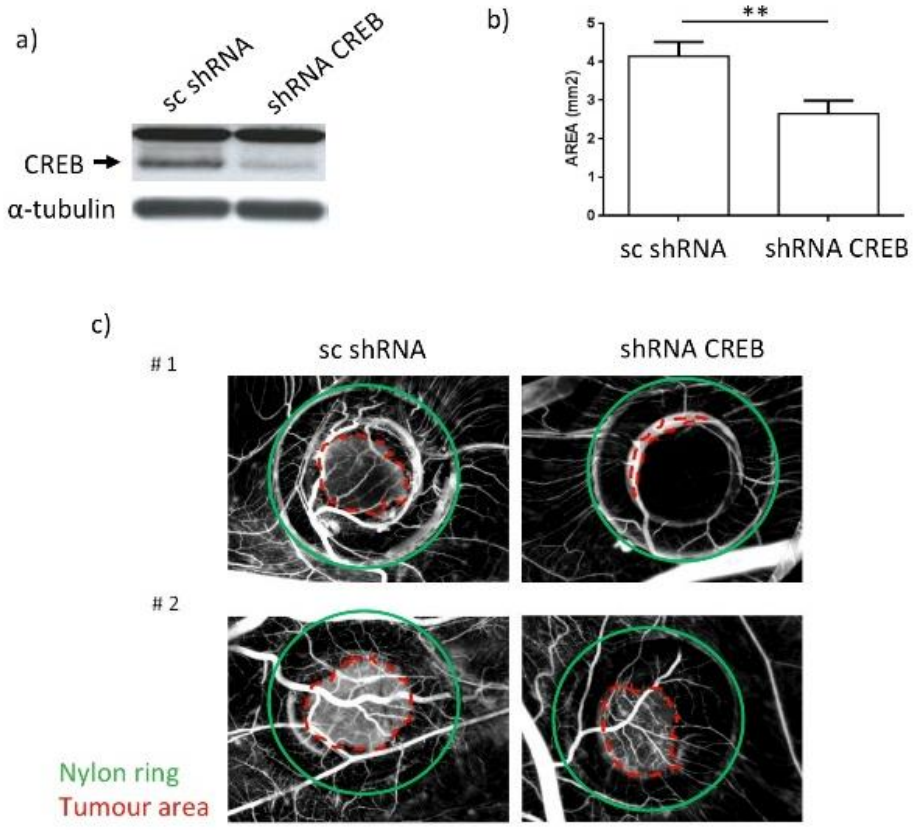


Figure 6. CREB inhibition reduces *in vivo* cell growth. a) Before inoculation of AGS cells in the chorioallantoic membrane (CAM), CREB protein knockdown was checked by western blotting; b) AGS cells transfected with shRNA against CREB give rise to small sized tumours compared with scramble transfected cells; c) images representing the different sizes (delimited by red dashed lines) of scramble and shRNA CREB tumours in two paired CAM experiments (#1 and #2). Values of $p<0.05$ were considered to be statistically significant.

DISCUSSION

Our results demonstrate that IL1B is able to activate CREB and C/EBPβ in GC cells. This process is mediated by ERK1/2 since its inhibition by U0126 reverted the effects induced by IL1B. This is in agreement with previously reported results showing that IL1B is able to induce GC cell proliferation in an ERK1/2 dependent manner [322]. We could also demonstrate that in GC cells CREB is able to transactivate C/EBPβ.

Our in vitro observations were reinforced by the analysis of the expression of CREB and C/EBPβ in a series of GC samples and normal gastric mucosa. We showed that in normal gastric mucosa CREB is expressed in the proliferative neck/isthmus region of the gastric glands, whereas in GC it is overexpressed in the majority of tumour samples. These results are in accordance with those published by Chen *et al* [331], showing that CREB mRNA levels are upregulated in GC samples when compared with adjacent normal mucosa.

CREB expression was also significantly associated with the expression of C/EBPβ in GC. The observation that in normal gastric mucosa CREB and C/EBPβ are expressed in a cellular compartment that includes progenitor cells, suggests that CREB and C/EBPβ may be involved in maintaining a proliferative phenotype in gastric epithelial cells. This would be in accordance with the observed pattern of overexpression of both proteins in the majority of the GC cases included in the present study. These results are also in keeping with our previous demonstration that C/EBPβ is overexpressed in preneoplastic lesions and in GC, suggesting that this protein might be relevant for transformation of gastric epithelial cells by inducing the expression of COX-2 [185] and by inhibiting the expression of the putative gastric tumor suppressor gene *TFF1* [263, 327]

In order to complement the aforementioned observations with a biological readout, we evaluated the role of CREB in mediating IL1B-induced changes in cell proliferation and apoptosis. Although no significant effect was observed in relation to apoptosis, our results show that CREB is an effector of IL1B-induced cell proliferation, since downregulation of CREB impairs the pro-mitogenic action of IL1B on GC cells *in vitro*. These results were further supported by the CAM assays showing that inhibition of CREB reduces the ability of GC cells to survive in this *in vivo* model.

Infection with *H. pylori* leads to chronic inflammation and increased risk of developing GC. Our results support the hypothesis that the effect of chronic inflammation on tumourigenesis includes modulation of critical signalling pathways that regulate survival in

epithelial cells. In this scenario, *H. pylori* infection leads to overexpression of IL1B which, in turn, activates CREB and C/EBPβ. This effect may be more pronounced in individuals that carry genetic susceptibility polymorphisms that have been demonstrated to be associated with enhanced chronic inflammation, such as those in the *IL1B* gene promoter. If one couples increased cell survival with increased likelihood to accumulate genetic mutations, this may help explain why individuals with pro-inflammatory genetic polymorphisms have an increased risk of developing GC.

DISCUSSION AND CONCLUSIONS

The onset and progression of GC, in analogy with other cancer models, must rely on the deregulation of critical signalling pathways and modulation of associated molecular effectors. However, the general lack of knowledge about those mechanisms remains a major gap in GC research. In that sense, the identification of the aberrant/deregulated mechanisms underlying gastric carcinogenesis could facilitate early diagnosis and help to inform about putative valuable therapeutic targets.

A consistent amount of evidences establishes chronic inflammation-associated with *H. pylori* infection with risk of GC. The association is even more significant in infected individuals that due to their genetic makeup, develop a more intense inflammatory response. So, chronic inflammation must have the ability to continually modulate crucial signalling pathways involved in processes such as cell proliferation and survival, In fact, inflammatory mediators deeply perturb the normal gastric homeostasis, inducing behavioural changes in gastric epithelial cells at the level of proliferation [322, 323, 332, 333], apoptosis [321, 334] and overall gene expression, caused by the differential modulation of cell signalling pathways [182, 325]. Ultimately, the deregulated signalling cascades will nurture the conditions to turn gastric epithelial cells more susceptible to genetic aberrations that will drive gastric carcinogenesis.

With this work we proposed to understand the biological roles of signalling events and associated molecular mechanisms underlying inflammation-driven GC development. We have previously reported the transcription factor C/EBPβ to be overexpressed in GC and also in early preneoplastic lesions of gastric epithelium, such as gastritis. Moreover, we found C/EBPβ to regulate the promoter activation of the inflammatory mediator COX2 [185]. Taken together, those results led us to hypothesize about a possible inflammation-driven role of C/EBPβ on gastric carcinogenesis. Additionally, and to understand the reason/s behind C/EBPβ deregulation, we wanted to determine the upstream signalling mechanisms and/or molecular events responsible for this phenomenon. In a first approach, *CEBPB* was the target of mutational screening conducted in a series of 35 GC cases to clarify if the up-regulation of C/EBPβ could have a genetic basis. Because only one mutation was found, although we did not explored the pathogenicity of the mutation, the reason behind C/EBPβ up-regulation must reside in aberrations in upstream regulatory mechanisms (unpublished data).

To understand the signalling mechanisms involved in C/EBPβ deregulation in GC, we first need to understand the mechanisms that control C/EBP expression in normal mucosa. In normal gastric mucosa, we observed that C/EBPβ positive cells also express the proliferation marker Ki67 [185]. That observation suggests C/EBPβ as playing a role in normal gastric

epithelial cell proliferation. Taking the advantage of the existence of a C/EBPβ null (C/EBPβ$^{-/-}$) mouse model [239] we observed, for the first time, the *in vivo* role of C/EBPβ on normal gastric epithelium proliferation. We observed that C/EBPβ$^{-/-}$ mice exhibited a reduction in the thickness of the epithelium and decreased amounts of Ki67 positive cells. Furthermore, gastric mucosa of C/EBPβ$^{-/-}$ mice constitutively showed decreased levels of cell-cycle inducers Cyclin A1, Cyclin D3, and Cyclin E1. These results give support to our first observations on human gastric tissue [185] and show that C/EBPβ is a necessary effector for normal gastric epithelial cell proliferation.

It is well known that RUNX3$^{-/-}$ mice develop gastric mucosa hyperplasia, although the mechanism underlying RUNX3-deficient phenotype remains unclear [90]. We found C/EBPβ strongly expressed in the hyper-proliferative gastric mucosa of RUNX3$^{-/-}$ mice. Furthermore, by crossing RUNX3$^{-/-}$ with C/EBPβ$^{-/-}$ mice and comparing with control mice, we found the expression of C/EBPβ to be a mandatory requisite for the hyperplastic phenotype of RUNX3 deficient mice. Besides being a gastric tumour suppressor [91], RUNX3 is also an important transcriptional effector of the TGFβ pathway [335], which exerts powerful anti-proliferative and anti-inflammatory actions on different cell types [128]. Thus, our results suggest an antagonistic relation between TGFβ signalling, mediated by RUNX3, and C/EBPβ in the modulation of proliferation of normal gastric epithelial cells. Giving support to our rationale, C/EBPβ was previously reported to be involved in the cytostatic activity of TGFβ [256]. To explore the possible duality between TGFβ and C/EBPβ, we performed a series of *in vitro* experiments in which we blocked TGFβ-dependent signalling with different chemical inhibitors (SIS3 – SMAD3 phosphorylation inhibitor; SB505124 – TGFBRI inhibitor). After 12 to 48 hours of chemical inhibition, we observed a significant increase in C/EBPβ protein levels (>3 fold increase), suggesting that TGFβ possibly exerts its anti-proliferative actions by controlling C/EBPβ protein levels (unpublished data).

C/EBPβ was previously reported to exert a repressive role on gastric differentiation genes, such as *TFF1* [121, 263, 327]. Validating those studies through an *in vivo* approach, we demonstrated that the gastric mucosa of C/EBPβ$^{-/-}$ mice express significantly higher levels of TFF1 protein. Additionally, we observed the levels of other two gastric differentiation markers (MUC5AC and MUC6) strongly up-regulated in the gastric mucosa of C/EBPβ$^{-/-}$ mice. It is possible that the repressive action of C/EBPβ upon gastric cell differentiation can be a direct consequence of its effect on cell growth, promoting the expression of proliferation markers, while inhibiting the expression of differentiation proteins. Future studies are needed to

understand whether the observed results are a direct or indirect action of C/EBPβ-dependent transcriptional activity.

Based on our previous results of C/EBPβ expression in preneoplastic lesions and GC [185] and in our *in vivo* results from C/EBPβ$^{-/-}$ mice, in which we revealed the proliferative role of this protein, we asked about its the true impact on GC cell biology. Through a series of *in vivo* tumorigenic assays with GC cell lines silenced for C/EBPβ we could observe that cells with C/EBPβ knockdown gave rise to smaller tumours when compared to control GC cells. Also, in addition to the impaired tumorigenic ability, GC cells with C/EBPβ knockdown also exhibited decreased proliferation and survival. Looking at our data, where C/EBPβ appears to be a crucial effector in normal and neoplastic cell proliferation, it seems logical to consider C/EBPβ as a gastric proliferative transcription factor that modulates biological processes that are transversal from normal to GC cell. Possibly these observations may have a more profound biological meaning, going deeper till the initial GC cell. It is generally accepted, at least conceptually, that the probability of occurrence of the necessary cancer-driver events is higher in a tissue progenitor cell – due to the increased lifespan – than in the derived differentiated cells [58]. Thus, one can hypothesize about a possible cellular origin of GC emerging from a C/EBPβ-positive gastric progenitor cell. However, this hypothesis was not yet addressed, deserving particular attention in future work.

The similarities of C/EBPβ biology between human and mouse gastric epithelial cells raised the question: to what extent the homeostatic and oncogenic C/EBPβ-dependent proliferation share common molecular mechanisms? After the comparison and validation of gene expression assays between C/EBPβ$^{-/-}$ mouse gastric mucosa and GC cases, we found RUNX1t1 to be up-regulated in C/EBPβ$^{-/-}$ gastric epithelium and downregulated in intestinal-type GC, revealing an inverse correlation with the expression of C/EBPβ. RUNX1t1 was previously reported as a potent inhibitor of adipogenesis by blocking C/EBPβ-dependent transcriptional activity through the direct physical interaction of the two proteins [313]. Moreover, RUNX1t1 expression was reported to play an essential role in the gastrointestinal system, regulating normal morphogenesis [315]. Thereby, our results suggest RUNX1t1 as a putative GC tumour suppressor, because its expression was lost in 38% of GC cases and it exerts an effective role in decreasing GC cell proliferation. Also, RUNX1t1 seems to play a tumour suppressor role in other cancer types, because it has been reported as an ovarian tumour suppressor [291], and its loss of expression was found to be necessary for liver metastization by pancreatic endocrine cancer cells [316]. An important aspect to be addressed in future work is to characterize the expression of RUNX1t1 in gastric preneoplastic lesions. By

doing this we expect to clarify if RUNX1t1 deregulation is a GC specific event or if it is an early GC development – as previously seen for C/EBPβ [185] – helping to reveal a possible reciprocal expression pattern and oncogenic mechanism between the two proteins.

From our first observations of C/EBPβ histological expression in normal gastric mucosa and its proliferative and anti-differentiation effects on gastric epithelial cells, we wonder about a possible factor that would play the opposite functions: stops cell proliferation and induces differentiation. The adipogenesis model seemed attractive to us, because preadipocytes highly express C/EBPβ during the first steps of differentiation, while in an immature proliferating state, diminishing the protein levels during the terminal phase of differentiation, a point in which C/EBPα starts to be highly expressed [235, 236]. So, we proposed to evaluate the expression pattern of C/EBPα on normal gastric mucosa and GC samples. Strikingly, we observed C/EBPα to be expressed in the differentiated epithelial compartment of the superficial foveolar region of the normal gastric mucosa, co-localizing with the differentiation marker TFF1. Moreover, we observed a downregulation or loss of C/EBPα expression in 30% of our cohort of GC cases. However, we could not observe an evident C/EBPα downregulation in preneoplastic lesions. These results suggested us that C/EBPα downregulation is a late-stage cancer-specific event in gastric carcinogenesis. Next, we wanted to disclose the biological impact of C/EBPα over GC cell biology. The *in vitro* modulation of C/EBPα expression allowed us to observe that this protein plays anti-proliferative (decreasing cyclin D1 levels, and increasing p27) and pro-differentiation (increasing the expression of TFF1) roles on GC cells. The results obtained by us with the GC model are similar with previous results obtained for different cancer cell models, suggesting C/EBPα as a tumour suppressor in different cellular contexts [247, 249, 251, 252]. Although we did not addressed how C/EBPα inhibits cell proliferation, it would be mechanistically relevant to determine if the inhibition occurs through induction of p21 expression [218], repression of E2F-dependent transcription [220], or interaction with SWI/SNF complex [221].

Curiously, in normal gastric mucosa, we observed a few C/EBPα positive cells located in the neck region of gastric glands – the glandular compartment where we had previously detected C/EBPβ-positive cells. We demonstrated by immunohistochemistry that TFF1, as initially expected, was not present in cells from the proliferative neck region. One possible explanation for this observation – sparse expression of C/EBPα and absence of TFF1 expression on neck region – can reside on a dominant inhibitor effect caused by the physical interaction between different proteins. It is known that C/EBP-family members can homo and heterodimerize [208, 209, 211], resulting in differential transactivation activities. Thus, C/EBPβ

can heterodimerize with C/EBPα in the epithelial cells of the neck zone, and exerting a dominant effect over C/EBPα can counteract the transcriptional potential of this last protein. However, this hypothesizes was not addressed in this work, deserving particular attention the next future.

An aspect that we have not explored in this work was the ascertainment of the underlying mechanism/s behind C/EBPα loss of expression in GC. From our previous work, we knew that, in opposition to leukemias [244, 245], genetic alterations are not the major mechanism for the observed C/EBPα downregulation, because we only found one mutated GC from a cohort of a hundred and forty-two GC cases [248]. A possible underlying mechanism, as observed in lung cancer, is promoter hypermethylation [250]. Alternatively, the loss of expression can reside in a transcriptional repressive effect of C/EBPβ (or other transcription repressor) over *CEBPA* promoter. So, further work is needed to decipher the causes behind C/EBPα loss of expression.

As mentioned above, in clear opposition to C/EBPα whose loss of expression appears to be a relatively late event in gastric carcinogenesis, C/EBPβ overexpression is observed even at very initial stages of the process, such as in gastritis. So, being chronic inflammation a risk factor for GC development and because we observed C/EBPβ deregulation in inflamed gastric mucosa, we asked if inflammatory mediators can regulate C/EBPβ expression. Strengthening our rationale, the results we obtained *in vitro* with TGFβ inhibitors and *in vivo* with RUNX3$^{-/-}$ mice strongly suggested that association. One of the most relevant pro-inflammatory cytokines in the context of *H. pylori* is IL1B, which is highly up-regulated in response to infection and contributes to the development of hypochlorhydria, gastric atrophy and other pre-cancerous lesions [36, 38]. However, the signalling mechanisms involved in IL1B signalling in GC are still very obscure. With this in our minds, we proposed to dissect the IL1B-induced signalling events in GC cells. From published works, we knew two critical points: first, transcription factors that are involved in progenitor cell homeostasis are target of inflammation-induced modulation [106]; second, in non-gastric cell models IL1B was able to induce the expression/activation of C/EBPβ [326]. So, after treating different GC cell lines with IL1B we observed an up-regulation in C/EBPβ protein levels. Next, and based on our results that indicated C/EBPβ has a gastric pro-mitogenic effector, we assessed if MAPK signalling would be involved in IL1B-dependent increase in C/EBPβ expression. Through chemical inhibition, we established a functional link between IL1B and C/EBPβ through an ERK1/2-dependent mechanism. Noteworthy, MAPK signalling pathway is one of the most consistently altered and biological relevant pathways in human cancers [83]. Also, activation of the MAPK pathway was found to be increased in *H.*

pylori-induced chronic inflammation [122] and in GC [123-125].Thus, we wonder if MAPK signalling could be a possible mechanism behind the differential pattern of expression of C/EBPα and C/EBPβ observed in normal gastric mucosa and in GC. After *in vitro* chemical inhibition of MAPK signalling we were able to observe a significant protein increase and nuclear accumulation of C/EBPα, suggesting that MAPK activation exerts a strong inhibitory effect over C/EBPα expression. Strikingly, this observation clearly contrasts with what we had observed for the role of MAPK signalling in the control of C/EBPβ expression, and reinforce the conception that the two C/EBP family members are inversely regulated and have antagonistic roles in the gastric carcinogenesis model.

Nevertheless, we faced with a crucial question: mechanistically, how does MAPK signalling induced the expression of C/EBPβ? Because ERK1/2 transduce signals through the phosphorylation of specific downstream proteins, we hypothesized that possibly ERK1/2 induced the expression of C/EBPβ not directly, but in an indirect way through the activation a transcription factor involved in the regulation of *CEBPB*. Based on a few studies: one in hepatocytes [174] and the other two in preadipocytes [175, 176]; and after a deep analysis of *CEBPB* promoter, we identified CREB as a putative transcriptional regulator of *CEBPB*. Strengthening our rationale, we observed that CREB and phosphorylated-CREB (pCREB) were also increased after IL1B stimulation through an ERK1/2-dependent action. Through a series of ChIP and gene silencing experiments we confirmed CREB as a direct transactivator of *CEBPB* in GC cells. Therefore, our results put CREB in an upstream signalling position in relation to C/EBPβ, suggesting that it may be involved not only in the transcription of *CEBPB* but also in control of a broader range of biological processes. In fact, that assumption was confirmed because we found CREB expression to be a mandatory requisite for both basal and IL1B-induced gastric cell proliferation. In addition to the crucial role in cell proliferation, we also found CREB as a pro-survival effector in GC cell lines (unpublished results).

CREB has been recently described as a real culprit in cancer, whose deregulated protein expression is frequently observed in different tumour types [192-195, 200, 201, 204]. So, based on the results of our *in vitro* experiments we asked about the histological expression pattern of CREB in human gastric primary tumours. In normal gastric mucosa, CREB was expressed in a region of the gastric gland within which is located the neck/isthmus – in analogy with C/EBPβ. However, when we observed the GC samples we found CREB overexpressed in the vast majority of the cohort studied (94%), particularly in intestinal and atypical cases. Moreover, the pattern of expression that we observed for CREB in GC was significantly associated with C/EBPβ expression. To evaluate if CREB acts as a pro-oncogenic effector, we

performed a series of *in vivo* tumourigenic experiments using the chicken CAM assay as an animal model. With this assay, we observed that GC cells with CREB knockdown gave rise to significantly smaller tumours when compared with control GC cells, indicating undoubtedly that CREB is a gastric pro-oncogenic factor.

Curiously, we observed more GC cases positive for CREB than for C/EBPβ. This observation suggests the action of a possible *CEBPB* transcriptional repressor in the cases negative for C/EBPβ, whose repressional action must be stronger than the CREB activity. One possible repressor of *CEBPB* transactivation could be the CREB-family inhibitor ICER, because it recognizes the same CRE-binding sites and has the ability to bind them with greater affinity than the other CREB family members [156]. Nevertheless, we did not assessed the expression of ICER in our GC cases. However, it is possible that, in accordance with other cancer models [195, 336, 337], ICER expression would be lost in a percentage of GC cases, with underlying biological implications This is a relevant point, with important functional impact that needs to be addressed in future work.

Our results revealed a mechanism through which CREB regulates C/EBPβ expression not only in the scenarios of inflammation and GC but also in normal gastric epithelium. As aforementioned, C/EBPβ was found to transactivate the expression of the powerful inflammatory mediator COX2, at least at the promoter level [185]. Additionally, CREB was also reported to be necessary for the expression of COX2 in intestinal epithelial cells [338]. Thus, it seemed logical that in a GC cell context and in combination with C/EBPβ, CREB could also be involved in COX2 expression. Our rationale was strengthened because in osteogenic cells the expression of the proto-oncogene *FOS* is modulated by a CREB–C/EBPβ dimer [339]. Thus, it was highly tempting for us to hypothesize about a possible CREB–C/EBPβ dimer that would underlie the expression of COX2 – and possible other important genes in GC biology. Pursuing this idea, we performed a series of co-immunoprecitation experiments in GC cells with the objective to detect CREB–C/EBPβ interactions in a GC cell context. However, we were unable to detect any physical interaction between the two proteins. These result suggested us that in a GC cell context, and in unstimulated conditions, CREB and C/EBPβ do not stably interact with each other. However, if they really interact, the process may be too short-lived to be detected and possibly may lack any biological implication (unpublished data). Nevertheless, and due to the possible relevant biological implications, this subject needs to be further explored in future work.

It has been reported that CREB acts as the final effector of various signalling pathways [144, 145], and our results indicate CREB as a critical effector of IL1B signalling in GC cells.

Further, according to Mantovani *et al.* (2008) transcription factors are at the crossing point between inflammatory and oncogenic signalling pathways [104], and CREB is possibly one of those transcription factors, which is involved in cell proliferation and survival in both extrinsic pathways. Regarding oncogenic activation, in HER2-positive breast tumours CREB was reported as a major cellular effector, whose expression is necessary for cellular proliferation, survival, and tumour formation ability [203]. Noteworthy, HER2 expression is also found deregulated in up to 20% of intestinal type GC [39, 69] and although we did not explored this subject, it is possible that GC cases that are HER2-positive could depend on the activity of CREB to confer them a growth and survival advantage. Although at low frequencies, alterations in other molecular targets have been reported in GC [39, 53, 69]. One example is *PTEN,* whose expression is lost in approximately 20% of GC cases [87]. It is possible that the 20% of GC cases in which PTEN expression is lost are the ones in which we observed a stronger immunoreactivity for C/EBPβ. The rationale for this possibility resides in the fact that PTEN, when translocated to the cell nucleus, acts as a potent inhibitor of CREB-dependent transcriptional activity [340]. So, in GC cases with loss of PTEN it should be possible to detect increased expression levels of C/EBPβ – because we found it to be a CREB transcriptional target in GC cells. Regarding target genes, CREB was reported to be involved in the expression of a plethora of genes in a cell-specific manner [171-173]. One cell-specific target gene is quite interesting: CREB itself – as reported in Sertoli cells where CREB was observed to directly transactivate its own gene [341]. Knowing this, we looked for a positive-feedback mechanism that, once activated by an oncogenic event could justify the observed overexpression of CREB in gastric primary tumours. In fact, after performing ChIP in a GC cell line, we detected the physically interaction of CREB with its own promoter (unpublished data). The underlying mechanisms behind this auto-regulatory mechanism can be of major biological relevance and will be the focus of research in future work.

Looking forward, and due to the limited number and frequency of molecular aberrations observed in GC that could potentially explain the functional basis by which CREB crosses the boundaries of the proliferative neck-zone in normal gastric mucosa and become overexpressed in the majority of GC cases, in future work we expect to apply high-throughput sequencing technology in a panel of GC cases to identify molecular alterations that could give a solid genetic support to our results.

A broad number of studies suggested that only a restricted number of transcription factors are overexpressed and/or overactive in most human cancers, making them tempting targets for the development of anticancer drugs. This rationale is even more attractive by

knowing that are more oncogenic signalling mediators upstream of those transcription factors than there are oncogenic transcription factors. Therefore, being transcription factors the final executors of malignant gene expression signatures, they occupy a central role in all classic hallmarks of carcinogenesis [139, 265, 266]. Thus, one effective anti-transcription factor drug could be able to fight and inhibit the action of various upstream-activated oncogenic signalling pathways [267]. CREB has been pointed as a central target for cancer therapy. In fact, a battery of small-molecule inhibitors of CREB activity was recently described [272, 273]. These small-molecule inhibitors, designated KIX-KID interaction inhibitors, abrogate CREB-dependent transcription by inhibiting the interaction between CREB and the transcriptional apparatus. Noteworthy, one of the referred inhibitors exhibited a strong effect in downregulating the proliferative and survival potential of the cancer cells but showed no impact on normal cells [273]. With this on our minds, we used this same small-molecule inhibitor in GC cell lines and we observed a significant decrease in cell proliferation and cell survival. The protein levels of cell-cycle inducer Cyclin D1 were also drastically decreased. Nevertheless, the use of small-molecule inhibitors of CREB activity was only reported in *in vitro* experiments, with no animal or human trials described to date. In future, and maybe after additional chemical refinements, some of these chemical inhibitors could open new promising therapeutic options in cancer treatment.

In conclusion, in this thesis we show that in normal gastric mucosa, C/EBPα is expressed in the differentiated foveolar gastric epithelium where it co-localizes with gastric differentiation marker TFF1. In primary gastric tumours, we observed a downregulation or loss of C/EBPα expression in 30% of the cases analysed. Further, through expression modulation we found C/EBPα to act as a potent anti-proliferative effector on GC cells. Moreover, C/EBPα expression was found to be negatively regulated by p38 and ERK1/2 signalling, two critical MAPK branches previously reported as activated in gastric inflammation and GC. On the other hand, we show that C/EBPβ is positively associated with cell proliferation and survival of both normal gastric epithelium and GC cell lines. Also, C/EBPβ expression was found to be necessary for the full tumorigenic ability of GC cells. Moreover, we observed that the transcriptional activity of C/EBPβ was inhibited through the physical interaction with RUNX1t1, whose expression was lost in 38% of primary gastric tumours. These results suggest that the biological function of RUNX1t1 is mechanistically connected to the suppression of the pro-oncogenic functions of C/EBPβ.

Exploring the association between inflammatory mediators and C/EBPβ expression, we demonstrate that IL1B is able to induce the expression of C/EBPβ through the activation of MAPK signalling cascade. Remarkably, we observed a differentially modulatory role of MAPK signalling over C/EBPα and C/EBPβ expressions, strengthening the conception that the two C/EBP family members are inversely regulated and have antagonistic roles in the gastric carcinogenesis model. Based on other cell models, we observed MAPK-activated CREB transcriptional activity as a mechanism underlying C/EBPβ expression in GC cells lines. We also demonstrate that in normal gastric mucosa C/EBPβ and CREB are expressed in a compartmentalized glandular region within which gastric progenitor cells are located, while in GC samples both proteins are associated and overexpressed in the majority of cases studied. Moreover, we demonstrate that CREB acts as a crucial effector in both basal and IL1B-induced GC cell proliferation and survival, and in *in vivo* tumorigenic ability of GC cells.

Finally, our results provide further support to the hypothesis that the effect of chronic inflammation on gastric carcinogenesis, as seen in the context of genetically susceptible individuals infected with *H*. *pylori*, includes modulation of signalling pathways that regulate critical biological mechanisms in gastric epithelial cells. Furthermore, we reinforce this view by identifying the MAPK-CREB-C/EBPβ signalling mechanism linking inflammation and GC. Our results may help inform new strategies for prevention and treatment of GC, including the control of chronic inflammation and the identification of new therapy targets.

REFERENCES

1. Crew, K.D. and A.I. Neugut, *Epidemiology of gastric cancer.* World J Gastroenterol, 2006. **12**(3): p. 354-62.
2. Parkin, D.M., P. Pisani, and J. Ferlay, *Estimates of the worldwide incidence of eighteen major cancers in 1985.* Int J Cancer, 1993. **54**(4): p. 594-606.
3. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.
4. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
5. Chandanos, E. and J. Lagergren, *Oestrogen and the enigmatic male predominance of gastric cancer.* Eur J Cancer, 2008. **44**(16): p. 2397-403.
6. Milne, A.N., et al., *Nature meets nurture: molecular genetics of gastric cancer.* Hum Genet, 2009. **126**(5): p. 615-28.
7. Parkin, D.M., *International variation.* Oncogene, 2004. **23**(38): p. 6329-40.
8. Cancer, I.-I.A.f.R.o., *Tumours of the Digestive System*. 2000, Lyon: IARC Press.
9. Faivre, J., et al., *Survival of patients with oesophageal and gastric cancers in Europe. EUROCARE Working Group.* Eur J Cancer, 1998. **34**(14 Spec No): p. 2167-75.
10. Lunet, N., F. Pina, and H. Barros, *Regional trends in Portuguese gastric cancer mortality (1984-1999).* Eur J Cancer Prev, 2004. **13**(4): p. 271-5.
11. 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**: p. 31-49.
12. Smith, M.G., et al., *Cellular and molecular aspects of gastric cancer.* World J Gastroenterol, 2006. **12**(19): p. 2979-2990.
13. Carneiro, F., et al., *Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening.* J Pathol, 2004. **203**(2): p. 681-7.
14. Correa, P., *Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention.* Cancer Res, 1992. **52**(24): p. 6735-40.
15. McMichael, A.J., et al., *Patterns of gastro-intestinal cancer in European migrants to Australia: the role of dietary change.* Int J Cancer, 1980. **25**(4): p. 431-7.
16. Jenab, M., et al., *Plasma and dietary vitamin C levels and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST).* Carcinogenesis, 2006. **27**(11): p. 2250-2257.

17. Kono, S. and T. Hirohata, *Nutrition and stomach cancer.* Cancer Causes Control, 1996. **7**(1): p. 41-55.
18. Joossens, J.V., et al., *Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group.* Int J Epidemiol, 1996. **25**(3): p. 494-504.
19. Tatematsu, M., et al., *Effects in rats of sodium chloride on experimental gastric cancers induced by N-methyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline-1-oxide.* J Natl Cancer Inst, 1975. **55**(1): p. 101-6.
20. Takahashi, M. and R. Hasegawa, *Enhancing effects of dietary salt on both initiation and promotion stages of rat gastric carcinogenesis.* Princess Takamatsu Symposia, 1985. **16**: p. 169-182.
21. Trédaniel, J., et al., *Tobacco smoking and gastric cancer: Review and meta-analysis.* International Journal of Cancer, 1997. **72**(4): p. 565-573.
22. González, C.A., et al., *Smoking and the risk of gastric cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC).* International Journal of Cancer, 2003. **107**(4): p. 629-634.
23. Koizumi, Y., et al., *Cigarette smoking and the risk of gastric cancer: a pooled analysis of two prospective studies in Japan.* Int J Cancer, 2004. **112**(6): p. 1049-55.
24. Marshall, B.J. and J.R. Warren, *Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration.* Lancet, 1984. **1**(8390): p. 1311-5.
25. Go, M.F., *Review article: natural history and epidemiology of Helicobacter pylori infection.* Aliment Pharmacol Ther, 2002. **16 Suppl 1**: p. 3-15.
26. Parkin, D.M., *The global health burden of infection-associated cancers in the year 2002.* International Journal of Cancer, 2006. **118**(12): p. 3030-3044.
27. Siurala, M., K. Varis, and M. Wiljasalo, *Studies of patients with atrophic gastritis: a 10-15-year follow-up.* Scand J Gastroenterol, 1966. **1**(1): p. 40-8.
28. Parsonnet, J., et al., *Helicobacter pylori infection and the risk of gastric carcinoma.* N Engl J Med, 1991. **325**(16): p. 1127-31.
29. Uemura, N., et al., *Helicobacter pylori infection and the development of gastric cancer.* N Engl J Med, 2001. **345**(11): p. 784-9.
30. Cancer, I.-I.A.f.R.o., *Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, Liver Flukes, and Helicobacter pylori*. 1994, Lyon: IARC Press.
31. Amieva, M.R. and E.M. El-Omar, *Host-bacterial interactions in Helicobacter pylori infection.* Gastroenterology, 2008. **134**(1): p. 306-23.

32. Figueiredo, C., et al., *Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma.* J Natl Cancer Inst, 2002. **94**(22): p. 1680-7.
33. Sakagami, T., et al., *Atrophic gastric changes in both Helicobacter felis and Helicobacter pylori infected mice are host dependent and separate from antral gastritis.* Gut, 1996. **39**(5): p. 639-648.
34. Wang, T.C., et al., *Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with Helicobacter felis infection.* Gastroenterology, 1998. **114**(4): p. 675-689.
35. D'Elios, M.M., et al., *T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease.* J Immunol, 1997. **158**(2): p. 962-7.
36. Basso, D., et al., *Helicobacter pylori infection enhances mucosal interleukin-1 beta, interleukin-6, and the soluble receptor of interleukin-2.* Int J Clin Lab Res, 1996. **26**(3): p. 207-10.
37. Calam, J., *Helicobacter pylori modulation of gastric acid.* Yale J Biol Med, 1999. **72**(2-3): p. 195-202.
38. Noach, L.A., et al., *Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with Helicobacter pylori infection.* Scand J Gastroenterol, 1994. **29**(5): p. 425-9.
39. Resende, C., A. Ristimaki, and J.C. Machado, *Genetic and epigenetic alteration in gastric carcinogenesis.* Helicobacter, 2010. **15 Suppl 1**: p. 34-9.
40. El-Omar, E.M., et al., *Interleukin-1 polymorphisms associated with increased risk of gastric cancer.* Nature, 2000. **404**(6776): p. 398-402.
41. Hurme, M., N. Lahdenpohja, and S. Santtila, *Gene polymorphisms of interleukins 1 and 10 in infectious and autoimmune diseases.* Ann Med, 1998. **30**(5): p. 469-73.
42. Pociot, F., et al., *A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro.* Eur J Clin Invest, 1992. **22**(6): p. 396-402.
43. Santtila, Savinainen, and Hurme, *Presence of the IL-1RA Allele 2 (IL1RN*2) is Associated with Enhanced IL-1β Production In Vitro.* Scandinavian Journal of Immunology, 1998. **47**(3): p. 195-198.
44. Hwang, I.-R., et al., *Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1β production in Helicobacter pylori infection.* Gastroenterology, 2002. **123**(6): p. 1793-1803.

45. Furuta, T., et al., *Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan.* Gastroenterology, 2002. **123**(1): p. 92-105.
46. Garza-Gonzalez, E., et al., *Role of the polymorphic IL-1B, IL-1RN and TNF-A genes in distal gastric cancer in Mexico.* Int J Cancer, 2005. **114**(2): p. 237-41.
47. Machado, J.C., et al., *Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma.* Gastroenterology, 2001. **121**(4): p. 823-829.
48. El-Omar, E.M., et al., *Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms.* Gastroenterology, 2003. **124**(5): p. 1193-201.
49. Beales, I.L. and J. Calam, *Interleukin 1 beta and tumour necrosis factor alpha inhibit acid secretion in cultured rabbit parietal cells by multiple pathways.* Gut, 1998. **42**(2): p. 227-34.
50. Machado, J.C., et al., *A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma.* Gastroenterology, 2003. **125**(2): p. 364-71.
51. Zhuang, W., et al., *Interleukin10 -592 Promoter Polymorphism Associated with Gastric Cancer Among Asians: A Meta-Analysis of Epidemiologic Studies.* Digestive Diseases and Sciences, 2010. **55**(6): p. 1525-1532.
52. Won, H.H., et al., *Interleukin 10 polymorphisms differentially influence the risk of gastric cancer in East Asians and Caucasians.* Cytokine, 2010. **51**(1): p. 73-7.
53. Resende, C., et al., *Gastric cancer: basic aspects.* Helicobacter, 2011. **16 Suppl 1**: p. 38-44.
54. Li, Z.W., et al., *Inflammatory cytokine gene polymorphisms increase the risk of atrophic gastritis and intestinal metaplasia.* World J Gastroenterol, 2010. **16**(14): p. 1788-94.
55. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.
56. Tysnes, B.B., *Tumor-initiating and -propagating cells: cells that we would like to identify and control.* Neoplasia, 2010. **12**(7): p. 506-515.
57. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome.* Nature, 2009. **458**(7239): p. 719-24.
58. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-11.
59. Hoffmann, W., *Regeneration of the Gastric Mucosa and its Glands from Stem Cells.* Current Medicinal Chemistry, 2008. **15**(29): p. 3133-3144.

60. Mills, J.C. and R.A. Shivdasani, *Gastric epithelial stem cells.* Gastroenterology, 2011. **140**(2): p. 412-24.
61. Suzuki, S., S. Tsuyama, and F. Murata, *Cells intermediate between mucous neck cells and chief cells in rat stomach.* Cell and Tissue Research, 1983. **233**(3): p. 475-484.
62. Thompson, M., et al., *Gastric endocrine cells share a clonal origin with other gut cell lineages.* Development, 1990. **110**(2): p. 477-81.
63. Leblond, C.P., C.E. Stevens, and R. Bogoroch, *Histological Localization of Newly-formed Desoxyribonucleic Acid.* Science, 1948. **108**(2811): p. 531-3.
64. Cheng, H. and C.P. Leblond, *Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types.* Am J Anat, 1974. **141**(4): p. 537-61.
65. Bjerknes, M. and H. Cheng, *Multipotential stem cells in adult mouse gastric epithelium.* Am J Physiol Gastrointest Liver Physiol, 2002. **283**(3): p. G767-77.
66. Starodub, O.T., et al., *Disruption of the Cox-1 gene slows repair of microscopic lesions in the mouse gastric epithelium.* Am J Physiol Cell Physiol, 2008. **294**(1): p. C223-32.
67. Faller, G. and T. Kirchner, *Immunological and morphogenic basis of gastric mucosa atrophy and metaplasia.* Virchows Arch, 2005. **446**(1): p. 1-9.
68. Cai, X., et al., *Helicobacter felis eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice.* Gastroenterology, 2005. **128**(7): p. 1937-52.
69. Grabsch, H.I. and P. Tan, *Gastric Cancer Pathology and Underlying Molecular Mechanisms.* Digestive Surgery, 2013. **30**(2): p. 150-158.
70. Oliveira, C., R. Seruca, and F. Carneiro, *Genetics, Pathology, and Clinics of Familial Gastric Cancer.* International Journal of Surgical Pathology, 2006. **14**(1): p. 21-33.
71. Machado, J.C., et al., *E-cadherin gene mutations provide a genetic basis for the phenotypic divergence of mixed gastric carcinomas.* European Journal of Cancer Prevention, 1999. **8**(4): p. 351.
72. Pinto, M., et al., *Somatic mutations in mismatch repair genes in sporadic gastric carcinomas are not a cause but a consequence of the mutator phenotype.* Cancer Genetics and Cytogenetics, 2008. **180**(2): p. 110-114.
73. Oliveira, C., et al., *The Clinicopathological Features of Gastric Carcinomas with Microsatellite Instability May Be Mediated by Mutations of Different "Target Genes": A Study of the TGFβ RII, IGFII R, and BAX Genes.* The American Journal of Pathology, 1998. **153**(4): p. 1211-1219.

74. Vauhkonen, M., et al., *Differences in genomic instability between intestinal- and diffuse-type gastric cancer.* Gastric Cancer, 2005. **8**(4): p. 238-244.
75. Machado, J.C., et al., *Pattern of pS2 protein expression in premalignant and malignant lesions of gastric mucosa.* Eur J Cancer Prev, 1996. **5**(3): p. 169-79.
76. Carvalho, R., et al., *Loss of Heterozygosity and Promoter Methylation, but not Mutation, May Underlie Loss of TFF1 in Gastric Carcinoma.* Lab Invest, 2002. **82**(10): p. 1319-1326.
77. Lefebvre, O., et al., *Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein.* Science, 1996. **274**(5285): p. 259-62.
78. Kuniyasu, H., et al., *Frequent amplification of the c-met gene in scirrhous type stomach cancer.* Biochemical and Biophysical Research Communications, 1992. **189**(1): p. 227-232.
79. Gravalos, C. and A. Jimeno, *HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target.* Annals of Oncology, 2008. **19**(9): p. 1523-1529.
80. Levine, A.J., J. Momand, and C.A. Finlay, *The p53 tumour suppressor gene.* Nature, 1991. **351**(6326): p. 453-456.
81. Tohdo, H., et al., *p53 gene mutations in gastric adenomas.* Virchows Archiv B, 1993. **63**(1): p. 191-195.
82. Ochiai, A., Y. Yamauchi, and S. Hirohashi, *p53 mutations in the non-neoplastic mucosa of the human stomach showing intestinal metaplasia.* International Journal of Cancer, 1996. **69**(1): p. 28-33.
83. Lee, K.-H., et al., *Clinicopathologic significance of the K-ras gene codon 12 point mutation in stomach cancer. An analysis of 140 cases.* Cancer, 1995. **75**(12): p. 2794-2801.
84. Brennetot, C., et al., *Frequent Ki-ras mutations in gastric tumors of the MSI phenotype.* Gastroenterology, 2003. **125**(4): p. 1282.
85. Corso, G., et al., *Oncogenic mutations in gastric cancer with microsatellite instability.* European Journal of Cancer, 2011. **47**(3): p. 443-451.
86. Myers, M.P., et al., *P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase.* Proceedings of the National Academy of Sciences, 1997. **94**(17): p. 9052-9057.
87. Kang, Y.-H., H.S. Lee, and W.H. Kim, *Promoter Methylation and Silencing of PTEN in Gastric Carcinoma.* Lab Invest, 2002. **82**(3): p. 285-291.

88. Oki, E., et al., *Genetic mutual relationship between PTEN and p53 in gastric cancer.* Cancer Letters, 2005. **227**(1): p. 33-38.
89. Ito, K., et al., *RUNX3, A Novel Tumor Suppressor, Is Frequently Inactivated in Gastric Cancer by Protein Mislocalization.* Cancer Research, 2005. **65**(17): p. 7743-7750.
90. Li, Q.-L., et al., *Causal Relationship between the Loss of RUNX3 Expression and Gastric Cancer.* Cell, 2002. **109**(1): p. 113-124.
91. Ito, K., et al., *Loss of Runx3 Is a Key Event in Inducing Precancerous State of the Stomach.* Gastroenterology, 2011. **140**(5): p. 1536-1546.e8.
92. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. **357**(9255): p. 539-45.
93. Pages, F., et al., *Immune infiltration in human tumors: a prognostic factor that should not be ignored.* Oncogene, 2009. **29**(8): p. 1093-1102.
94. Chiba, T., H. Marusawa, and T. Ushijima, *Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation.* Gastroenterology, 2012. **143**(3): p. 550-563.
95. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-85.
96. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
97. Wang, D. and R.N. Dubois, *Eicosanoids and cancer.* Nat Rev Cancer, 2010. **10**(3): p. 181-93.
98. Ziech, D., et al., *Reactive oxygen species (ROS)--induced genetic and epigenetic alterations in human carcinogenesis.* Mutat Res, 2011. **711**(1-2): p. 167-73.
99. Kraus, S. and N. Arber, *Inflammation and colorectal cancer.* Curr Opin Pharmacol, 2009. **9**(4): p. 405-10.
100. Barrett, M.T., et al., *Evolution of neoplastic cell lineages in Barrett oesophagus.* Nat Genet, 1999. **22**(1): p. 106-9.
101. Kou, T., et al., *Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis.* Int J Cancer, 2007. **120**(3): p. 469-76.
102. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis, 2009. **30**(7): p. 1073-81.
103. Brentnall, T.A., et al., *Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis.* Cancer Res, 1996. **56**(6): p. 1237-40.
104. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-44.

105. Okumura, T., et al., *K-ras mutation targeted to gastric tissue progenitor cells results in chronic inflammation, an altered microenvironment, and progression to intraepithelial neoplasia.* Cancer Res, 2010. **70**(21): p. 8435-45.
106. Chen, X., et al., *Integration of external signaling pathways with the core transcriptional network in embryonic stem cells.* Cell, 2008. **133**(6): p. 1106-17.
107. Darnell, J.E., *Validating Stat3 in cancer therapy.* Nat Med, 2005. **11**(6): p. 595-6.
108. Apte, R.N., et al., *The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions.* Cancer Metastasis Rev, 2006. **25**(3): p. 387-408.
109. Apte, R.N., et al., *Opposing effects of IL-1 alpha and IL-1 beta on malignancy patterns. Tumor cell-associated IL-1 alpha potentiates anti-tumor immune responses and tumor regression, whereas IL-1 beta potentiates invasiveness.* Adv Exp Med Biol, 2000. **479**: p. 277-88.
110. Horai, R., et al., *Production of Mice Deficient in Genes for Interleukin (IL)-1α, IL-1β, IL-1α/β, and IL-1 Receptor Antagonist Shows that IL-1β Is Crucial in Turpentine-induced Fever Development and Glucocorticoid Secretion.* J Exp Med, 1998. **187**(9): p. 1463-1475.
111. Voronov, E., et al., *IL-1 is required for tumor invasiveness and angiogenesis.* Proceedings of the National Academy of Sciences, 2003. **100**(5): p. 2645-2650.
112. Jin, L., et al., *Expression of interleukin-1beta in human breast carcinoma.* Cancer, 1997. **80**(3): p. 421-34.
113. Miller, L.J., et al., *Interleukin-1 family expression in human breast cancer: interleukin-1 receptor antagonist.* Cancer Invest, 2000. **18**(4): p. 293-302.
114. Maihofner, C., et al., *Expression of cyclooxygenase-2 parallels expression of interleukin-1beta, interleukin-6 and NF-kappaB in human colorectal cancer.* Carcinogenesis, 2003. **24**(4): p. 665-71.
115. Kaler, P., L. Augenlicht, and L. Klampfer, *Macrophage-derived IL-1beta stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3.* Oncogene, 2009. **28**(44): p. 3892-902.
116. Kaler, P., et al., *Tumor Associated Macrophages Protect Colon Cancer Cells from TRAIL-Induced Apoptosis through IL-1β- Dependent Stabilization of Snail in Tumor Cells.* PLoS ONE, 2010. **5**(7): p. e11700.
117. Li, Y., et al., *IL-1beta promotes stemness and invasiveness of colon cancer cells through Zeb1 activation.* Molecular Cancer, 2012. **11**(1): p. 87.

118. Corley, D.A., et al., *Race, ethnicity, sex and temporal differences in Barrett's oesophagus diagnosis: a large community-based study, 1994-2006.* Gut, 2009. **58**(2): p. 182-8.
119. Falk, G.W., *Barrett's esophagus.* Gastroenterology, 2002. **122**(6): p. 1569-91.
120. Quante, M., et al., *Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia.* Cancer Cell, 2012. **21**(1): p. 36-51.
121. Dossinger, V., et al., *Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors.* Cell Physiol Biochem, 2002. **12**(4): p. 197-206.
122. Jackson, C.B., et al., *Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression.* J Pathol, 2007. **213**(2): p. 140-51.
123. Kiyokawa, E., et al., *Overexpression of ERK, an EPH Family Receptor Protein Tyrosine Kinase, in Various Human Tumors.* Cancer Research, 1994. **54**(14): p. 3645-3650.
124. Liang, B., et al., *Increased expression of mitogen-activated protein kinase and its upstream regulating signal in human gastric cancer.* World J Gastroenterol, 2005. **11**(5): p. 623-8.
125. Yang, D., et al., *Significance of decoy receptor 3 (Dcr3) and external-signal regulated kinase 1/2 (Erk1/2) in gastric cancer.* BMC Immunol, 2012. **13**: p. 28.
126. Shigematsu, Y., et al., *Interleukin-1beta induced by Helicobacter pylori infection enhances mouse gastric carcinogenesis.* Cancer Lett, 2013. **340**(1): p. 141-7.
127. Tu, S., et al., *Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice.* Cancer Cell, 2008. **14**(5): p. 408-19.
128. Roberts, A.B., *The ever-increasing complexity of TGF-β signaling.* Cytokine & Growth Factor Reviews, 2002. **13**(1): p. 3-5.
129. Datto, M.B., et al., *The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B.* Mol Cell Biol, 1997. **17**(4): p. 2030-7.
130. Yagi, K., et al., *c-myc Is a Downstream Target of the Smad Pathway.* Journal of Biological Chemistry, 2002. **277**(1): p. 854-861.
131. Kim, S.J., et al., *Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis.* Cytokine Growth Factor Rev, 2000. **11**(1-2): p. 159-68.
132. Kang, S.H., et al., *Transcriptional repression of the transforming growth factor-beta type I receptor gene by DNA methylation results in the development of TGF-beta resistance in human gastric cancer.* Oncogene, 1999. **18**(51): p. 7280-6.

133. Calin, G.A., et al., *Genetic progression in microsatellite instability high (MSI-H) colon cancers correlates with clinico-pathological parameters: A study of the TGRbetaRII, BAX, hMSH3, hMSH6, IGFIIR and BLM genes.* Int J Cancer, 2000. **89**(3): p. 230-5.
134. Wang, L.H., et al., *Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression.* Clin Cancer Res, 2007. **13**(1): p. 102-10.
135. Li, M.O. and R.A. Flavell, *TGF-β: A Master of All T Cell Trades.* Cell, 2008. **134**(3): p. 392-404.
136. Bornstein, S., et al., *Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation.* J Clin Invest, 2009. **119**(11): p. 3408-19.
137. Lu, S.L., H. Herrington, and X.J. Wang, *Mouse models for human head and neck squamous cell carcinomas.* Head Neck, 2006. **28**(10): p. 945-54.
138. Chetty, R., *Gastrointestinal cancers accompanied by a dense lymphoid component: an overview with special reference to gastric and colonic medullary and lymphoepithelioma-like carcinomas.* J Clin Pathol, 2012. **65**(12): p. 1062-5.
139. Konstantinopoulos, P.A. and A.G. Papavassiliou, *Seeing the future of cancer-associated transcription factor drug targets.* JAMA, 2011. **305**(22): p. 2349-50.
140. Montminy, M.R. and L.M. Bilezikjian, *Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene.* Nature, 1987. **328**(6126): p. 175-178.
141. Shaywitz, A.J. and M.E. Greenberg, *CREB: A Stimulus-Induced Transcription Factor Activated by A Diverse Array of Extracellular Signals.* Annual Review of Biochemistry, 1999. **68**(1): p. 821-861.
142. Gonzalez, G.A. and M.R. Montminy, *Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133.* Cell, 1989. **59**(4): p. 675-680.
143. Brindle, P., T. Nakajima, and M. Montminy, *Multiple protein kinase A-regulated events are required for transcriptional induction by cAMP.* Proceedings of the National Academy of Sciences, 1995. **92**(23): p. 10521-10525.
144. Mayr, B. and M. Montminy, *Transcriptional regulation by the phosphorylation-dependent factor CREB.* Nat Rev Mol Cell Biol, 2001. **2**(8): p. 599-609.
145. Johannessen, M., M.P. Delghandi, and U. Moens, *What turns CREB on?* Cellular Signalling, 2004. **16**(11): p. 1211-1227.
146. Montminy, M.R., et al., *Identification of a cyclic-AMP-responsive element within the rat somatostatin gene.* Proceedings of the National Academy of Sciences, 1986. **83**(18): p. 6682-6686.

147. Chrivia, J.C., et al., *Phosphorylated CREB binds specifically to the nuclear protein CBP.* Nature, 1993. **365**(6449): p. 855-859.
148. Arias, J., et al., *Activation of cAMP and mitogen responsive genes relies on a common nuclear factor.* Nature, 1994. **370**(6486): p. 226-9.
149. Conkright, M.D., et al., *TORCs: transducers of regulated CREB activity.* Mol Cell, 2003. **12**(2): p. 413-23.
150. Hai, T.W., et al., *Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers.* Genes Dev, 1989. **3**(12B): p. 2083-90.
151. Foulkes, N.S., E. Borrelli, and P. Sassone-Corsi, *CREM gene: Use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription.* Cell, 1991. **64**(4): p. 739-749.
152. de Groot, R.P., et al., *Multiple and cooperative phosphorylation events regulate the CREM activator function.* EMBO J, 1993. **12**(10): p. 3903-11.
153. Quinn, P.G., *Distinct activation domains within cAMP response element-binding protein (CREB) mediate basal and cAMP-stimulated transcription.* Journal of Biological Chemistry, 1993. **268**(23): p. 16999-7009.
154. Shaywitz, A.J. and M.E. Greenberg, *CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals.* Annu Rev Biochem, 1999. **68**: p. 821-61.
155. Foulkes, N.S. and P. Sassone-Corsi, *More is better: Activators and repressors from the same gene.* Cell, 1992. **68**(3): p. 411-414.
156. Molina, C.A., et al., *Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor.* Cell, 1993. **75**(5): p. 875-86.
157. Rudolph, D., et al., *Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein.* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4481-6.
158. Bird, A.D., et al., *cAMP Response Element Binding Protein Is Required for Differentiation of Respiratory Epithelium during Murine Development.* PLoS ONE, 2011. **6**(3): p. e17843.
159. Bleckmann, S.C., et al., *Activating transcription factor 1 and CREB are important for cell survival during early mouse development.* Mol Cell Biol, 2002. **22**(6): p. 1919-25.

160. Kitazawa, S., et al., *A p.D116G mutation in CREB1 leads to novel multiple malformation syndrome resembling CrebA knockout mouse.* Hum Mutat, 2012. **33**(4): p. 651-4.
161. Long, F., et al., *The CREB family of activators is required for endochondral bone development.* Development, 2001. **128**(4): p. 541-550.
162. Desdouets, C., et al., *Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM.* Mol Cell Biol, 1995. **15**(6): p. 3301-9.
163. D'Amico, M., et al., *The Integrin-linked Kinase Regulates the Cyclin D1 Gene through Glycogen Synthase Kinase 3β and cAMP-responsive Element-binding Protein-dependent Pathways.* Journal of Biological Chemistry, 2000. **275**(42): p. 32649-32657.
164. Riccio, A., et al., *Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons.* Science, 1999. **286**(5448): p. 2358-61.
165. Bonni, A., et al., *Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms.* Science, 1999. **286**(5443): p. 1358-62.
166. Jaworski, J., et al., *Inducible cAMP early repressor, an endogenous antagonist of cAMP responsive element-binding protein, evokes neuronal apoptosis in vitro.* J Neurosci, 2003. **23**(11): p. 4519-26.
167. Mioduszewska, B., et al., *Inducible cAMP early repressor (ICER)-evoked delayed neuronal death in the organotypic hippocampal culture.* J Neurosci Res, 2008. **86**(1): p. 61-70.
168. Yamamoto, K.K., et al., *Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB.* Nature, 1988. **334**(6182): p. 494-8.
169. Craig, J.C., et al., *Consensus and Variant cAMP-regulated Enhancers Have Distinct CREB-binding Properties.* Journal of Biological Chemistry, 2001. **276**(15): p. 11719-11728.
170. Iguchi-Ariga, S.M. and W. Schaffner, *CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation.* Genes & Development, 1989. **3**(5): p. 612-619.
171. Impey, S., et al., *Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions.* Cell, 2004. **119**(7): p. 1041-54.
172. Zhang, X., et al., *Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4459-64.

173. Cha-Molstad, H., et al., *Cell-type-specific binding of the transcription factor CREB to the cAMP-response element.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13572-7.
174. Niehof, M., M.P. Manns, and C. Trautwein, *CREB controls LAP/C/EBP beta transcription.* Mol Cell Biol, 1997. **17**(7): p. 3600-13.
175. Reusch, J.E., L.A. Colton, and D.J. Klemm, *CREB activation induces adipogenesis in 3T3-L1 cells.* Mol Cell Biol, 2000. **20**(3): p. 1008-20.
176. Zhang, J.W., et al., *Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis.* J Biol Chem, 2004. **279**(6): p. 4471-8.
177. Beaupre, D.M., et al., *Autocrine interleukin-1beta production in leukemia: evidence for the involvement of mutated RAS.* Cancer Res, 1999. **59**(12): p. 2971-80.
178. Potter, S., et al., *NF-IL6 and CRE elements principally account for both basal and interleukin-1β-induced transcriptional activity of the proximal 528 bp of the PGHS-2 promoter in amnion-derived AV3 cells: evidence for involvement of C/EBPβ.* Molecular Human Reproduction, 2000. **6**(9): p. 771-778.
179. Basbaum, C., et al., *Control of mucin transcription by diverse injury-induced signaling pathways.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 2): p. S44-8.
180. Song, K.S., et al., *Interleukin-1β and Tumor Necrosis Factor-α Induce MUC5AC Overexpression through a Mechanism Involving ERK/p38 Mitogen-activated Protein Kinases-MSK1-CREB Activation in Human Airway Epithelial Cells.* Journal of Biological Chemistry, 2003. **278**(26): p. 23243-23250.
181. Song, K.S., et al., *Induction of MUC8 Gene Expression by Interleukin-1β Is Mediated by a Sequential ERK MAPK/RSK1/CREB Cascade Pathway in Human Airway Epithelial Cells.* Journal of Biological Chemistry, 2003. **278**(37): p. 34890-34896.
182. Kudo, T., et al., *Pattern of Transcription Factor Activation in Helicobacter pylori–Infected Mongolian Gerbils.* Gastroenterology, 2007. **132**(3): p. 1024-1038.
183. Eckmann, L., et al., *Role of intestinal epithelial cells in the host secretory response to infection by invasive bacteria. Bacterial entry induces epithelial prostaglandin h synthase-2 expression and prostaglandin E2 and F2alpha production.* J Clin Invest, 1997. **100**(2): p. 296-309.
184. Ristimaki, A., et al., *Expression of cyclooxygenase-2 in human gastric carcinoma.* Cancer Res, 1997. **57**(7): p. 1276-80.
185. Regalo, G., et al., *C/EBPbeta is over-expressed in gastric carcinogenesis and is associated with COX-2 expression.* The Journal of Pathology, 2006. **210**(4): p. 398-404.

186. Juttner, S., et al., *Helicobacter pylori stimulates host cyclooxygenase-2 gene transcription: critical importance of MEK/ERK-dependent activation of USF1/-2 and CREB transcription factors.* Cell Microbiol, 2003. **5**(11): p. 821-34.
187. Siu, Y.T. and D.Y. Jin, *CREB--a real culprit in oncogenesis.* FEBS J, 2007. **274**(13): p. 3224-32.
188. Zucman, J., et al., *EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts.* Nat Genet, 1993. **4**(4): p. 341-5.
189. Antonescu, C.R., et al., *EWS-CREB1: a recurrent variant fusion in clear cell sarcoma--association with gastrointestinal location and absence of melanocytic differentiation.* Clin Cancer Res, 2006. **12**(18): p. 5356-62.
190. Brown, A.D., et al., *Promoters containing ATF-binding sites are de-regulated in cells that express the EWS/ATF1 oncogene.* Oncogene, 1995. **10**(9): p. 1749-1756.
191. Bosilevac, J.M., et al., *Tumor cell viability in clear cell sarcoma requires DNA binding activity of the EWS/ATF1 fusion protein.* J Biol Chem, 1999. **274**(49): p. 34811-8.
192. Crans-Vargas, H.N., et al., *Expression of cyclic adenosine monophosphate response-element binding protein in acute leukemia.* Blood, 2002. **99**(7): p. 2617-9.
193. Shankar, D.B., et al., *The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia.* Cancer Cell, 2005. **7**(4): p. 351-362.
194. Kinjo, K., et al., *The Role of CREB as a Proto-oncogene in Hematopoiesis.* Cell Cycle, 2005. **4**(9): p. 1134-1135.
195. Pigazzi, M., et al., *cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression.* Haematologica, 2007. **92**(10): p. 1435-1437.
196. Cheng, J.C., et al., *CREB is a critical regulator of normal hematopoiesis and leukemogenesis.* Blood, 2008. **111**(3): p. 1182-1192.
197. Trapman, J. and A.O. Brinkmann, *The androgen receptor in prostate cancer.* Pathol Res Pract, 1996. **192**(7): p. 752-60.
198. Hellawell, G.O., et al., *Expression of the Type 1 Insulin-like Growth Factor Receptor Is Up-Regulated in Primary Prostate Cancer and Commonly Persists in Metastatic Disease.* Cancer Research, 2002. **62**(10): p. 2942-2950.
199. Genua, M., et al., *Role of cyclic AMP response element-binding protein in insulin-like growth factor-i receptor up-regulation by sex steroids in prostate cancer cells.* Cancer Res, 2009. **69**(18): p. 7270-7.

200. Wu, D., et al., *cAMP-responsive element-binding protein regulates vascular endothelial growth factor expression: implication in human prostate cancer bone metastasis.* Oncogene, 2007. **26**(35): p. 5070-7.
201. Chhabra, A., et al., *Expression of transcription factor CREB1 in human breast cancer and its correlation with prognosis.* Oncol Rep, 2007. **18**(4): p. 953-8.
202. Son, J., et al., *cAMP-response-element-binding protein positively regulates breast cancer metastasis and subsequent bone destruction.* Biochem Biophys Res Commun, 2010. **398**(2): p. 309-14.
203. Steven, A., et al., *HER-2/neu mediates oncogenic transformation via altered CREB expression and function.* Mol Cancer Res, 2013. **11**(11): p. 1462-77.
204. Seo, H.S., et al., *Cyclic AMP response element-binding protein overexpression: a feature associated with negative prognosis in never smokers with non-small cell lung cancer.* Cancer Res, 2008. **68**(15): p. 6065-73.
205. Johnson, P.F. and S.L. McKnight, *Eukaryotic Transcriptional Regulatory Proteins.* Annual Review of Biochemistry, 1989. **58**(1): p. 799-839.
206. Osada, S., et al., *DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family.* J Biol Chem, 1996. **271**(7): p. 3891-6.
207. Hurst, H.C., *Transcription factors 1: bZIP proteins.* Protein Profile, 1995. **2**(2): p. 101-68.
208. Ramji, D.P. and P. Foka, *CCAAT/enhancer-binding proteins: structure, function and regulation.* Biochem J, 2002. **365**(Pt 3): p. 561-75.
209. Cao, Z., R.M. Umek, and S.L. McKnight, *Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells.* Genes Dev, 1991. **5**(9): p. 1538-52.
210. Vinson, C.R., P.B. Sigler, and S.L. McKnight, *Scissors-grip model for DNA recognition by a family of leucine zipper proteins.* Science, 1989. **246**(4932): p. 911-6.
211. Williams, S.C., C.A. Cantwell, and P.F. Johnson, *A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro.* Genes Dev, 1991. **5**(9): p. 1553-67.
212. Nerlov, C. and E.B. Ziff, *Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter.* Genes Dev, 1994. **8**(3): p. 350-62.
213. Yamanaka, R., et al., *CCAAT/enhancer binding protein ε is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by*

alternative use of promoters and differential splicing. Proceedings of the National Academy of Sciences, 1997. **94**(12): p. 6462-6467.

214. Descombes, P. and U. Schibler, *A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA.* Cell, 1991. **67**(3): p. 569-79.
215. Roman, C., et al., *Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP.* Genes & Development, 1990. **4**(8): p. 1404-1415.
216. Lekstrom-Himes, J. and K.G. Xanthopoulos, *Biological role of the CCAAT/enhancer-binding protein family of transcription factors.* J Biol Chem, 1998. **273**(44): p. 28545-8.
217. Hendricks-Taylor, L.R. and G.J. Darlington, *Inhibition of cell proliferation by C/EBPα occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen.* Nucleic Acids Research, 1995. **23**(22): p. 4726-4733.
218. Timchenko, N.A., et al., *CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein.* Genes & Development, 1996. **10**(7): p. 804-815.
219. Wang, H., et al., *C/EBPα Arrests Cell Proliferation through Direct Inhibition of Cdk2 and Cdk4.* Molecular Cell, 2001. **8**(4): p. 817-828.
220. Porse, B.T., et al., *E2F Repression by C/EBPα Is Required for Adipogenesis and Granulopoiesis In Vivo.* Cell, 2001. **107**(2): p. 247-258.
221. Müller, C., et al., *The CCAAT Enhancer-binding Protein α (C/EBPα) Requires a SWI/SNF Complex for Proliferation Arrest.* Journal of Biological Chemistry, 2004. **279**(8): p. 7353-7358.
222. Loomis, K.D., et al., *Genetic ablation of CCAAT/enhancer binding protein alpha in epidermis reveals its role in suppression of epithelial tumorigenesis.* Cancer Res, 2007. **67**(14): p. 6768-76.
223. Wang, N.D., et al., *Impaired energy homeostasis in C/EBP alpha knockout mice.* Science, 1995. **269**(5227): p. 1108-12.
224. Flodby, P., et al., *Increased Hepatic Cell Proliferation and Lung Abnormalities in Mice Deficient in CCAAT/Enhancer Binding Protein α.* Journal of Biological Chemistry, 1996. **271**(40): p. 24753-24760.
225. Johnson, P.F., *Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors.* J Cell Sci, 2005. **118**(Pt 12): p. 2545-55.

226. Zhang, D.E., et al., *Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 569-74.
227. Martis, P.C., et al., *C/EBPα is required for lung maturation at birth.* Development, 2006. **133**(6): p. 1155-1164.
228. Christy, R.J., et al., *Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes.* Genes Dev, 1989. **3**(9): p. 1323-35.
229. Zhu, S., et al., *CCAAT/enhancer binding protein-β is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling.* Proceedings of the National Academy of Sciences, 2002. **99**(1): p. 207-212.
230. Greenbaum, L.E., et al., *CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy.* The Journal of Clinical Investigation, 1998. **102**(5): p. 996-1007.
231. Seagroves, T.N., et al., *C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland.* Genes & Development, 1998. **12**(12): p. 1917-1928.
232. Robinson, G.W., et al., *The C/EBPβ transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland.* Genes & Development, 1998. **12**(12): p. 1907-1916.
233. Bundy, L.M. and L. Sealy, *CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture.* Oncogene, 2003. **22**(6): p. 869-83.
234. LaMarca, H.L., et al., *CCAAT/enhancer binding protein beta regulates stem cell activity and specifies luminal cell fate in the mammary gland.* Stem Cells, 2010. **28**(3): p. 535-44.
235. Yeh, W.C., et al., *Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins.* Genes & Development, 1995. **9**(2): p. 168-181.
236. Calkhoven, C.F. and G. Ab, *Multiple steps in the regulation of transcription-factor level and activity.* Biochem. J., 1996. **317**(2): p. 329-342.
237. Poli, V., F.P. Mancini, and R. Cortese, *IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP.* Cell, 1990. **63**(3): p. 643-53.

238. Tanaka, T., et al., *Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages.* Cell, 1995. **80**(2): p. 353-61.
239. Sterneck, E., et al., *Conditional ablation of C//EBP[beta] demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis.* Oncogene, 2005. **25**(8): p. 1272-1276.
240. Didon, L., et al., *Lung Epithelial CCAAT/Enhancer-binding Protein-β Is Necessary for the Integrity of Inflammatory Responses to Cigarette Smoke.* American Journal of Respiratory and Critical Care Medicine, 2011. **184**(2): p. 233-242.
241. Zauberman, A., S. Lapter, and D. Zipori, *Smad proteins suppress CCAAT/enhancer-binding protein (C/EBP) beta- and STAT3-mediated transcriptional activation of the haptoglobin promoter.* J Biol Chem, 2001. **276**(27): p. 24719-25.
242. Yu, S.J., et al., *Attenuation of haptoglobin gene expression by TGFbeta requires the MAP kinase pathway.* Biochem Biophys Res Commun, 1999. **259**(3): p. 544-9.
243. Chang, Y.J., et al., *Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by Helicobacter pylori involves TLR2/TLR9 and c-Src-dependent nuclear factor-kappaB activation.* Mol Pharmacol, 2004. **66**(6): p. 1465-77.
244. Pabst, T., et al., *Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia.* Nat Genet, 2001. **27**(3): p. 263-70.
245. Gombart, A.F., et al., *Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias.* Blood, 2002. **99**(4): p. 1332-40.
246. Bereshchenko, O., et al., *Hematopoietic Stem Cell Expansion Precedes the Generation of Committed Myeloid Leukemia-Initiating Cells in C/EBPα Mutant AML.* Cancer Cell, 2009. **16**(5): p. 390-400.
247. Hackanson, B., et al., *Epigenetic modification of CCAAT/enhancer binding protein alpha expression in acute myeloid leukemia.* Cancer Res, 2008. **68**(9): p. 3142-51.
248. Resende, C., et al., *Genetic changes of CEBPA in cancer: mutations or polymorphisms?* J Clin Oncol, 2007. **25**(17): p. 2493-4; author reply 2494-5.
249. Halmos, B., et al., *Down-regulation and antiproliferative role of C/EBPalpha in lung cancer.* Cancer Res, 2002. **62**(2): p. 528-34.
250. Tada, Y., et al., *Epigenetic modulation of tumor suppressor CCAAT/enhancer binding protein alpha activity in lung cancer.* J Natl Cancer Inst, 2006. **98**(6): p. 396-406.

251. Gery, S., et al., *Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer.* Clin Cancer Res, 2005. **11**(9): p. 3184-90.
252. Kumagai, T., et al., *Epigenetic regulation and molecular characterization of C/EBPalpha in pancreatic cancer cells.* Int J Cancer, 2009. **124**(4): p. 827-33.
253. Yoon, K., et al., *Decreased survival of C//EBP[beta]-deficient keratinocytes is due to aberrant regulation of p53 levels and function.* Oncogene, 2006. **26**(3): p. 360-367.
254. Zahnow, C.A., et al., *Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer.* J Natl Cancer Inst, 1997. **89**(24): p. 1887-91.
255. Zahnow, C.A., et al., *A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation.* Cancer Res, 2001. **61**(1): p. 261-9.
256. Gomis, R.R., et al., *C/EBPβ at the core of the TGFβ cytostatic response and its evasion in metastatic breast cancer cells.* Cancer Cell, 2006. **10**(3): p. 203-214.
257. Kagan, B.L., et al., *Complex regulation of the fibroblast growth factor-binding protein in MDA- MB-468 breast cancer cells by CCAAT/enhancer-binding protein beta.* Cancer Res, 2003. **63**(7): p. 1696-705.
258. Yokota, T., et al., *Differential Regulation of Elafin in Normal and Tumor-Derived Mammary Epithelial Cells Is Mediated by CCAAT/Enhancer Binding Protein β.* Cancer Research, 2007. **67**(23): p. 11272-11283.
259. Albergaria, A., et al., *CCAAT/Enhancer Binding Protein β (C/EBPβ) Isoforms as Transcriptional Regulators of the Pro-Invasive <italic>CDH3</italic>/P-Cadherin Gene in Human Breast Cancer Cells.* PLoS ONE, 2013. **8**(2): p. e55749.
260. Park, B.H., et al., *An isoform of C/EBPbeta, LIP, regulates expression of the chemokine receptor CXCR4 and modulates breast cancer cell migration.* J Biol Chem, 2013. **288**(40): p. 28656-67.
261. Damiano, L., et al., *Oncogenic targeting of BRM drives malignancy through C/EBP[beta]-dependent induction of [alpha]5 integrin.* Oncogene, 2013.
262. Lamb, J., et al., *A Mechanism of Cyclin D1 Action Encoded in the Patterns of Gene Expression in Human Cancer.* Cell, 2003. **114**(3): p. 323-334.
263. Sankpal, N.V., et al., *Overexpression of CEBPbeta correlates with decreased TFF1 in gastric cancer.* Oncogene, 2006. **25**(4): p. 643-9.
264. Milne, A.N.A., et al., *Early-onset gastric cancers have a different molecular expression profile than conventional gastric cancers.* Mod Pathol, 2006. **19**(4): p. 564-572.

265. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
266. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
267. Darnell, J.E., *Transcription factors as targets for cancer therapy.* Nat Rev Cancer, 2002. **2**(10): p. 740-749.
268. Blume-Jensen, P. and T. Hunter, *Oncogenic kinase signalling.* Nature, 2001. **411**(6835): p. 355-65.
269. Gibbs, J.B., *Mechanism-based target identification and drug discovery in cancer research.* Science, 2000. **287**(5460): p. 1969-73.
270. Tilley, W.D., et al., *Hormones and cancer: new insights, new challenges.* Trends Endocrinol Metab, 2001. **12**(5): p. 186-8.
271. Brivanlou, A.H. and J.E. Darnell, Jr., *Signal transduction and the control of gene expression.* Science, 2002. **295**(5556): p. 813-8.
272. Best, J.L., et al., *Identification of small-molecule antagonists that inhibit an activator:coactivator interaction.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(51): p. 17622-17627.
273. Li, B.X., K. Yamanaka, and X. Xiao, *Structure–activity relationship studies of naphthol AS-E and its derivatives as anticancer agents by inhibiting CREB-mediated gene transcription.* Bioorganic & Medicinal Chemistry, 2012. **20**(23): p. 6811-6820.
274. Takemoto, S., et al., *Expression of activated signal transducer and activator of transcription-3 predicts poor prognosis in cervical squamous-cell carcinoma.* Br J Cancer, 2009. **101**(6): p. 967-72.
275. Gu, F.M., et al., *Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3.* World J Gastroenterol, 2011. **17**(34): p. 3922-32.
276. Horiguchi, A., et al., *STAT3 inhibitor WP1066 as a novel therapeutic agent for renal cell carcinoma.* Br J Cancer, 2010. **102**(11): p. 1592-1599.
277. Leong, P.L., et al., *Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4138-43.
278. Sen, M., et al., *First-in-human trial of a STAT3 decoy oligonucleotide in head and neck tumors: implications for cancer therapy.* Cancer Discov, 2012. **2**(8): p. 694-705.
279. Baeuerle, P.A. and T. Henkel, *Function and activation of NF-kappa B in the immune system.* Annu Rev Immunol, 1994. **12**: p. 141-79.
280. Umezawa, K., *Inhibition of tumor growth by NF-kappaB inhibitors.* Cancer Sci, 2006. **97**(10): p. 990-5.

281. Sasaki, N., et al., *Nuclear Factor-κB p65 (RelA) Transcription Factor Is Constitutively Activated in Human Gastric Carcinoma Tissue.* Clinical Cancer Research, 2001. **7**(12): p. 4136-4142.
282. Chua, H.L., et al., *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): p. 711-24.
283. Tew, G.W., et al., *SmgGDS regulates cell proliferation, migration, and NF-kappaB transcriptional activity in non-small cell lung carcinoma.* J Biol Chem, 2008. **283**(2): p. 963-76.
284. Vilimas, T., et al., *Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia.* Nat Med, 2007. **13**(1): p. 70-7.
285. Matsumoto, G., et al., *Targeting of nuclear factor kappaB Pathways by dehydroxymethylepoxyquinomicin, a novel inhibitor of breast carcinomas: antitumor and antiangiogenic potential in vivo.* Clin Cancer Res, 2005. **11**(3): p. 1287-93.
286. Taipale, J. and P.A. Beachy, *The Hedgehog and Wnt signalling pathways in cancer.* Nature, 2001. **411**(6835): p. 349-354.
287. Ewan, K., et al., *A Useful Approach to Identify Novel Small-Molecule Inhibitors of Wnt-Dependent Transcription.* Cancer Research, 2010. **70**(14): p. 5963-5973.
288. Mitelman, F., B. Johansson, and F. Mertens, *Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer.* Nat Genet, 2004. **36**(4): p. 331-334.
289. Forster, S., et al., *THBS4, a novel stromal molecule of diffuse-type gastric adenocarcinomas, identified by transcriptome-wide expression profiling.* Mod Pathol, 2011. **24**(10): p. 1390-403.
290. Astrosini, C., et al., *REG1A expression is a prognostic marker in colorectal cancer and associated with peritoneal carcinomatosis.* International Journal of Cancer, 2008. **123**(2): p. 409-413.
291. Yeh, K.T., et al., *Aberrant TGFbeta/SMAD4 signaling contributes to epigenetic silencing of a putative tumor suppressor, RunX1T1 in ovarian cancer.* Epigenetics, 2011. **6**(6): p. 727-39.
292. Peek, R.M., Jr. and J.E. Crabtree, *Helicobacter infection and gastric neoplasia.* J Pathol, 2006. **208**(2): p. 233-48.

293. Nerlov, C., *The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control.* Trends in Cell Biology, 2007. **17**(7): p. 318-324.
294. Rosen, E.D., *The transcriptional basis of adipocyte development.* Prostaglandins, Leukotrienes and Essential Fatty Acids, 2005. **73**(1): p. 31-34.
295. Payne, V.A., et al., *Sequential regulation of diacylglycerol acyltransferase 2 expression by CAAT/enhancer-binding protein beta (C/EBPbeta) and C/EBPalpha during adipogenesis.* J Biol Chem, 2007. **282**(29): p. 21005-14.
296. Tang, Q.Q., J.W. Zhang, and M. Daniel Lane, *Sequential gene promoter interactions of C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis.* Biochem Biophys Res Commun, 2004. **319**(1): p. 235-9.
297. Schuster, M.B. and B.T. Porse, *C/EBPalpha: a tumour suppressor in multiple tissues?* Biochim Biophys Acta, 2006. **1766**(1): p. 88-103.
298. Nerlov, C., *C/EBPalpha mutations in acute myeloid leukaemias.* Nat Rev Cancer, 2004. **4**(5): p. 394-400.
299. Costa, D.B., et al., *Immunohistochemical analysis of C/EBPalpha in non-small cell lung cancer reveals frequent down-regulation in stage II and IIIA tumors: a correlative study of E3590.* Lung Cancer, 2007. **56**(1): p. 97-103.
300. Shim, M., et al., *Diminished Expression of C/EBPα in Skin Carcinomas Is Linked to Oncogenic Ras and Reexpression of C/EBPα in Carcinoma Cells Inhibits Proliferation.* Cancer Research, 2005. **65**(3): p. 861-867.
301. Anastasov, N., et al., *C/EBPβ expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway.* Haematologica, 2010. **95**(5): p. 760-767.
302. Bonzheim, I., et al., *Identification of C/EBPbeta target genes in ALK+ anaplastic large cell lymphoma (ALCL) by gene expression profiling and chromatin immunoprecipitation.* PLoS ONE, 2013. **8**(5): p. e64544.
303. Lamb, J. and M.E. Ewen, *Cyclin D1 and molecular chaperones: implications for tumorigenesis.* Cell Cycle, 2003. **2**(6): p. 525-7.
304. Pal, R., et al., *C/EBPbeta regulates transcription factors critical for proliferation and survival of multiple myeloma cells.* Blood, 2009. **114**(18): p. 3890-8.
305. Rask, K., et al., *Increased expression of the transcription factors CCAAT-enhancer binding protein-beta (C/EBBeta) and C/EBzeta (CHOP) correlate with invasiveness of human colorectal cancer.* Int J Cancer, 2000. **86**(3): p. 337-43.

306. Watanabe-Okochi, N., et al., *The shortest isoform of C/EBPbeta, liver inhibitory protein (LIP), collaborates with Evi1 to induce AML in a mouse BMT model.* Blood, 2013. **121**(20): p. 4142-55.
307. Forones, N.M., et al., *Expression of COX-2 in stomach carcinogenesis.* J Gastrointest Cancer, 2008. **39**(1-4): p. 4-10.
308. Saukkonen, K., et al., *Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma.* Clin Cancer Res, 2001. **7**(7): p. 1923-31.
309. Oshima, H., et al., *Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice.* EMBO J, 2004. **23**(7): p. 1669-78.
310. Carneiro, P., et al., *E-cadherin dysfunction in gastric cancer--cellular consequences, clinical applications and open questions.* FEBS Lett, 2012. **586**(18): p. 2981-9.
311. Ito, Y., *RUNX3 is expressed in the epithelium of the gastrointestinal tract.* EMBO Molecular Medicine, 2012. **4**(7): p. 541-542.
312. Levanon, D., et al., *Absence of Runx3 expression in normal gastrointestinal epithelium calls into question its tumour suppressor function.* EMBO Molecular Medicine, 2011. **3**(10): p. 593-604.
313. Rochford, J.J., et al., *ETO/MTG8 Is an Inhibitor of C/EBPβ Activity and a Regulator of Early Adipogenesis.* Molecular and Cellular Biology, 2004. **24**(22): p. 9863-9872.
314. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
315. Calabi, F., R. Pannell, and G. Pavloska, *Gene targeting reveals a crucial role for MTG8 in the gut.* Mol Cell Biol, 2001. **21**(16): p. 5658-66.
316. Nasir, A., et al., *RUNX1T1: a novel predictor of liver metastasis in primary pancreatic endocrine neoplasms.* Pancreas, 2011. **40**(4): p. 627-33.
317. Nimer, S.D. and M.A. Moore, *Effects of the leukemia-associated AML1-ETO protein on hematopoietic stem and progenitor cells.* Oncogene, 2004. **23**(24): p. 4249-54.
318. Davis, J.N., L. McGhee, and S. Meyers, *The ETO (MTG8) gene family.* Gene, 2003. **303**: p. 1-10.
319. Lichtinger, M., et al., *RUNX1 reshapes the epigenetic landscape at the onset of haematopoiesis.* The EMBO Journal, 2012. **31**(22): p. 4318-4333.
320. Canedo, P., et al., *The interferon gamma receptor 1 (IFNGR1) −56C/T gene polymorphism is associated with increased risk of early gastric carcinoma.* Gut, 2008. **57**(11): p. 1504-1508.

321. Konturek, P.C., et al., *H. pylori infection, atrophic gastritis, cytokines, gastrin, COX-2, PPAR gamma and impaired apoptosis in gastric carcinogenesis.* Med Sci Monit, 2003. **9**(7): p. SR53-66.
322. Beales, I.L., *Effect of interlukin-1beta on proliferation of gastric epithelial cells in culture.* BMC Gastroenterol, 2002. **2**: p. 7.
323. Yasunaga, Y., et al., *Increased production of interleukin 1 beta and hepatocyte growth factor may contribute to foveolar hyperplasia in enlarged fold gastritis.* Gut, 1996. **39**(6): p. 787-794.
324. Newton, K. and V.M. Dixit, *Signaling in Innate Immunity and Inflammation.* Cold Spring Harbor Perspectives in Biology, 2012. **4**(3).
325. Naumann, M. and J.E. Crabtree, *Helicobacter pylori-induced epithelial cell signalling in gastric carcinogenesis.* Trends in Microbiology, 2004. **12**(1): p. 29-36.
326. Giltiay, N.V., et al., *Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin-1β in hepatocytes.* Journal of Lipid Research, 2005. **46**(11): p. 2497-2505.
327. Sankpal, N.V., M.W. Mayo, and S.M. Powell, *Transcriptional repression of TFF1 in gastric epithelial cells by CCAAT/enhancer binding protein-β.* Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 2005. **1728**(1–2): p. 1-10.
328. Zhao, D., *Protein kinase Cδ-mediated CREB activation regulates ghrelin-induced cyclooxygenase-2 expression and prostaglandin E2 production in human colonic epithelial cells.* Journal of Cellular Biochemistry, 2007. **102**(5): p. 1245-1255.
329. Sun, H., et al., *Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis.* Cancer Prev Res (Phila), 2008. **1**(5): p. 316-28.
330. Aggarwal, S., et al., *Growth suppression of lung cancer cells by targeting cyclic AMP response element-binding protein.* Cancer Res, 2008. **68**(4): p. 981-8.
331. Chen, L., et al., *MicroRNA-181b targets cAMP responsive element binding protein 1 in gastric adenocarcinomas.* IUBMB Life, 2012. **64**(7): p. 628-35.
332. Lynch, D.A., et al., *Cell proliferation in Helicobacter pylori associated gastritis and the effect of eradication therapy.* Gut, 1995. **36**(3): p. 346-50.
333. Peek, J.R.M., et al., *Helicobacter pylori alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils.* Gastroenterology, 2000. **118**(1): p. 48-59.

334. Ding, S.-Z., et al., *Helicobacter pylori Infection Induces Oxidative Stress and Programmed Cell Death in Human Gastric Epithelial Cells.* Infection and Immunity, 2007. **75**(8): p. 4030-4039.
335. Vogiatzi, P., et al., *How does the human RUNX3 gene induce apoptosis in gastric cancer? Latest data, reflections and reactions.* Cancer Biology & Therapy, 2006. **5**(4): p. 371-374.
336. Pigazzi, M., et al., *ICER expression inhibits leukemia phenotype and controls tumor progression.* Leukemia, 2008. **22**(12): p. 2217-25.
337. Yehia, G., et al., *The Expression of Inducible cAMP Early Repressor (ICER) Is Altered in Prostate Cancer Cells and Reverses the Transformed Phenotype of the LNCaP Prostate Tumor Cell Line.* Cancer Research, 2001. **61**(16): p. 6055-6059.
338. Pham, H., et al., *Ang II and EGF synergistically induce COX-2 expression via CREB in intestinal epithelial cells.* J Cell Physiol, 2008. **214**(1): p. 96-109.
339. Chen, Y., et al., *Synergism between Calcium and Cyclic GMP in Cyclic AMP Response Element-Dependent Transcriptional Regulation Requires Cooperation between CREB and C/EBP-β.* Molecular and Cellular Biology, 2003. **23**(12): p. 4066-4082.
340. Gu, T., et al., *CREB Is a Novel Nuclear Target of PTEN Phosphatase.* Cancer Research, 2011. **71**(8): p. 2821-2825.
341. Meyer, T.E., et al., *The promoter of the gene encoding 3',5'-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription.* Endocrinology, 1993. **132**(2): p. 770-780.

APPENDIX: PUBLICATIONS

Article I

Laboratory Investigation (2010) 90, 1132–1139

C/EBPα expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis

Gonçalo Regalo[1,2], Carlos Resende[1], Xiaogang Wen[1], Bárbara Gomes[1,2], Cecília Durães[1], Raquel Seruca[1,2], Fátima Carneiro[1,2] and José C Machado[1,2]

Transcription factors from the CCAAT/enhancer-binding protein (C/EBP) family are fundamental for the control of differentiation and proliferation of many adult tissues. C/EBPα has a crucial role in inducing terminal differentiation and is an established tumor suppressor gene in several cancer models. The objective of this study was to analyze the putative role of C/EBPα in gastric carcinoma (GC). We analyzed the expression of C/EBPα in normal and neoplastic gastric tissues, and assessed the role of C/EBPα on proliferation and differentiation of GC cells. In normal gastric mucosa, C/EBPα is expressed in the foveolar epithelium and co-localizes with the gastric differentiation marker trefoil factor 1 (TFF1). The expression of C/EBPα was found to be lost in 30% of GC cases. To evaluate the role of C/EBPα in cell proliferation and differentiation, we transfected GC cells with a full-length C/EBPα protein. We observed a significant decrease in proliferation in C/EBPα-transfected cells. This was accompanied by a decrease in Cyclin D1, an increase in P27 expression, and an increased expression of TFF1. Finally, we showed that inhibition of the Ras/MAPK pathway leads to increased C/EBPα and TFF1 expression, and decreased cell proliferation and cyclin D1 expression in GC cells. Our results suggest that C/EBPα (together with other members of the C/EBP family) has an active role in the control of differentiation and proliferation in normal gastric mucosa. In GC, loss of C/EBPα may be associated with the switch from a cellular differentiation to a cellular proliferation program, presumably as a consequence of Ras/MAPK pathway activation.
Laboratory Investigation (2010) **90,** 1132–1139; doi:10.1038/labinvest.2010.79; published online 12 April 2010

KEYWORDS: gastric cancer; C/EBPα; C/EBPβ; TFF1; stomach

Gastric carcinoma (GC) is still one of the most common cancers worldwide, despite its decreasing incidence in the developed countries. The continued inflammation of the gastric epithelium by chronic *Helicobacter pylori* infection is a major contributor to carcinogenesis, most likely by promoting disruption of the balance between proliferation and differentiation in the regenerating inflamed mucosa. Although this process has been well characterized phenotipically, the main molecular players in gastric neoplastic transformation are largely unknown.[1]

Proteins of the CCAAT/enhancer-binding protein (C/EBP) family are important transcription factors that link gene expression to proliferation/differentiation control.[2] We have recently shown that C/EBPβ is over-expressed in pre-neoplastic lesions and GC.[3] Most notably, C/EBPβ over-expression is associated with loss of trefoil factor 1 (TFF1), an established differentiation marker, and a putative gastric tumor suppressor.[4]

Members of the C/EBP family are known to heterodimerize among themselves, giving rise to different functional transcriptional complexes. Moreover, they often act with a high degree of coordination.[5] This is well demonstrated in adipogenesis, where sequential expression of different C/EBP members underlies the process of differentiation from pre-adipocytes to fully mature adipocytes.[6] After the differentiation stimulus is given, C/EBPβ is expressed in immature pre-adipocytes and primes cells to differentiate by inducing C/EBPα expression.[7] Once active, C/EBPα drastically reduces cell proliferation, and promotes the expression of peroxisome proliferator-activated receptor γ.[8] In this and other models, C/EBPα is a crucial effector of lineage commitment and terminal differentiation programs.

The disruption of these programs has been shown to be oncogenic in several cellular contexts. For instance, C/EBPα is a consensual tumor suppressor in acute myeloid leukemia

[1]IPATIMUP—Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal and [2]Faculty of Medicine, University of Porto, Porto, Portugal
Correspondence: Professor JC Machado, PhD, Institute of Molecular Pathology and Immunology, University of Porto (IPATIMUP), Faculty of Medicine, Rua Dr. Roberto Frias s/n, Porto 4200-465, Portugal.
E-mail: josem@ipatimup.pt

Received 29 June 2009; revised 20 January 2010; accepted 10 February 2010

where deleterious mutations have been described in a proportion of cases.[9] C/EBPα may also have a role in other cancer models[10] such as lung cancer, where it was found downregulated by methylation.[11,12] However, the expression pattern and functional relevance of C/EBPα in normal stomach and in GC has never been described.

In this study, we characterized the expression of C/EBPα in the normal gastric mucosa and in GC. Furthermore, we investigated the effects of expressing C/EBPα in GC cells, and aimed at clarifying the link between pathways of C/EBPα modulation and gastric carcinogenesis.

MATERIALS AND METHODS

Tissue Material

Surgical specimens from 54 GC were resected and diagnosed at Hospital S. João/Faculty of Medicine, Porto, Portugal. Tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 3 μm were obtained from each block and used for routine staining with hematoxylin and eosin and immunohistochemistry.

The procedures followed in this study were in accordance with the institutional ethical standards. All the samples enrolled in this study were delinked and unidentified from their donors.

Immunohistochemistry

Tissue sections were first treated with 10 mmol/l citrate buffer (pH 6.0) for 40 min at 99°C. Unspecific endogenous peroxidase activity was eliminated with a Hydrogen Peroxide Block solution (Labvision, UK) for 10 min. After washing, slides were incubated with monoclonal mouse antibody anti-C/EBPα (1:300, Cell Signaling Technology, MA, USA) or C/EBPβ (1:100, Santa Cruz Biotechnology, CA, USA) 1 h at room temperature (RT). Sections were then washed and incubated with Dako Real Emvision/HRP Rabbit/Mouse solution (DAKO, Denmark) for 30 min (RT). The slides were then developed for 10 min in Dako Real diaminobenzidine (DAB) (0.05%, DAKO) and sections counterstained with hematoxylin, dehydrated, and mounted. For immunofluorescence, after the primary C/EBPα antibody incubation, sections were incubated with a biotinylated secondary antibody and signal was obtained with Alexa Fluor (Molecular probes, Invitrogen, CA, USA) incubation.

For double TFF1 and C/EBPα staining, two independent reactions were performed on the same slides. Sections were blocked for 15 min in 10% BSA with anti-mouse serum and incubated overnight in monoclonal antibody anti-C/EBPα (1:100, Cell Signaling Technology). After washing, samples were incubated with anti-rabbit secondary antibody (1:200, DAKO) for 30 min and washed again. A final 1 h incubation with avidine-biotin-peroxidase (1:100, DAKO) was performed. Slides were then developed with DAB (DAKO). After a washing step of 30 min in PBS at 60°C, slides were again incubated overnight with monoclonal antibody anti-TFF1 (1:100, Zymed, CA, USA) and developed with alkaline phosphatase (DAKO) and Fast Red (Sigma-Aldrich, MO, USA).

Slides were reviewed by a pathologist, tumors were classified according to Laurén, and the sections were semi-quantitatively scored according to the intensity of staining when compared with the positive control: intense staining was classified as III; moderate intensity as II; and weak intensity or negativity as I. Cases were classified as 'downregulated' when $>50\%$ of the tumor cells were classified as I. All washing steps were performed in PBS buffer. Normal gastric mucosa was used as a positive control, and negative controls were performed by substitution of the primary antibody with immunoglobulins of the same class and concentration.

Cell Culture, Transfections, and Blotting

AGS and MKN28 cells were grown in RPMI medium with 10% FBS (GIBCO, Invitrogen, CA, USA). AGS cells were grown until 60–80% confluence in six-well plates, and then transfected using 3 μg of Plenti-C/EBPα expression vector with an appropriate TFX-50 (Promega, WI, USA) concentration and volume. For western blot analysis, cells were scrapped in PBS and then lysed in RIPA buffer with protease and phosphatase inhibitors. A measure of 40 μg of total protein were loaded into acrylamide gels and separated by electrophoresis. The proteins were then transferred to Hybond membranes (Amersham Biosciences, UK). For dot blot, 20 μg of denatured protein extract were directly pipeted into Hybond membranes. After blocking, blots where incubated 1 h with primary antibodies anti-C/EBPα (1:100, Cell Signaling), anti-P27 and anti Cyclin D1 (1:100, Santa Cruz Biotechnology), anti-tubulin (1:15 000, Sigma-Aldrich), and in the case of the dot blot with anti-TFF1 (1:100, Zymed) in PBS plus 5% non-fat dried milk and 0.5% tween-20. The blots were then washed three times in the same solution and incubated 45 min with an HRP-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology) in PBS 0.5% tween-20. Blots were then washed three times in PBS 0.5% tween-20 and signal was detected with chemiluminescence using ECL (Amersham Biosciences). For MAPK inhibition experiments, MKN28 cells were grown until 50–60% confluence and treated for 24 h with 10 μM SB239063 or PD98059 (Sigma-Aldrich).

BRDU Incorporation Assay and Immunocytochemistry

AGS cells were harvested in 24-well plates with glass slides, and transfected using TFX50 (Invitrogen) with empty vector and full-length C/EBPα expression vectors in OPTIMEM medium (GIBCO). After 1 h, complete RPMI medium was added and cells were left growing for 48 h. MKN28 cells were grown in six-well plates with glass slides and treated with MAPK inhibitors as described above. After incubating 1 h in 5-bromo-2′-deoxy-uridine (BRDU), cells in the glass slides were fixed in 4% paraformaldehyde, washed with PBS two times, and quenched by incubation with 2 M HCl for 20 min. After washing, slides were incubated with anti-BRDU

antibody (1:100, DAKO) for 1 h. For simple immunocytochemistry, MKN28 cells treated and untreated with MAPK inhibitors were blocked in PBS with 4% BSA and incubated in C/EBPα (1:100, Cell Signaling) antibody for 1 h. In both procedures, cells were finally incubated with secondary anti-mouse FITC (1:100, DAKO)-conjugated antibody for 30 min. After washing, cells were mounted in vectashield (Vector Laboratories) with DAPI blue and scored for BRDU incorporation or C/EBPα expression on a fluorescence microscope.

Inhibition of C/EBPα by siRNA

MKN28 cells were grown until 50% confluence and preincubated in serum-free medium. The appropriate anti-C/EBPα target sequence (100 nM) as well as scrambled control siRNA (Qiagen) were mixed with Metafectene (Biontex laboratories GmbH, Germany) in serum-free medium, incubated for 20 min and added to the cells. After overnight incubation, the medium was changed to complete RPMI and cells left to grow for 48 h, after which BRDU incorporation and protein expression analyses were performed.

Statistical Analysis

Comparison of GC cases regarding their clinicopathological features was performed using Fisher's and χ^2 test. Three independent measurements were performed for the BRDU incorporation experiments and results were compared by Student's *t*-test.

RESULTS

Immunohistochemical Analysis of C/EBPα Expression

In the normal mucosa of the stomach, C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity and mostly localized in the mucous surface epithelium (Figure 1a). This expression pattern contrasts with that of C/EBPβ whose expression is concentrated to the neck zone

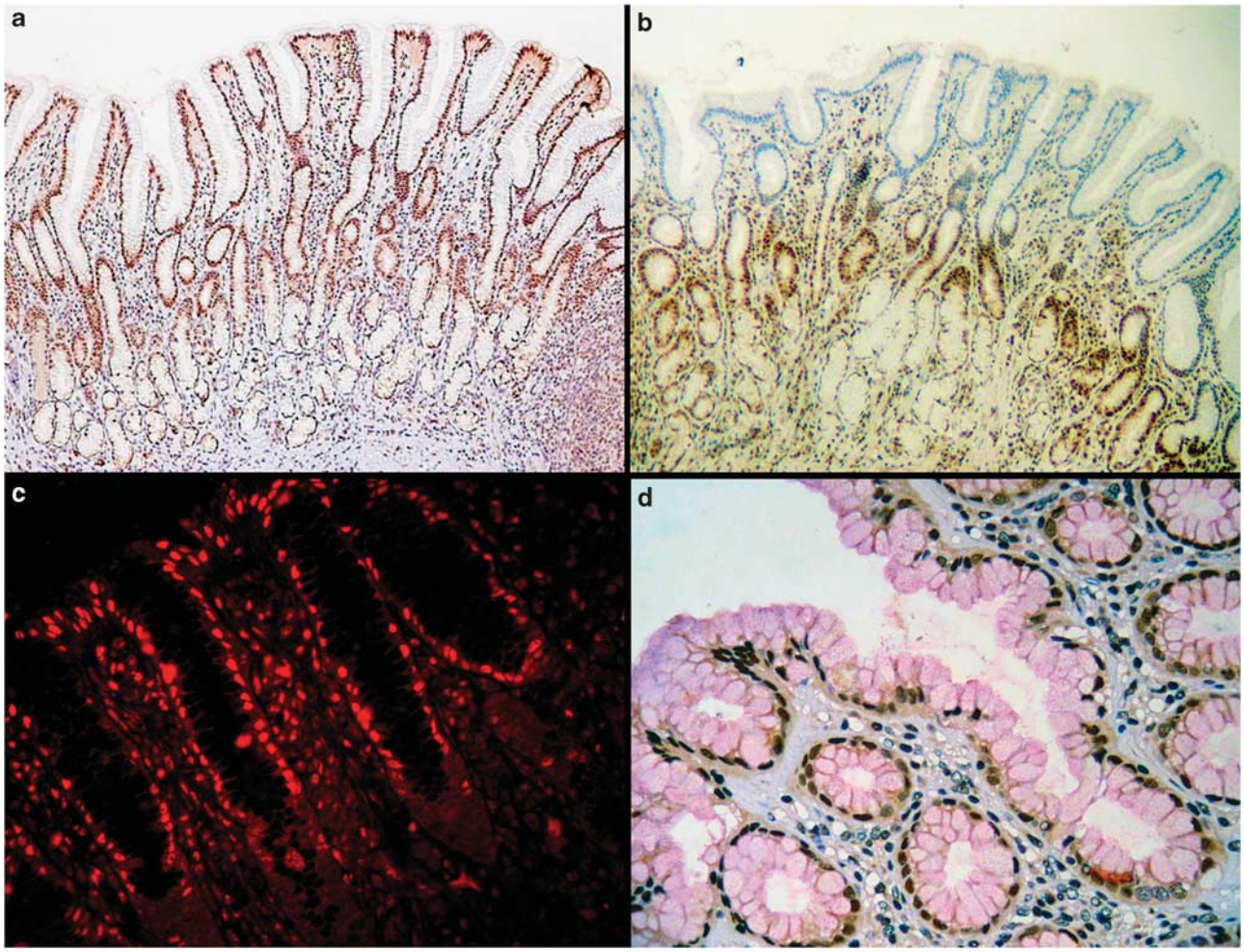


Figure 1 C/EBP expression in normal gastric mucosa. (**a**) C/EBPα immunostaining in non-neoplastic mucosa, showing strong expression in the superficial epithelium. (**b**) C/EBPβ expression in normal gastric mucosa of the antrum, showing strong localization in the neck zone. (**c**) C/EBPα immunofluorescence, showing expression in differentiated gastric foveolae, and few positive cells toward the neck zone. (**d**) C/EBPα (brown) and TFF1 (red) double staining, showing co-expression of the two proteins in gastric foveolae.

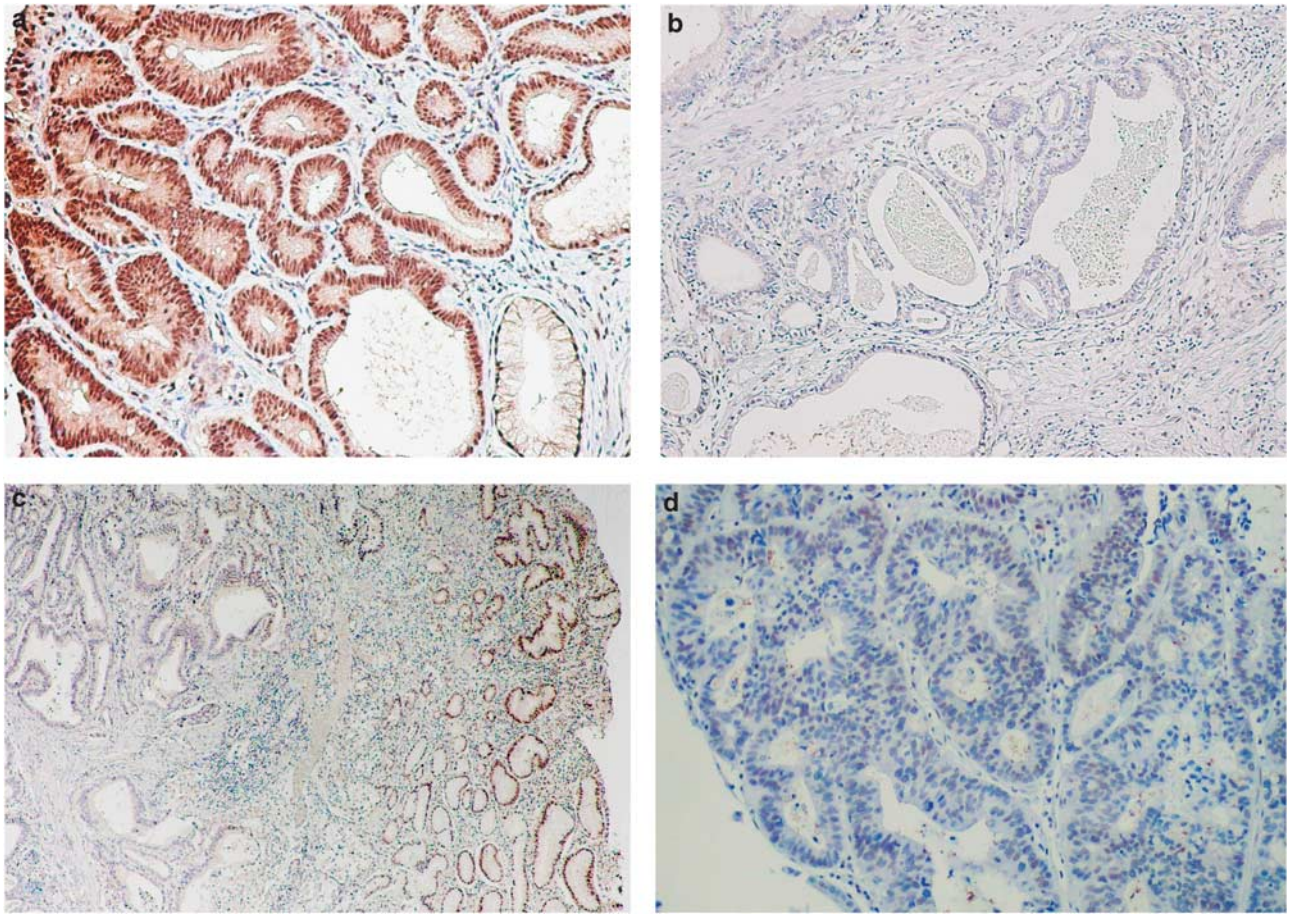


Figure 2 C/EBPα staining in intestinal-type GC. (**a**) C/EBPα positive tumor. (**b**) Tumor showing complete loss of C/EBPα expression. (**c**) GC displaying downregulation of C/EBPa expression (positive cells to the right are located in non-neoplastic gastric epithelium). (**d**) GC negative for C/EBPα expression.

(Figure 1b). This expression pattern was confirmed using immunofluorescence, where C/EBPα staining was again found to be stronger in the foveolar and surface epithelium, with fewer positive cells observed in the neck zone (Figure 1c). As described earlier, infiltrating inflammatory cells were also found to express C/EBPα. To confirm that C/EBPα expression does correlate with the differentiation status of the gastric epithelium, we performed double staining with TFF1, a well-established gastric differentiation marker. A clear overlap was observed between TFF1 and C/EBPα in the surface epithelium (Figure 1d).

Similarly to what was observed in the normal gastric mucosa, in GC C/EBPα staining was mostly nuclear with some residual cytoplasmic positivity (Figure 2a). In GC, C/EBPα was considered downregulated in 30% of the tumors (Figures 2b–d). No statistical significant relationships were found between C/EBPα expression and any clinicopathological features of the cases (Table 1).

Effect of C/EBPα Expression on Cell Proliferation and Differentiation

To assess the effect of C/EBPα on the proliferation status of GC cells, we transfected the C/EBPα-negative GC cell line AGS with an expression vector for the full-length C/EBPα gene and measured the incorporation of BRDU after 48 h. We observed that re-expression of C/EBPα on AGS cells led to a 15% reduction ($P=0.001$) in cell proliferation in comparison with the control (Figure 3a). Conversely, inhibition of C/EBPα by siRNA in the MKN28 cell line led to an increase ($P<0.001$) in cell proliferation in comparison with the control (Figure 3b).

To confirm this inhibitory effect of C/EBPα on proliferation, we analyzed by western blotting the expression of two cell-cycle proteins typically associated with the control of gastric epithelial cell division. We observed decreased expression of Cyclin D1, a cell-cycle inductor, and increased expression of P27, a cyclin-dependent kinase inhibitor (Figures 4a and c). Both these changes are consistent with an inhibitory effect on proliferation.

The results on the effect of C/EBPα on proliferation, together with its expression pattern in the normal gastric mucosa, suggested C/EBPα to have a role both on proliferation arrest and on the differentiation of gastric epithelial cells. That being the case, increased expression of TFF1 would be expected in the presence of higher levels of C/EBPα. In accordance with this hypothesis, after transfection of AGS

Table 1 Relationship between the clinicopathological features of GC and C/EBPα expression scoring

		C/EBPα downregulation		
	No. of cases (%)	Yes	No	*P*-value
Age (years)				
≤40	2 (4)	1 (50)	1 (50)	NS
40–65	18 (36)	2 (11.1)	16 (88.9)	NS
≥65	30 (60)	12 (40)	18 (60)	NS
Gender				
Male	30 (60)	11 (36.7)	19 (63.3)	NS
Female	20 (40)	4 (20)	16 (80)	NS
Histological type				
Intestinal	27 (54)	11 (40.7)	16 (59.3)	NS
Diffuse	16 (32)	1 (6.3)	15 (93.7)	NS
Atypical	7 (14)	3 (42.9)	4 (57.1)	NS
Depth of invasion				
T1	2 (4)	0 (0)	2 (100)	NS
T2	26 (52)	10 (38.5)	16 (61.5)	NS
≥T3	22 (44)	5 (22.7)	17 (77.3)	NS
Vascular invasion				
Absent	15 (30)	5 (33.3)	10 (66.7)	NS
Present	35 (70)	10 (28.6)	25 (71.4)	NS
Metastasis				
Absent	12 (24)	6 (50)	6 (50)	NS
Present	38 (76)	9 (23.7)	29 (76.3)	NS
Total	50 (100)	15 (30)	35 (70)	

NS, non-significant.

Cases are classified according to the intensity and percentage of positive cells. Cases classified as 'downregulated' present >50% of tumor cells classified as I.

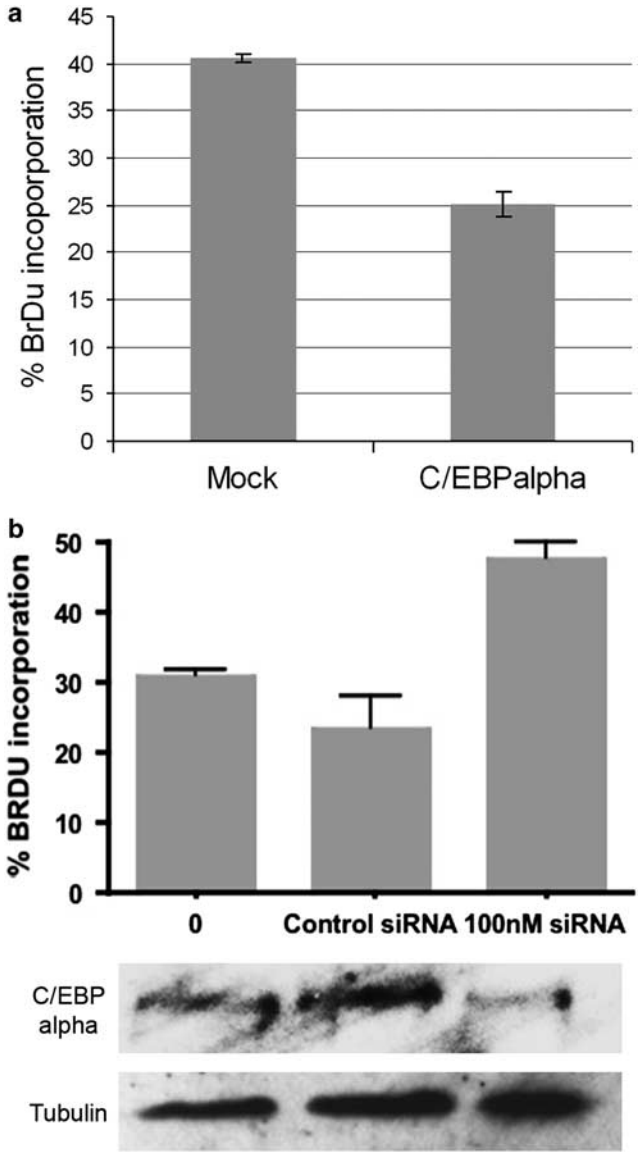


Figure 3 BRDU incorporation assay in GC cells. (**a**) Decreased proliferation rates of C/EBPα-transfected AGS cells in comparison with the control ($P = 0.001$). (**b**) C/EBPα inhibition by siRNA leads to increased BRDU incorporation in MKN28 cells. In all, 1000 cells were counted and BRDU incorporation expressed as the rate between DAPI and BRDU positive cells. The *y* axis represents the % of BRDU positive cells. Error bars represent s.d. Tubulin was used as protein-loading control.

cells with the C/EBPα expression vector, we observed an increase in the expression of TFF1 (Figures 4b and c).

Effect of MAPK Inhibitors on the Expression of C/EBPα and Cell Proliferation

The Ras/MAPK signaling pathway is one of the most consistently altered in human cancers. In GC, the Ras/MAPK pathway is constitutively activated through mutation of several of its receptors and signal-transducing members.[13] To explore the possibility of C/EBPα regulation by the Ras/MAPK pathway in GC, we treated MKN28 cells, which express C/EBPα, with specific p38 (SB239063) and ERK1/2 (PD98059) inhibitors. Treatment with both inhibitors led to a marked increase in C/EBPα expression and nuclear localization as detected by immunocytochemistry (Figure 5). This increase in C/EBPα expression was further confirmed by western blotting, and shown to be accompanied by an increase in TFF1 expression (Figures 6a and c). Concomitantly, we observed a decrease in cell proliferation by BRDU incorporation (Figure 6b) both in cells treated with p38 inhibitor ($P = 0.009$) and in cells treated with ERK1/2 inhibitor ($P = 0.003$). This decrease in proliferation was accompanied by a decrease in Cyclin D1 expression (Figure 6c).

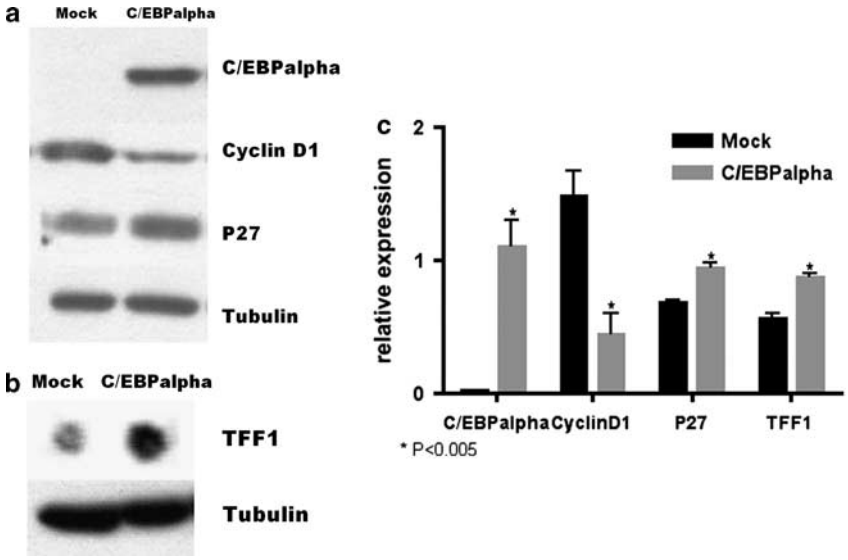


Figure 4 Effect of C/EBPα expression on AGS cells. (**a**) Western blot for cell-cycle proteins, showing increased P27 and decreased cyclin D1 expression after transfection with C/EBPα. (**b**) Dot blot showing increased TFF1 expression in C/EBPα-transfected cells. (**c**) Expression of C/EBPα, cyclin D1, p27, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between mock- and C/EBPα-transfected cells.

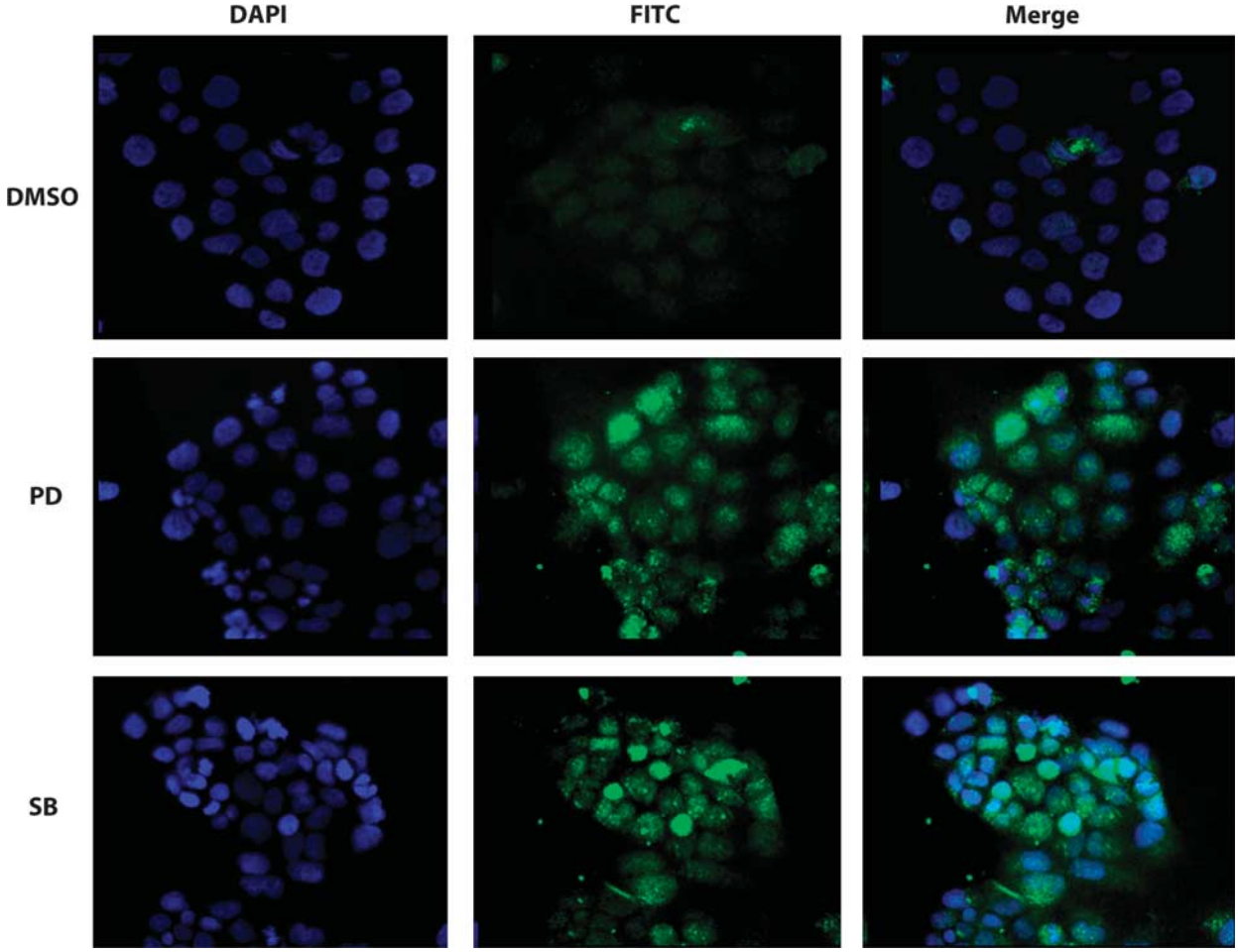


Figure 5 Treatment of MKN28 cells with p38 (SB) and ERK1/2 (PD) inhibitors leads to an increase in C/EBPα expression with nuclear localization. C/EBPα is stained green with FITC and nuclei are stained blue with DAPI for contrast.

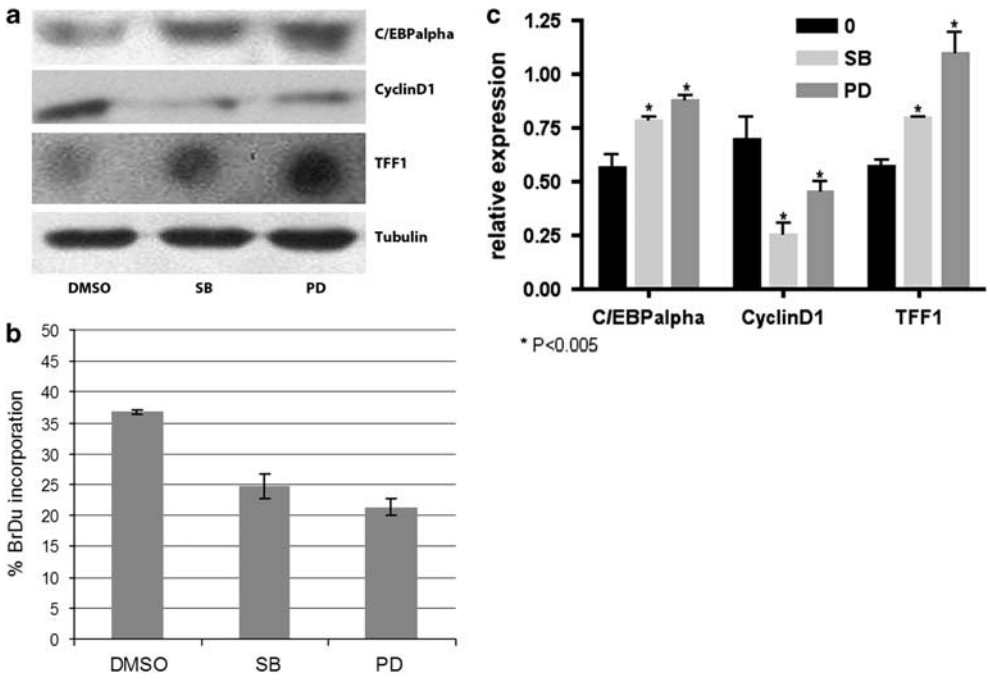


Figure 6 Effects of the treatment of MKN28 cells with a p38 (SB) and an ERK1/2 (PD) inhibitor in cellular proliferation and differentiation. (**a**) Western blot showing that treatment of MKN28 cells with SB and PD leads to an increase in C/EBPα and TFF1 expression and to a decrease in Cyclin D1 levels. (**b**) Decrease of cell proliferation by BRDU incorporation assay of MKN28 cells treated with SB ($P = 0.009$) and PD ($P = 0.003$) inhibitors. (**c**) Expression of C/EBPα, cyclin D1, and TFF1 shown as ratios to loading controls. Error bars represent s.d. *represents statistically significant differences between treated and non-treated cells.

DISCUSSION

We have shown that C/EBPα is expressed in the differentiated epithelial compartment of the superficial gastric mucosa. This expression pattern mirrors that described for C/EBPβ, which is expressed in the proliferative neck zone of the normal gastric mucosa. We have previously argued that C/EBPβ may have a role in maintaining a balance between proliferation and differentiation in the normal gastric mucosa.[3] In the proposed model, C/EBPβ would have a pro-proliferative activity in gastric epithelial stem-like cells. The presence of C/EBPα in differentiated cells, together with its ability to reduce cell proliferation and to upregulate the gastric differentiation marker TFF1, suggest that C/EBPβ and C/EBPα may have complementary roles in maintaining a balance between proliferation and differentiation in the normal gastric mucosa. By analogy to the model of adipogenesis,[6] one feels tempted to speculate that C/EBPβ is expressed in gastric epithelial stem-like cells and may prime gastric epithelial cells to differentiate by inducing C/EBPα expression. Once active, C/EBPα would reduce cell proliferation, and promote the expression of gastric differentiation markers such as TFF1.

C/EBPα was first described as a tumor suppressor gene in acute myeloid leukemias. In normal hematopoiesis, C/EBPα has a key role in defining cell lineages through interaction with other transcription factors. C/EBPα disruption by mutation leaves bone marrow cells in an undifferentiated, hyperproliferative state being this event causal for a large percentage of leukemias.[14] Downregulation of C/EBPα was additionally found in several epithelial tumor types, namely lung, breast, and skin carcinomas.[11,12,15,16] In all these examples, a role for impaired C/EBPα function in tumourigenesis was strengthened by the observation that C/EBPα re-expression is able to inhibit tumourigenesis both *in vivo* and *in vitro*.[15,16]

In our study, we observed downregulation of C/EBPα in about 30% of GC cases. In an earlier study, we have described a frameshift mutation of C/EBPα in a GC. This mutation was deleterious and absent from adjacent non-neoplastic tissue.[17] These results in the GC model are in keeping with the aforedescribed role of C/EBPα in tumourigenesis, whereby loss of C/EBPα would be associated to loss of differentiation and sustained proliferation of tumor cells. On top of C/EBPα loss of expression, we have shown earlier that C/EBPβ is over-expressed in cells retaining a proliferative phenotype such as those seen in dysplastic and cancer lesions. C/EBPβ is able to counteract, either by heterodimerization or repression of expression, the differentiating activity of C/EBPα. Altogether, either aberrant over-expression of C/EBPβ or loss of expression of C/EBPα are present in the majority of GC cases. Hence, these results suggest that changes in expression/function of both C/EBPα and C/EBPβ may be pieces of the same puzzle rather than independent events in gastric carcinogenesis. This possibility, together with other putative mechanisms of post-translational or protein–protein interaction, would help explaining why expression of C/EBPα is still seen in about 70% of GC cases.

In other cancer models, loss of C/EBPα has been linked with oncogenic Ras activation.[16] In GC, activating RAS mutations do occur in a subset of microsatellite unstable tumors.[13] By using specific inhibitors for p38 and ERK1/2, both downstream effectors of Ras signaling, we were able to show that inhibition of C/EBPα expression was dependent on the activation of this pathway. Moreover, inhibition of p38 and ERK1/2 increased TFF1 expression and strongly reduced MKN28 cell proliferation and Cyclin D1 levels, in a set of alterations most likely linked with the observed increase in C/EBPα expression.

In summary, we show that in normal gastric mucosa, C/EBPα is expressed mainly in the differentiated foveolar epithelium where it co-localizes with TFF1. We show that C/EBPα is downregulated in a considerable percentage of GC. We additionally show that C/EBPα re-expression in a C/EBPα-negative cell line leads to a reduction in proliferation that is accompanied by an increase in P27 and reduction of cyclin D1 levels. In parallel, we show an increase in the expression of TFF1 in C/EBPα-transfected cells. Finally, we show that treatment of a C/EBPα expressing cell line with MAPK inhibitors leads to increased C/EBPα and TFF1 expression, and a concomitant reduction on cell proliferation and Cyclin D1 expression. Overall, these results substantiate the role of the C/EBP transcription factor family in homeostasis of the gastric epithelium and in the process of gastric carcinogenesis.

ACKNOWLEDGEMENT

This work was supported by Sixth Research Framework Programme of the European Union, Project INCA (LSHC-CT-2005-018704).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Peek Jr RM, Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol 2006;208:233–248.
2. Nerlov C. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. Trends Cell Biol 2007;17:318–324.
3. Regalo G, Canedo P, Suriano G, *et al.* C/EBPbeta is over-expressed in gastric carcinogenesis and is associated with COX-2 expression. J Pathol 2006;210:398–404.
4. Dossinger V, Kayademir T, Blin N, *et al.* Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors. Cell Physiol Biochem 2002;12:197–206.
5. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002;365:561–575.
6. Rosen ED. The transcriptional basis of adipocyte development. Prostaglandins Leukot Essent Fatty Acids 2005;73:31–34.
7. Payne VA, Au WS, Gray SL, *et al.* Sequential regulation of diacylglycerol acyltransferase 2 expression by CAAT/enhancer-binding protein beta (C/EBPbeta) and C/EBPalpha during adipogenesis. J Biol Chem 2007;282:21005–21014.
8. Tang QQ, Zhang JW, Daniel Lane M. Sequential gene promoter interactions by C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis. Biochem Biophys Res Commun 2004;318:213–218.
9. Pabst T, Mueller BU, Zhang P, *et al.* Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet 2001;27: 263–270.
10. Schuster MB, Porse BT. C/EBPalpha: a tumor suppressor in multiple tissues? Biochim Biophys Acta 2006;1766:88–103.
11. Costa DB, Li S, Kocher O, *et al.* Immunohistochemical analysis of C/EBPalpha in non-small cell lung cancer reveals frequent down-regulation in stage II and IIIA tumors: a correlative study of E3590. Lung Cancer 2007;56:97–103.
12. Tada Y, Brena RM, Hackanson B, *et al.* Epigenetic modulation of tumor suppressor CCAAT/enhancer binding protein alpha activity in lung cancer. J Natl Cancer Inst 2006;98:396–406.
13. Brennetot C, Duval A, Hamelin R, *et al.* Frequent Ki-ras mutations in gastric tumors of the MSI phenotype. Gastroenterology 2003;125:1282.
14. Nerlov C. C/EBPalpha mutations in acute myeloid leukaemias. Nat Rev Cancer 2004;4:394–400.
15. Gery S, Tanosaki S, Bose S, *et al.* Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer. Clin Cancer Res 2005;11:3184–3190.
16. Shim M, Powers KL, Ewing SJ, *et al.* Diminished expression of C/EBPalpha in skin carcinomas is linked to oncogenic Ras and reexpression of C/EBPalpha in carcinoma cells inhibits proliferation. Cancer Res 2005;65:861–867.
17. Resende C, Regalo G, Durães C, *et al.* Genetic changes of CEBPA in cancer: mutations or polymorphisms? J Clin Oncol 2007;25: 2493–2494.

Article II

C/EBPβ/RUNX1t1 regulatory loop controls cell proliferation in gastric cancer.

Goncalo Regalo[1*], Susann Förster[1], Carlos Resende[4], Bianca Bauer[5], Barbara Fleige[6], Wolfgang Kemmner[1], Peter M Schlag[7], Kosei Ito[8], Suk-Chul Bae[9], Thomas F. Meyer[5], Yoshiaki Ito[10], José C Machado[4], Achim Leutz[1, 2, 3*]

[1] Max-Delbrueck-Center for Molecular Medicine, 13125 Berlin, Germany

[2] Humboldt-University of Berlin, Institute of Biology, 10115 Berlin, Germany

[3] Berlin-Brandenburg Center for Regenerative Therapies, 13353 Berlin, Germany

[4] Institute of Pathology and Molecular Immunology of the University of Porto, 4200-465 Porto, Portugal

[5] Max-Planck Institute for Infection Biology, 10117 Berlin, Germany

[6] Institut für Gewebediagnostik Berlin am MVZ des HELIOS Klinikum, 13125 Berlin, Germany

[7] Charité Comprehensive Cancer Centers, Charité – Universitätsmedizin, 10117 Berlin, Germany

[8] Department of Molecular Bone Biology, Nagasaki University, 852-8588 Nagasaki, Japan

[9] Department of Biochemistry, Chungbuk National University, 361-763 Cheongju, South Korea

[10] Cancer Science Institute of Singapore, National University of Singapore, 117599 Singapore

*Correspondence:
Goncalo Regalo & Achim Leutz
goncalo.regalo@mdc-berlin.de
aleutz@mdc-berlin.de

Mailing address:
Max-Delbrueck-Center for Molecular Medicine
Robert-Roessle-Str.10
13125 BERLIN
Germany

Phone: +49 30 9406 3735
Fax: +49 30 9406 3298

Abstract

Background: The transcription factor C/EBPβ represents a central hub in cell differentiation/proliferation control and is overexpressed in gastric cancer. Here we aim at understanding the relevance of C/EBPβ expression to gastric homeostasia and tumorigenesis, and to unravel novel related molecular pathways in gastric carcinogenesis.

Methods: C/EBPβ and Runx3 single and double knockout mice. Cross-species gene expression profiling with human gastric cancer samples and mouse knockout stomachs. ShRNA-based knockdown of C/EBPβ expression in gastric cancer cells.

Results: We show that the murine C/EBPβ knockout stomach displays changes in the homeostatic balance between cell differentiation and proliferation. Tumorigenesis was suppressed by knockdown of C/EBPβ in human-murine xenograft tumor models and by C/EBPβ deletion in a mouse model of gastric hyperproliferation. Cross-species comparison of gene expression profiles revealed a subset of tumors that are characterized by a strong C/EBPβ-regulation. Within this tumor set expression signature the tumor suppressor RUNX1t1 was identified, and shown to be downregulated in 38% of gastric tumors. Finally we show that the tumor suppressor activity of RUNX1t1 is mechanistically connected to C/EBPβ functions in gastric cancer cells

Conclusions: C/EBPβ expression is confirmed as being mandatory for gastric cancer cell proliferation, and a new C/EBPβ-related tumor suppressor gene, RUNX1t1 is identified, as well as of a subset of gastric cancer with a clear genetic signature and a potential molecular mechanism.

Keywords: C/EBPβ; Gastric Cancer; Transcription factor; RUNX1t1; proliferation

Background

Gastric cancer is among the leading causes of death by cancer worldwide, yet the molecular pathways in the etiology of gastric cancer remain elusive [1]. The majority of the gastric tumors belong to the intestinal sub-type, characterized by expansive growth and maintenance of a glandular structure. Diffuse-type gastric cancer is associated with loss of the adhesion protein E-Cadherin, however, despite the predominance and histological coherence of the intestinal type of gastric cancer no central common molecular pathway has been convincingly shown as aberrantly regulated [2-4].

The transcription factor C/EBPβ has been suggested to play a pro-oncogenic role [5-14]. In intestinal-type gastric cancer, C/EBPβ is highly expressed and associated with both, enhanced Cyclooxygenase-2 (COX2) expression and loss of the mucous-associated protein Trefoil Factor 1 (TFF1) [15, 16] [17-20]. Mice that over-express COX2 or are deficient for TFF1 develop gastric tumors, underscoring the destabilizing potential of the enhanced expression of C/EBPβ in gastric carcinogenesis [21, 22]. Nevertheless, a causal role of C/EBPβ in the development of gastric cancer has never been determined.

Here, we examined the functions of C/EBPβ in the murine stomach. Our results show that C/EBPβ controls the balance between proliferation and differentiation in the murine stomach. C/EBPβ expression is also mandatory for hyperproliferation in the RUNX3 KO mucosa. Cross-species analysis of gene expression between mouse C/EBPβ KO stomachs and human gastric cancer identified a C/EBPβ regulated gene signature in a sub-group of intestinal-type tumors. Within this signature, RUNX1t1 stood out as a potential tumor suppressor. RUNX1t1 inhibits C/EBPβ functions and ectopic expression of RUNX1t1 reduced pr oliferation in gastric cancer cell lines. The RUNX1t1 promoter was found to be frequently hypermethylated in human gastric cancer cases. Our data suggest C/EBPβ activation and RUNX1t1 silencing as important events in the process of gastric carcinogenesis.

Methods

Human gastric cancer samples and microarray data

Human tissue samples were derived from patients that had undergone resection for sporadic gastric adenocarcinoma at the Robert Roessle Hospital (1995–2003). The selection of samples, the procedure for histological classification and staging, the second blinded evaluation by an independent pathologist including assessment of tumor content in the pieces that RNA was extracted from, as well as RNA extraction and microarray procedure has been described elsewhere [25].

Transgenic mice

C/EBPβ knockout (KO) animals were previously established in C57-Bl6 background [36]. Bl6 RUNX3 KO mice were obtained from the group of Prof. Ito [26], and crossed with C/EBPβ KO mice. Due to the lethal phenotype of the single RUNX3 KO, C/EBPβ/RUNX3 heterozygote animals were bred and the phenotype analyzed in the offspring at birth. Animals were bred and kept according to the institutional guidelines, and genotyped by PCR as previously described [26, 36].

*C/EBP*β *knockdown cells and in vivo tumorigenic assay*

MKN45 and MKN74 cells were infected with lentivirus containing GFP-tagged control shRNA and shRNA against C/EBPβ. Knockdown efficiency was assessed by Western Blot and proliferation was measured by BrdU incorporation assay. The effect of C/EBPβ expression on tumor formation was examined by subcutaneously implanting 3×10^6 cells of both control MKN74/45 and ShRNA-mediated C/EBPβ-silenced MKN74/45 into 6-8-week-old male NIH(s) II-nu/nu nude mice, four mice per group. The animals were monitored weekly for tumor formation for 20 days after inoculation. Tumor sizes in two dimensions were measured with calipers, and volumes were calculated with the formula (a × b2) × 0.5, wherein “a” is the long axis and “b” is the short axis (in millimeters). Mice were maintained and sacrificed according to institutional guidelines, and at termination of the experiment

tumors were excised, fixed, embedded and analyzed by immunohistochemistry for Ki67 and C/EBPβ expression.

Co-immunoprecipitation

Flag-tagged RUNX1t1 was expressed in MKN28 and MKN45 cell lines. Cells were harvested and lysed in buffer containing 50 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 0.2% NP-40, 5 mM $MgCl_2$, 50 μM, $ZiCl_2$ and protease inhibitor cocktail (Roche®). Protein lysates were incubated at 4 °C with Protein A sepharose beads (Sigma®) for 1h. Beads were then washed 4 times in lysis buffer and examined by Western Blot analysis.

Immunohistochemistry

Stomachs were obtained from three month-old C/EBPβ knockout (KO) mice, and newborn C/EBPβ -, RUNX3 - and compound C/EBPβ/RUNX3 KO animals. Stomachs were longitudinally excised, formalin-fixed and embedded in paraffin. Gastric cancer tissue microarrays were obtained as described elsewhere [37].

Serial sections were obtained, deparaffinized and stained with Hematoxylin and Eosin, examined by a pathologist, and measured. An additional group of sections were treated with 2M (I always use 10mM) citrate buffer and stained with 1:100 anti-Ki67 (DAKO), 1:500 anti-C/EBPβ,1:50 anti-TFF1 (Santa Cruz Biotechnology), or 1:500 anti-RUNX1t1 (Sigma®) antibody. After washing with PBS with 0.02% Tween and incubation with horseradish peroxidase-bound secondary antibody (GE Healthcare®) development was performed using di-amido-benzidine.

BrdU assay

Cells with stable C/EBPβ knockdown were sorted and plated to 40% confluence. Cells were also transfected with RUNX1t1 and analyzed for BrdU incorporation after 48 h. Briefly, cells were incubated with 1M Bromo-deoxy-uridine for 20 min. and then trypsinized and harvested in ice-cold PBS. Cells were then fixed, permeabilized, and stained with fluorescent anti-BrdU antibody according to the APC-BrdU flow kit protocol (BD Biosciences®).

Dead cells were stained with 7-AAD and BrdU-positivity was then assessed by flow cytometry.

Total RNA extraction, cDNA synthesis and quantitative real-time PCR

For RNA extraction from mouse tissue, stomach sections were frozen in liquid nitrogen after excision, and finely grinded in a mortar. For RNA extraction from gastric cancer cells, these were harvested in ice-cold PBS and pelleted at 2000 rpm. Lysis buffer was then added to the obtained powder or to the pellet which was then vigorously resuspended using a 3ml syringe. RNA was extracted using a universal RNA extraction kit (Roboklon®). RNA was quantified, cDNA synthesized by standard methods and SYBER green quantitative real-time PCR performed (see supplementary table 4 for primer sequences).

Plasmids

For the construction of C/EBPβ isoform expression vectors, LAP*, LAP and LIP were cloned from human cDNA by PCR, following digestion with restriction enzymes, ligation into pcDNA3-flagged plasmid and ampicillin selection. TFF1-luciferase reporter plasmid was similarly cloned from human cDNA into a pGL3-basic plasmid. RUNX1t1 expression plasmid (pCMV-3xFlag-ETO) was obtained from ADDGENE® (ref: #12507).

For the construction of C/EBPβ knockdown vectors, shRNA (5'-gccgcgacaaggccaagatgc-3') was inserted into a pLVTH-M lentivral vector.

Tissue culture, transfection, and luciferase assays

MKN28, MKN45 and MKN74 cell lines were grown in RPMI medium (Gibco®). For transfection, cells were trypsinized, seeded, and grown to 50-60% confluence. C/EBPβ isoform plasmids and/or RUNX1t1 plasmid were resuspended in serum-free medium with transIT (Myrus®) transfection reagent and added to the cells. Protein and RNA were extracted after 48 h and analyzed by Western Blot and real-time PCR.

RUNX1T1 promoter methylation analysis

Methylation analysis of the RUNX1t1 promoter was determined by methylation-specific PCR (MSP), as previously described [31]. MSP method distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracil. Subsequently, PCR using primers specific to either methylated or unmethylated DNA was performed. Genomic DNA (350ng) was bisulfite-treated and purified with EZ DNA Methylation Kit Gold (Zymo Research, CA, USA®). The primer sequences of RUNX1t1, for both methylated and unmethylated reactions were as previously described [31]. 100ng of bisulfite-modified DNA was used in each PCR. Amplification was carried out for 36 cycles (30 s at 95 °C, 30 s at 56 °C, and then 30 s at 72 °C). Control PCRs lacking genomic DNA were performed for each set of reactions. Amplified products were separated by electrophoresis in a 2.5% agarose gel.

Electrophoretic Mobility Shift Assay (EMSA)

MKN28 and MKN45 cells were transfected with increasing amounts of RUNX1t1. Nuclear extracts were prepared from transfected cells, quantified, and incubated with previously radioactively labeled (α-32P dCTPs) nucleotides, containing an optimized C/EBPβ binding sequence. Protein/Labeled-DNA complexes were then run in a 15% acrylamide gel in non-denaturing conditions and binding intensity assessed by intensity of radioactive signal. Anti-C/EBPβ antibody was added to the protein/labeled-DNA complex as a control, and a supershift was observable, confirming that it was C/EBPβ what bond to DNA. Competition with non-labeled C/EBPβ binding sequence, confirmed the specificity of the observed signal.

Bioinformatic microarray data analysis and statistical analysis

The raw data files (.text files for murine Agilent Technologies® arrays and .cel files for human Affymetrix GeneChips®) were imported into GeneSpring GX 12.1 software (Agilent Technologies®) as two separate species-specific experiments. All subsequent microarray data analyses were performed using this software. Preprocessing (background correction, normalization and probe summarization) was performed according to the RMA algorithm followed by

baseline transformation to the median of all samples (in one experiment). Quality control was done by assessment of inter-array correlation analysis calculating the correlation coefficient of each array to every other one. By this means, one array of the murine gene expression experiment was identified to show relatively weak correlation to most of the other samples and thus excluded from further analysis. The human arrays yielded correlation coefficients between 0.829 and 0.972, with an arithmetic mean of 0.917 and the murine arrays between 0.991 and 0.924 with a mean of 0.9. In the murine array experiment, only probes owning “detected” flags in at least 3 arrays (34,150 probes) were used for further analyses. Genes whose expression between groups of samples was significantly different were identified by Welch-test with $p \leq 0.01$ being the significance cut-off. The fold change (FC) of expression between groups was calculated as the fold difference between group means. Gene annotation information was obtained from GeneSpring GX software (state of 08/2012). For hierarchical clustering, ‘Euclidean distance’ and ‘complete linkage’ were used as distance metric and linkage algorithm. The migration of genes between the murine and human microarray experiment was performed using the Orthology Search Tool of bioDBnet athttp://biodbnet.abcc.ncifcrf.gov/.

Microarray data is available at:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59585

Results

C/EBPβ knockdown reduces the tumorigenic potential of gastric cancer cells

C/EBPβ expression in gastric cancer was evaluated by real-time PCR, and shown to be enhanced predominantly in intestinal-type gastric cancer (Figure 1A), confirming the previously published data [15,16]. The functional importance of high C/EBPβ expression in gastric cancer was examined by stable knockdown in human gastric cancer cell lines using a viral-based GFP-

tagged short hairpin RNA. C/EBPβ-isoform knockdown efficiency in two cell lines approximated 70%, as confirmed by protein immunoblotting (Figure 1B). Proliferation in two cell lines (MKN74, MKN45) were examined by BrdU incorporation and, as shown in Figure 1C, proliferation of both cell lines was reduced after C/EBPβ knockdown.

The tumorigenic potential of both cell lines, before and after C/EBPβ knockdown, was compared by xenotransplantation in immune-compromised mice, as shown in Figure 1D. Equal numbers of freshly sorted control and knockdown MKN74 or MKN45 cells were injected. Twenty days post-injection, C/EBPβ knockdown cells formed markedly smaller tumors than parental cells, with less weight and volume (Figure 1D). The difference was more pronounced in MKN74 than in MKN45 cell line. Ki67 staining showed reduction of cell proliferation in tumors originating from C/EBPβ knockdown cells in comparison to controls (Figure 1C). Interestingly, proliferation in tumors was accompanied by re-expression of C/EBPβ and, in tissue culture knockdown cells required frequent sorting to prevent overgrowth of cells that regained C/EBPβ expression, suggesting selection for C/EBPβ re-expression. These results show that C/EBPβ plays an important role in gastric cancer cell proliferation.

C/EBPβ knockout mice display imbalanced differentiation/proliferation of the gastric mucosa

Analysis of nullizygous C/EBPβ stomachs (n=5) revealed a small but significant ($p<0.001$) reduction in the thickness of the antral gastric mucosa and diminished numbers of Ki67-positive cells, as compared to the wild type (WT) (n=8). No other histological abnormalities were observed, being the corpus region from the knockout largely indistinguishable from the WT. To gain further insight into the causes of reduced mucosa thickness, expression of cell cycle-related genes and apoptosis rates were examined. As shown in Figure 2C, reduction of Ki67 and of proliferating cell nuclear antigen (PCNA) in the KO antral mucosa was evident by quantitative PCR (qPCR) in accordance to histological observations. Additionally, reduced expression of

Cyclin A1, Cyclin D3 and Cyclin E1, and increased expression of the CDK inhibitor p15 was found. Apoptosis rate of the C/EBPβ KO mucosa, as revealed by TUNEL assay, showed enhanced cell death in C/EBPβ KO mice (n=5) compared to WT (n=8) (supplementary Figure 1A) and qPCR expression analysis showed decreased expression of BCL2 and BIRC5 (survivin) (supplementary figure 1B).

C/EBPβ has previously been suggested to repress the gastric differentiation marker and tumor suppressor TFF1 [23, 24]. Similarly to human gastric mucosa, expression of TFF1 was excluded from proliferating cells of the neck zone in murine WT gastric epithelium and expression of C/EBPβ and TFF1 was mutually exclusive (Figure 2D, upper panel). Increased expression of TFF1 in C/EBPβ KO mucosa was confirmed by qPCR, similar to the gastric differentiation marker MUC5AC (Figure 2D, lower panel). Taken together, these data confirmed a general repressive role of C/EBPβ on gastric differentiation genes expression [23, 24] and regulation of apoptosis in the normal gastric mucosa.

Cross-species gene expression profiling reveals a subset of intestinal-type gastric tumors with a C/EBPβ regulated signature

The apparent similarities between human and murine gastric C/EBPβ biology raised the question whether the homeostatic and oncogenic C/EBPβ-dependent proliferation share common molecular mechanisms. We therefore compared the gene expression profiles derived from C/EBPβ KO mice with previously analyzed human gastric adenocarcinoma samples [25].

Differentially expressed genes between the C/EBPβ KO (n=5) and WT (n=4) mice were identified by Welch-test. Significance in differential expression was accepted at $p \leq 0.01$ and a fold change (FC) of larger than >1.5. These cut-off criteria yielded 171/25 annotated/non-annotated unique transcripts (represented in 233 probes) as upregulated in the C/EBPβ KO and 79/12 annotated/non-annotated unique transcripts (represented in 135 probes) as

downregulated (supplementary Table 1 and 2 show the 20 most significantly regulated genes).

Next, the combined list of up- and downregulated genes (FC>1.5, p≤0.01) derived from the C/EBPβ KO mouse profiling data was used to cluster human gastric cancer microarray samples. The resulting gene expression heatmap suggested that the majority of genes did not show any overt deregulation in human cancers (whitish spots in heatmap). However, a group of genes showed explicit regulation (indicated by dark bluish and reddish spots in the heatmap) across the human cancer samples (Supplementary Figure 2, regulated gene cluster, indicated by box). Genes contained in this subset were then used to re-cluster the human cancer samples. The resultant cancer sample dendrogram and expression heatmap (Figure 3) revealed a group of cancer samples (Figure 3, black box) that exhibit downregulation of the majority of these genes. The group consisted of 16 of the original 59 (≈27%) samples and contained primarily cancers of the intestinal histological type. Importantly, genes downregulated in this particular cancer subgroup are mostly upregulated in the C/EBPβ KO gastric mucosa (changes ranging from 1.5 to 2.3 fold; Table 1), identifying them as C/EBPβ repressed genes.

In order to validate the results obtained by microarray comparison, we selected three C/EBPβ repressed genes, FOG2, SPARCL1, and RUNX1t1, and analyzed their expression by qPCR. Examination of WT and C/EBPβ KO stomach confirmed upregulation of these genes in the gastric mucosa of C/EBPβ KO mice (5 animals/group; supplementary figure 3B). It was also important to examine the expression of FOG2, SPARCL1, and RUNX1t1 in normal human gastric mucosa as no normal tissue samples were available for the initial human gastric cancer microarray analysis [25]. As shown in supplementary Figure 3A, expression of all three genes was downregulated in intestinal-type gastric cancer in comparison to normal tissue, however, a subset of diffuse-type tumors overexpressed RUNX1t1, compliant with the different etiology of these tumors.

C/EBPβ expression is mandatory for the hyperplastic phenotype in the RUNX3 KO mice stomach

The RUNX3 KO mouse is an established model of early gastric cancer initiation and hyperproliferation [26], although the mechanism underlying the RUNX3-deficient neoplastic phenotype remains under debate [27, 28]. As RUNX3 KO mice die shortly after birth, all the analysis was performed in newborn mice. As shown in Figure 4A, Ki67 staining confirmed increased proliferation of the epithelial stomach layer of newborn RUNX3-null mice and E-Cadherin staining confirmed the epithelial nature of the proliferating cells. Staining of serial longitudinal sections showed high expression and colocalization of C/EBPβ and Ki67 in the hyperproliferative gastric mucosa of the RUNX3 KO (Figure 4A).

C/EBPβ null animals were crossed with RUNX3 KO mice to determine the functional contribution of C/EBPβ in the neoplastic RUNX3 KO stomach tissue. Analysis of the stomach tissue of single RUNX3 KO and the compound C/EBPβ/RUNX3 KO showed that in the double KO animals stomach wall thickness was reduced back to WT levels. Ki67 staining confirmed almost complete reversion of the hyperproliferative phenotype by removal of C/EBPβ in RUNX3 KO (Figure 4A and 4B) that was accompanied by a substantial increase in the number of apoptotic cells (supplementary Figure 4A and 4B). These results strongly suggest that expression of C/EBPβ is mandatory for the neoplastic gastric phenotype of RUNX3 deficient mice.

In order to understand if downstream gene regulation associated with the reversion of gastric hyperproliferation by deletion of C/EBPβ would reflect the gene signature we previously identified by cross species gene expression analysis, we compared expression of C/EBPβ target genes in RUNX3 KO and compound C/EBPβ/RUNX3 KO. Whereas mucosal expression of all three genes (FOG2, SPARCL1, RUNX1t1) was enhanced in C/EBPβ KO (data not shown) only RUNX1t1 also displayed reduced expression in the hyperproliferative RUNX3 KO mucosa. Importantly, RUNX1t1 expression was partially rescued by removal of C/EBPβ in the compound KO, as shown in Figure 4C, suggesting RUNX1t1 to be inversely correlated with C/EBPβ in

association with proliferation control. Indeed, transfecting C/EBPβ isoforms (LAP*, LAP and LIP) into MKN28 and MKN45 cell lines led to the repression of RUNX1t1 expression (Figure 4D), further suggesting C/EBPβ mediated repression of RUNX1t1.

RUNX1t1 plays a tumor suppressive role in human gastric cancer and modulates C/EBPβ activity

Expression of RUNX1t1 protein was evaluated by tissue microarray immunohistochemistry on 64 human gastric cancer samples. Nuclear staining was classified as strong, moderate, weak or absent, relative to the expression of RUNX1t1 in the normal mucosa (classified as moderate). From the analyzed tumors, 25 out of 64 (38%) showed weak or absent RUNX1t1 protein staining (Figure 5A). To further assess whether C/EBPβ is responsible for downregulation of RUNX1t1 in gastric tumors, we selected tumor-RNAs showing reduced levels of RUNX1t1 (supplementary Figure 3A). The majority of cases (7 out of 10, Figure 5B), however, failed to show a convincing inverse correlation between low RUNX1t1 and high C/EBPβ expression, suggesting alternative means of RUNX1t1 downregulation in gastric cancer. Sequencing of RUNX1t1 from 26 gastric cancer patients failed to disclose mutations that would explain loss of RUNX1t1 protein (data not shown), however, analysis of the RUNX1t1 promoter by methylation-specific PCR revealed hypermethylation in the majority of the gastric cancer DNA samples (Figure 5C). Next, we examined the functional consequences of RUNX1t1 downregulation in gastric cancer. As shown in Figure 5D, overexpression of RUNX1t1 in MKN28 and MKN45 gastric cancer cell lines led to decreased cell proliferation, as determined by BrdU incorporation. These data suggest that RUNX1t1 inhibits proliferation and is frequently downregulated in gastric cancer.

RUNX1t1 has previously been reported to interact with C/EBPβ, to inhibit its DNA binding, and to block its pro-proliferative functions during the clonal expansion phase in adipogenic differentiation (Rochford et al, MCB 2004).

Ectopic expression of flag-tagged RUNX1t1 in MKN28 and MKN45 cell lines and subsequent immunoprecipitation showed that RUNX1t1 interacts with all endogenous C/EBPβ isoforms in both cell lines (Figure 5E). Electrophoretic mobility shift assay (EMSA) led to a dose dependent decrease of C/EBPβ binding to its DNA consensus sequence in both cell lines, although RUNX1t1 did not significantly alter nuclear C/EBPβ expression (Figure 5F). These results suggest that the tumor-suppressive function of RUNX1t1 is mechanistically connected to the suppression of pro-oncogenic C/EBPβ functions.

Discussion

Our data suggest a causal function of C/EBPβ in the development of a subset of gastric tumours. Comparison of gene expression profiles from C/EBPβ KO mice and human gastric cancer samples provided mechanistic insight in C/EBPβ-related molecular mechanisms.

Data presented here suggest that the function of C/EBPβ in gastric cancer is embedded in the homeostatic regulation of the gastric mucosa. Absence of C/EBPβ from the murine stomach shifts the balance from epithelial proliferation towards differentiation and apoptosis. Deregulation of pathways that sustain C/EBPβ functions such as inflammatory signals may favor uncontrolled proliferation and repression of differentiation genes such as TFF1 that ultimately promotes tumor development [22]

C/EBPβ is mandatory for the hyperproliferative phenotype of the RUNX3 KO mice and for the tumorigenic potential of gastric cancer cell lines. Expression profiling data of human gastric cancer samples and comparison with C/EBPβ KO mouse-derived expression data identified a subset of tumors with a C/EBPβ-regulated signature. These tumors mostly belong to the intestinal type and may define a novel subtype. One of the deregulated genes characterizing this tumor cluster, RUNX1t1, has previously been connected to gastrointestinal abnormalities [29] and to suppression of C/EBPβ functions

[30] and was consistently downregulated in the murine RUNX3 KO tumor model. RUNX1t1 is also a candidate tumor suppressor in ovarian cancer [31] and loss of RUNX1t1 expression has been associated with metastasis in pancreatic cancer [32]. Downregulation of RUNX1t1 during homeostasis and initially in gastric cancer may occur through C/EBPβ, however, analysis of DNA methylation showed that the RUNX1t1 promoter was frequently methylated in human gastric cancer samples, similarly to ovarian cancer [31] and suggests alternative routes of RUNX1t1 gene silencing in gastric carcinogenesis.

RUNX1t1, also known as MTG8 or ETO, is the recurrent t(8;21) translocation partner of the AML-ETO (RUNX1/MTG8) fusion protein. AML-ETO accounts for 15% of acute myeloid leukemia and 40% of M2-type leukemia, probably by interference with the differentiation inducing functions of C/EBPα and PU.1 [33] [34]. Few reports have focused on RUNX1t1 independently of the AML-ETO context, yet suggested involvement of RUNX1t1 in several corepressor complexes [34]. Our results support the notion of RUNX1t1 as a suppressor of gastric cancer development and suggest a regulatory loop between C/EBPβ and RUNX1t1 in homeostasis and disruption in cancer. High expression of C/EBPβ leads to reduction of RUNX1t1 expression and high RUNX1t1 expression leads to the inhibition of C/EBPβ functions. Antagonism between both proteins was reported in the adipogenic clonal expansion phase, which requires balanced expression of C/EBPβ and RUNX1t1 to prevent premature induction of C/EBPα and terminal differentiation [30]. The connection between C/EBPβ and RUNX1t1 may also be relevant in hematopoietic malignancies involving the AML-ETO translocation product. It has recently been shown that RUNX1 and C/EBPβ bind to all hematopoietic genes in embryonic stem cells that are committed to hematopoietic differentiation [35]. It thus appears that the fusion of RUNX1 and RUNX1t1 in the t(8;21) AML-ETO translocation may counteract distinct functions of C/EBPβ in earmarking lineage commitment and expression of differentiation genes.

Conclusions

In this paper a subset of gastric tumors characterized by a strong C/EBPβ regulation is described. Our genetic data firmly establishes a functional role of C/EBPβ in proliferation control, as well as a major factor bridging gastric homeostasis and tumorigenesis. RUNX1t1 is identified as a novel gastric tumor suppressor gene in close functional and regulatory connection with C/EBPβ. The identification of novel molecular targets for potential therapies, as well as previously unknown pathways of oncogenic transformation is of outmost importance in the study of a disease lacking solid knowledge on molecular mechanisms of development, such as it is the case of gastric cancer.

Competing Interests

The authors declare there they have no competing interests.

Author contributions

GR and AL conceptualized the project. GR designed, performed all the experiments except the ones executed by BB and CR, and wrote the manuscript. SF did the microarray analysis and revised the manuscript. CR did the tumorigenic assay of C/EBPβ knockdown cell, and the sequencing and methylation analysis of RUNX1t1. BB produced the stable knockdown of C/EBPβ. BF did the pathological analysis of cancer samples. WK, PMS, and JCM provided human gastric cancer samples. KI, SB and YI generated the RUNX3 KO mouse. AL supervised the project and revised the manuscript.

Acknowledgments

The authors would like to acknowledge Aline Ruderisch for technical assistance; Johanna Schiller, Frauke Debus and Elisabeth Kowenz-Leutz for providing vectors and probes; and Klaus Wethmar, Jeske Smink and Valérie Bégay for primers and editing the manuscript. This work was supported by a grant from the Berliner Krebsgesellschaft (LEFF-2012-10).

References

1. Parkin DM, Bray F, Ferlay J, Pisani P: **Global cancer statistics, 2002**. *CA Cancer J Clin* 2005, **55**(2):74-108.
2. Tahara E: **Genetic pathways of two types of gastric cancer**. *IARC Sci Publ* 2004(157):327-349.
3. Resende C, Thiel A, Machado JC, Ristimaki A: **Gastric cancer: basic aspects**. *Helicobacter* 2011, **16 Suppl 1**:38-44.
4. Carneiro P, Fernandes MS, Figueiredo J, Caldeira J, Carvalho J, Pinheiro H, Leite M, Melo S, Oliveira P, Simoes-Correia J *et al*: **E-cadherin dysfunction in gastric cancer--cellular consequences, clinical applications and open questions**. *FEBS Lett* 2012, **586**(18):2981-2989.
5. Zahnow CA, Cardiff RD, Laucirica R, Medina D, Rosen JM: **A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation**. *Cancer Res* 2001, **61**(1):261-269.
6. Bundy LM, Sealy L: **CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture**. *Oncogene* 2003, **22**(6):869-883.
7. Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC: **CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling**. *Proc Natl Acad Sci U S A* 2002, **99**(1):207-212.
8. Rask K, Thorn M, Ponten F, Kraaz W, Sundfeldt K, Hedin L, Enerback S: **Increased expression of the transcription factors CCAAT-enhancer binding protein-beta (C/EBBeta) and C/EBzeta (CHOP) correlate with invasiveness of human colorectal cancer**. *Int J Cancer* 2000, **86**(3):337-343.
9. Watanabe-Okochi N, Yoshimi A, Sato T, Ikeda T, Kumano K, Taoka K, Satoh Y, Shinohara A, Tsuruta T, Masuda A *et al*: **The shortest isoform of C/EBPbeta, liver inhibitory protein (LIP), collaborates with Evi1 to induce AML in a mouse BMT model**. *Blood* 2013, **121**(20):4142-4155.
10. Pal R, Janz M, Galson DL, Gries M, Li S, Johrens K, Anagnostopoulos I, Dorken B, Mapara MY, Borghesi L *et al*: **C/EBPbeta regulates transcription factors critical for proliferation and survival of multiple myeloma cells**. *Blood* 2009, **114**(18):3890-3898.
11. Lamb J, Ewen ME: **Cyclin D1 and molecular chaperones: implications for tumorigenesis**. *Cell Cycle* 2003, **2**(6):525-527.
12. Bonzheim I, Irmler M, Klier-Richter M, Steinhilber J, Anastasov N, Schafer S, Adam P, Beckers J, Raffeld M, Fend F *et al*: **Identification of C/EBPbeta Target Genes in ALK+ Anaplastic Large Cell Lymphoma (ALCL) by Gene Expression Profiling and Chromatin Immunoprecipitation**. *PLoS One* 2013, **8**(5):e64544.
13. Anastasov N, Bonzheim I, Rudelius M, Klier M, Dau T, Angermeier D, Duyster J, Pittaluga S, Fend F, Raffeld M *et al*: **C/EBPbeta expression in ALK-positive anaplastic large cell lymphomas is required for**

cell proliferation and is induced by the STAT3 signaling pathway. *Haematologica* 2010, **95**(5):760-767.
14. Nerlov C: **The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control**. *Trends Cell Biol* 2007, **17**(7):318-324.
15. Regalo G, Canedo P, Suriano G, Resende C, Campos ML, Oliveira MJ, Figueiredo C, Rodrigues-Pereira P, Blin N, Seruca R *et al*: **C/EBPbeta is over-expressed in gastric carcinogenesis and is associated with COX-2 expression**. *J Pathol* 2006, **210**(4):398-404.
16. Sankpal NV, Moskaluk CA, Hampton GM, Powell SM: **Overexpression of CEBPbeta correlates with decreased TFF1 in gastric cancer**. *Oncogene* 2006, **25**(4):643-649.
17. Forones NM, Kawamura KY, Segreto HR, Artigiani Neto R, Focchi GR, Oshima CT: **Expression of COX-2 in stomach carcinogenesis**. *J Gastrointest Cancer* 2008, **39**(1-4):4-10.
18. Saukkonen K, Nieminen O, van Rees B, Vilkki S, Harkonen M, Juhola M, Mecklin JP, Sipponen P, Ristimaki A: **Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma**. *Clin Cancer Res* 2001, **7**(7):1923-1931.
19. Machado JC, Carneiro F, Blin N, Sobrinho-Simoes M: **Pattern of pS2 protein expression in premalignant and malignant lesions of gastric mucosa**. *Eur J Cancer Prev* 1996, **5**(3):169-179.
20. Wu MS, Shun CT, Wang HP, Lee WJ, Wang TH, Lin JT: **Loss of pS2 protein expression is an early event of intestinal-type gastric cancer**. *Jpn J Cancer Res* 1998, **89**(3):278-282.
21. Oshima H, Oshima M, Inaba K, Taketo MM: **Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice**. *EMBO J* 2004, **23**(7):1669-1678.
22. Lefebvre O, Chenard MP, Masson R, Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P, Rio MC: **Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein**. *Science* 1996, **274**(5285):259-262.
23. Dossinger V, Kayademir T, Blin N, Gott P: **Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors**. *Cell Physiol Biochem* 2002, **12**(4):197-206.
24. Sankpal NV, Mayo MW, Powell SM: **Transcriptional repression of TFF1 in gastric epithelial cells by CCAAT/enhancer binding protein-beta**. *Biochim Biophys Acta* 2005, **1728**(1-2):1-10.
25. Forster S, Gretschel S, Jons T, Yashiro M, Kemmner W: **THBS4, a novel stromal molecule of diffuse-type gastric adenocarcinomas, identified by transcriptome-wide expression profiling**. *Mod Pathol* 2011, **24**(10):1390-1403.
26. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB *et al*: **Causal relationship between the loss of RUNX3 expression and gastric cancer**. *Cell* 2002, **109**(1):113-124.
27. Ito Y: **RUNX3 is expressed in the epithelium of the gastrointestinal tract**. *EMBO Mol Med* 2012, **4**(7):541-542; author reply 543-544.
28. Levanon D, Bernstein Y, Negreanu V, Bone KR, Pozner A, Eilam R, Lotem J, Brenner O, Groner Y: **Absence of Runx3 expression in**

normal gastrointestinal epithelium calls into question its tumour suppressor function. *EMBO Mol Med* 2011, **3**(10):593-604.
29. Calabi F, Pannell R, Pavloska G: **Gene targeting reveals a crucial role for MTG8 in the gut**. *Mol Cell Biol* 2001, **21**(16):5658-5666.
30. Rochford JJ, Semple RK, Laudes M, Boyle KB, Christodoulides C, Mulligan C, Lelliott CJ, Schinner S, Hadaschik D, Mahadevan M *et al*: **ETO/MTG8 is an inhibitor of C/EBPbeta activity and a regulator of early adipogenesis**. *Mol Cell Biol* 2004, **24**(22):9863-9872.
31. Yeh KT, Chen TH, Yang HW, Chou JL, Chen LY, Yeh CM, Chen YH, Lin RI, Su HY, Chen GC *et al*: **Aberrant TGFbeta/SMAD4 signaling contributes to epigenetic silencing of a putative tumor suppressor, RunX1T1 in ovarian cancer**. *Epigenetics* 2011, **6**(6):727-739.
32. Nasir A, Helm J, Turner L, Chen DT, Strosberg J, Hafez N, Henderson-Jackson EB, Hodul P, Bui MM, Nasir NA *et al*: **RUNX1T1: a novel predictor of liver metastasis in primary pancreatic endocrine neoplasms**. *Pancreas* 2011, **40**(4):627-633.
33. Nimer SD, Moore MA: **Effects of the leukemia-associated AML1-ETO protein on hematopoietic stem and progenitor cells**. *Oncogene* 2004, **23**(24):4249-4254.
34. Davis JN, McGhee L, Meyers S: **The ETO (MTG8) gene family**. *Gene* 2003, **303**:1-10.
35. Lichtinger M, Ingram R, Hannah R, Muller D, Clarke D, Assi SA, Lie ALM, Noailles L, Vijayabaskar MS, Wu M *et al*: **RUNX1 reshapes the epigenetic landscape at the onset of haematopoiesis**. *EMBO J* 2012, **31**(22):4318-4333.
36. Sterneck E, Tessarollo L, Johnson PF: **An essential role for C/EBPbeta in female reproduction**. *Genes Dev* 1997, **11**(17):2153-2162.
37. Astrosini C, Roeefzaad C, Dai YY, Dieckgraefe BK, Jons T, Kemmner W: **REG1A expression is a prognostic marker in colorectal cancer and associated with peritoneal carcinomatosis**. *Int J Cancer* 2008, **123**(2):409-413.

Figure Legends

Figure 1. C/EBPβ controls gastric cancer cell proliferation. A) RNA expression of C/EBPβ in intestinal and diffuse gastric cancer cases as determined by real-time PCR. Tumor versus normal ratios were established for each case. Values above 1 entail upregulation, whereas expression below 1 refers to downregulation (p value refers to normal vs Intestinal comparison). B) Stable knockdown of C/EBPβ in gastric cancer cell lines evaluated by protein blotting (left panel MKN28, right panel MKN45), showing reduction of all the C/EBPβ isoforms (LAP*, LAP and LIP). C) Cell proliferation was determined by BrdU analysis. Cells were labeled with BrdU and incorporation was determined by flow cytometry (FACS) and plotted against 7-AAD-positive cells, as a measure of DNA content. Depicted FACS plots are representative of one of three replicates, and show a reduced percentage of BrdU incorporation in gastric cells with C/EBPβ KO. S-phase percentages are highlighted in the FACS plots. D) Gastric cell lines with stable C/EBPβ KO were injected into nude mice and tumor volume and weight was assessed at different time points. Tumors originated from C/EBPβ KO cells were smaller than tumors in the controls ($p<0.005$). E) Ki67 staining revealed reduction of proliferation in the KO-derived tumors.

Figure 2. Analysis of the gastric phenotype of the C/EBPβ knockout (KO) mouse. A) Quantification of the C/EBPβ KO mice and WT antral gastric mucosa thickness showing small but significant ($p<0.001$) reduction. Adjacent immunohistochemical panel depicts the reduction of Ki67-positive cells in the C/EBPβ KO mucosa. Lower panels show qPCR evaluation of Ki67, PCNA, Cyclin A1, D3, E1 and p15 in the gastric mucosa of WT and C/EBPβ KO mouse stomach (5 animals/group, 3 months old). Values are presented as fold of WT expression, and asterisks refer to p-value of 0.05 or inferior. D) Mutually exclusive expression of TFF1 and C/EBPβ in the normal human (upper panel) and mouse (lower panel) stomach epithelium; C/EBPβ is expressed in proliferative cells of the neck zone and TFF1 in differentiated

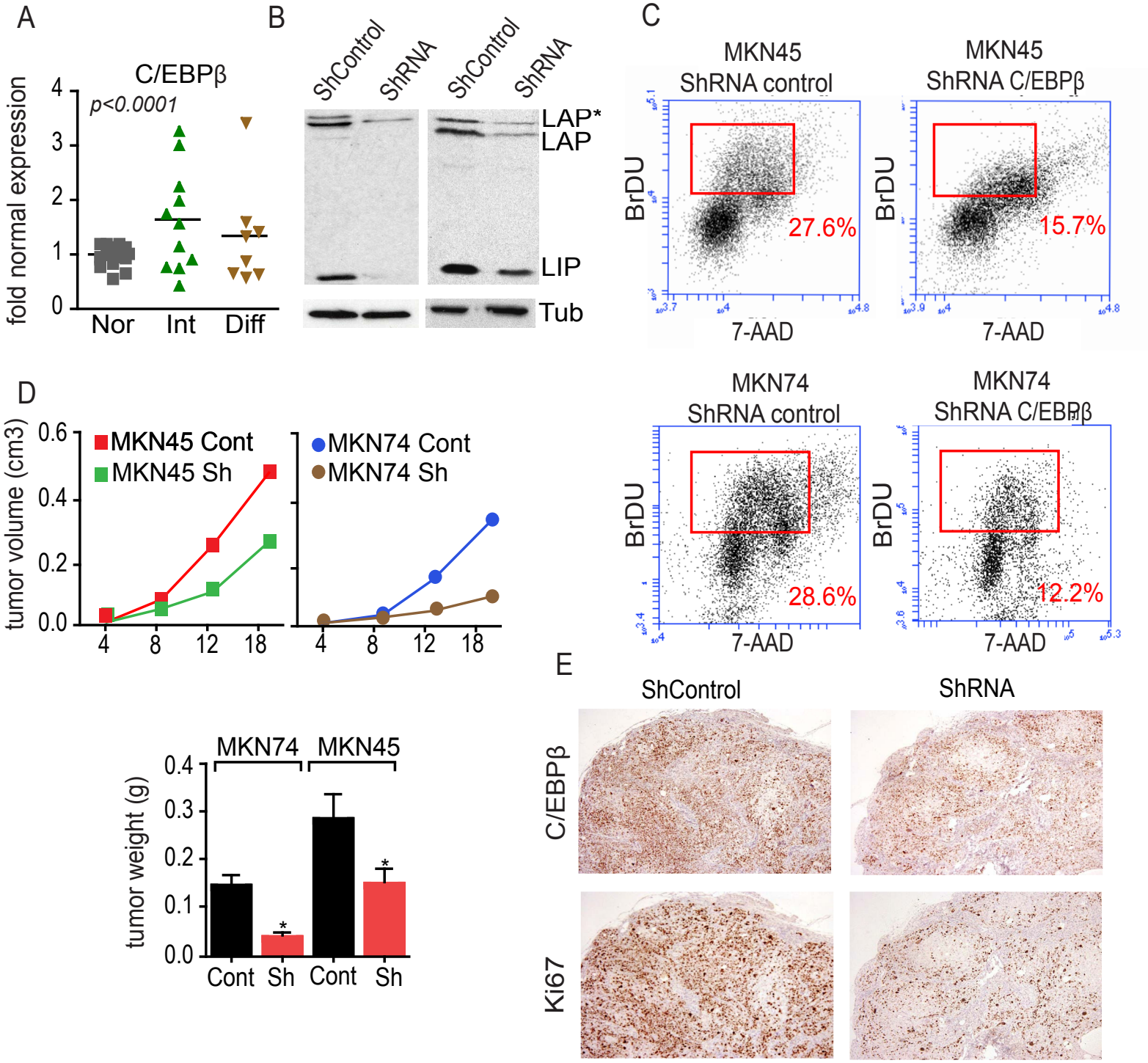
mucous epithelium. Increased expression of mRNA of differentiation proteins TFF1 and MUC5AC in the C/EBPβ KO mouse mucosa as measured by qPCR.

Figure 3. Cross-species comparison of gene expression. Two-way hierarchical clustering was performed using a strongly-regulated gene cluster (shown in Supplementary Figure 2) from microarray-derived murine genes that differed between C/EBPβ KO and WT stomach ($p \leq 0.01$, $FC \geq 1.5$) and human gastric cancer samples. Depicted are the resultant gene and sample dendrograms and the corresponding expression intensity heat map. The black box indicates a tumor cluster in which most of the genes show downregulation (bluish spots). This tumor group consisted of 16 of the original 59 (≈27%) samples and contained primarily cancers of the intestinal histological type.

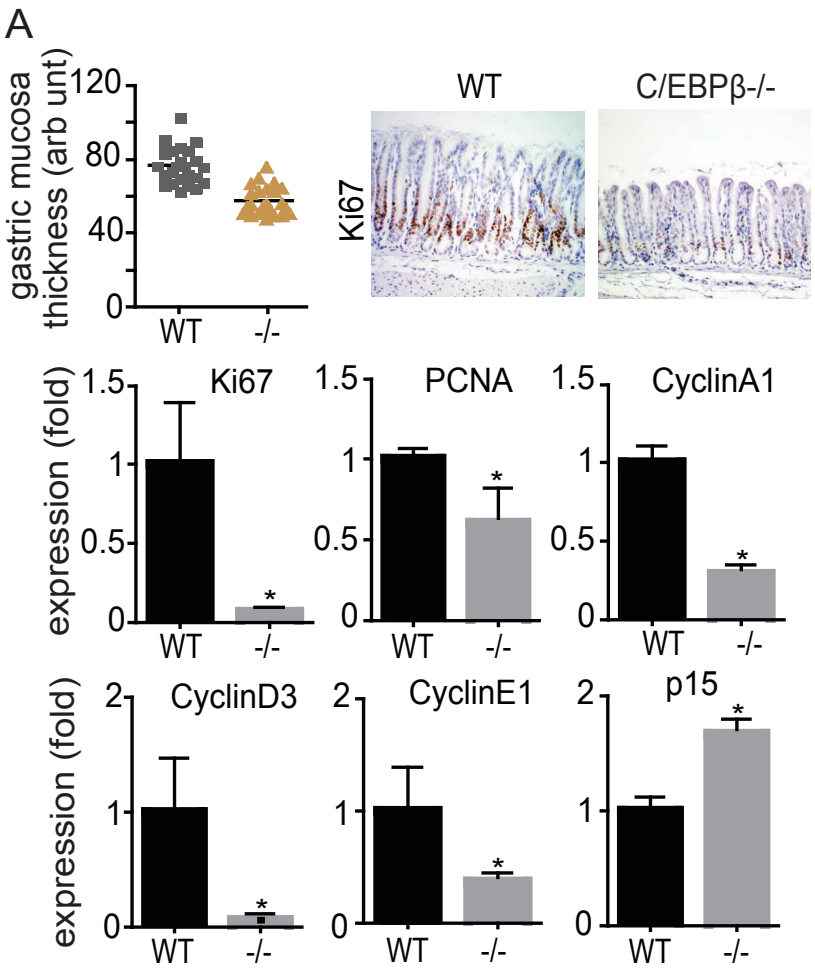
Figure 4. C/EBPβ in the RUNX3 knockout (KO) mouse. A) Immuno-staining of longitudinal sections of newborn stomachs shows increased C/EBPβ and Ki67 expression in the RUNX3 KO mouse stomach and reversion in the C/EBPβ/RUNX3 double KO. E-cadherin staining shows that hyperproliferation is confined to the epithelial compartment. B) Quantification of the mucosal thickness and Ki67 expression ($p<0.05$) in the wild type (WT) and RUNX3 KO and reversal of the mucosal thickness and hyperproliferative phenotype in the compound C/EBPβ/RUNX3 double KO. C) qPCR analysis of RUNX1t1, FOG2, and SPARCL1 in RUNX3 KO and C/EBPβ/RUNX3 KO stomachs shows that only RUNX1t1 is downregulated in the hyperproliferative mucosa of the RUNX3 KO ($p<0.005$) and reverted to almost WT levels in the compound C/EBPβ/RUNX3 KO stomach. D) Transfection of C/EBPβ isoforms LAP*, LAP, and LIP into gastric cell lines MKN28 and MKN45 repressed RUNX1t1 expression as measured by quantitative PCR.

Figure 5. RUNX1t1 and gastric cancer. A) RUNX1t1 expression was evaluated by immunohistochemistry in 64 human gastric cancer samples, and staining was classified by comparison to the expression in the normal mucosa

(left panel). 38% of the cases showed reduced expression of RUNX1t1 (right panel). B) In 10 gastric tumors with reduced RUNX1t1 RNA levels were examined for C/EBPβ expression by qPCR. Only 3 out of 10 cases showed higher C/EBPβ expression as compared to WT. C) The methylation status of the RUNX1t1 promoter was evaluated by methylation-specific PCR. Bisulfite treatment of tumor DNA converts unmethylated but not methylated cytosines to uracil, and subsequent methylation-specific PCR detects either methylated (M) or unmethylated (U) DNA. 90% of the analyzed human gastric cancer cases (rows a-b, columns 1-5) present some degree of RUNX1t1 promoter hypermethylation. D) Ectopic expression of RUNX1t1 in MKN28 and MKN45 gastric cancer cell lines reduces gastric cancer cell proliferation as measured by BrdU incorporation assay. S-phase percentages are indicated in the FACS plots. E) Immunoprecipitation of flag-tagged RUNX1t1 co-precipitates C/EBPβ. Visible in the input Western Blot is also that RUNX1t1 does not affect C/EBPβ expression. F) Electrophoretic mobility shift assay (EMSA) using a radiolabeled C/EBPβ DNA probe and nuclear extracts from MKN28 and MKN45 cells. Transfection of RUNX1t1 reduces the binding of C/EBPβ to DNA in both cell lines in a dose-dependent manner. Arrow indicates the super-shift.



Regalo et al. Figure 1



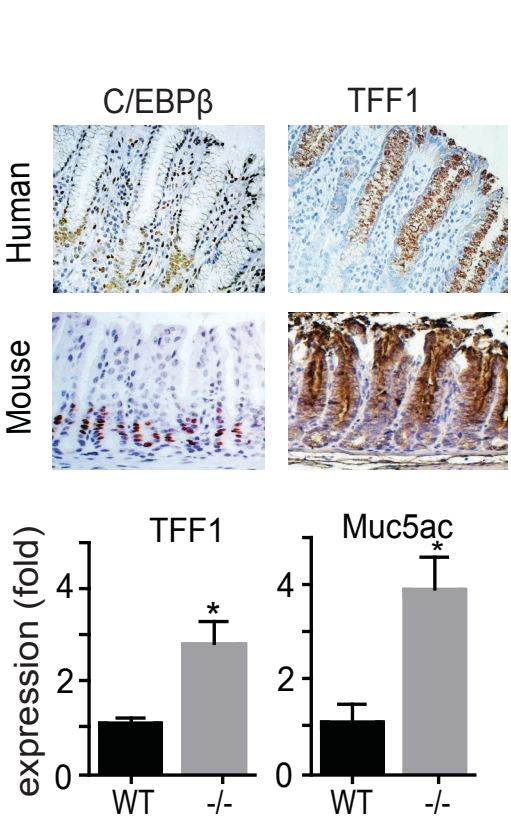


Figure 2 Regalo et al

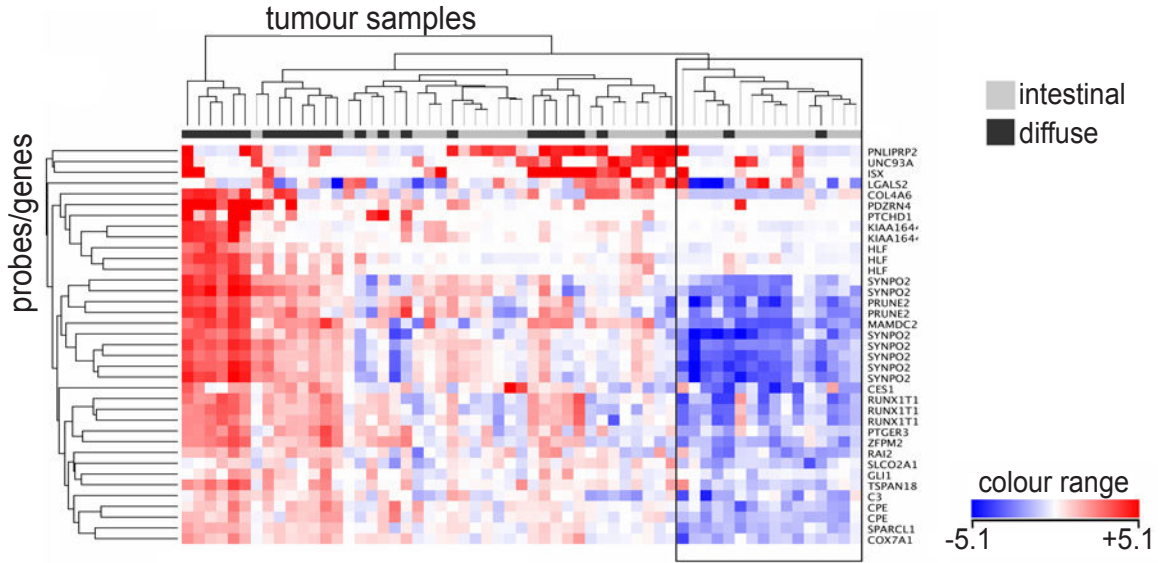

tumour samples
probes/genes
intestinal
diffuse
PNLIPRP2
UNC93A
ISX
LGALS2
COL4A6
PDZRN4
PTCHD1
KIAA164
KIAA164
HLF
HLF
HLF
SYNPO2
SYNPO2
PRUNE2
PRUNE2
MAMDC2
SYNPO2
SYNPO2
SYNPO2
SYNPO2
SYNPO2
CES1
RUNX1T1
RUNX1T1
RUNX1T1
PTGER3
ZFPM2
RAI2
SLCO2A1
GLI1
TSPAN18
C3
CPE
CPE
MARCL1
COX7A1
colour range
-5.1
+5.1

Figure 3

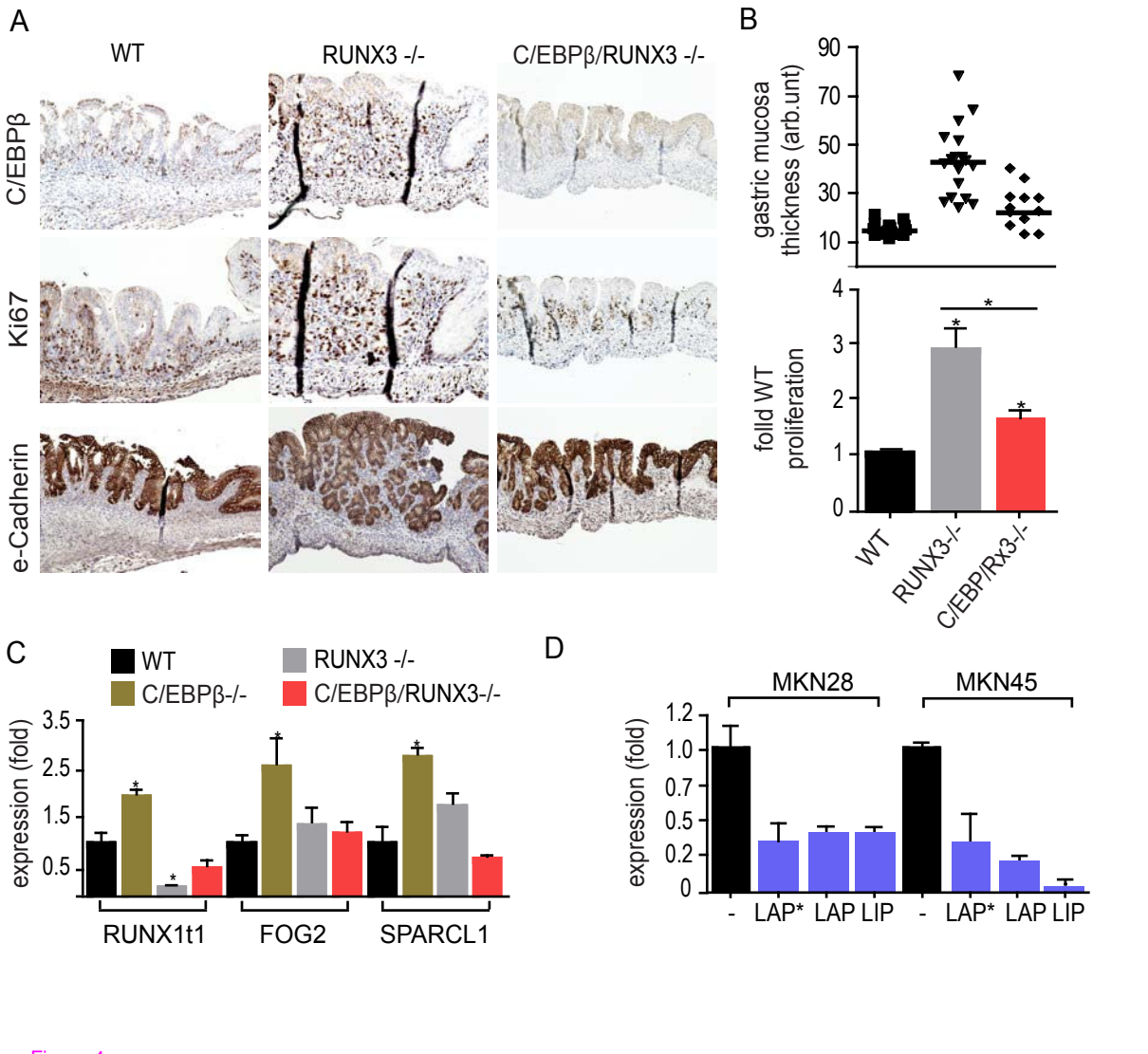

A
WT
RUNX3 -/-
C/EBPβ/RUNX3 -/-
C/EBPβ
Ki67
e-Cadherin
B
gastric mucosa thickness (arb.unt)
90
70
50
30
10
fold WT proliferation
4
3
2
1
0
*
WT
RUNX3-/-
C/EBP/Rx3-/-
C
WT
C/EBPβ-/-
RUNX3 -/-
C/EBPβ/RUNX3-/-
expression (fold)
3.5
2.5
1.5
0.5
RUNX1t1
FOG2
SPARCL1
D
MKN28
MKN45
expression (fold)
1.2
1.0
0.7
0.5
0.2
0
-
LAP*
LAP
LIP

Figure 4

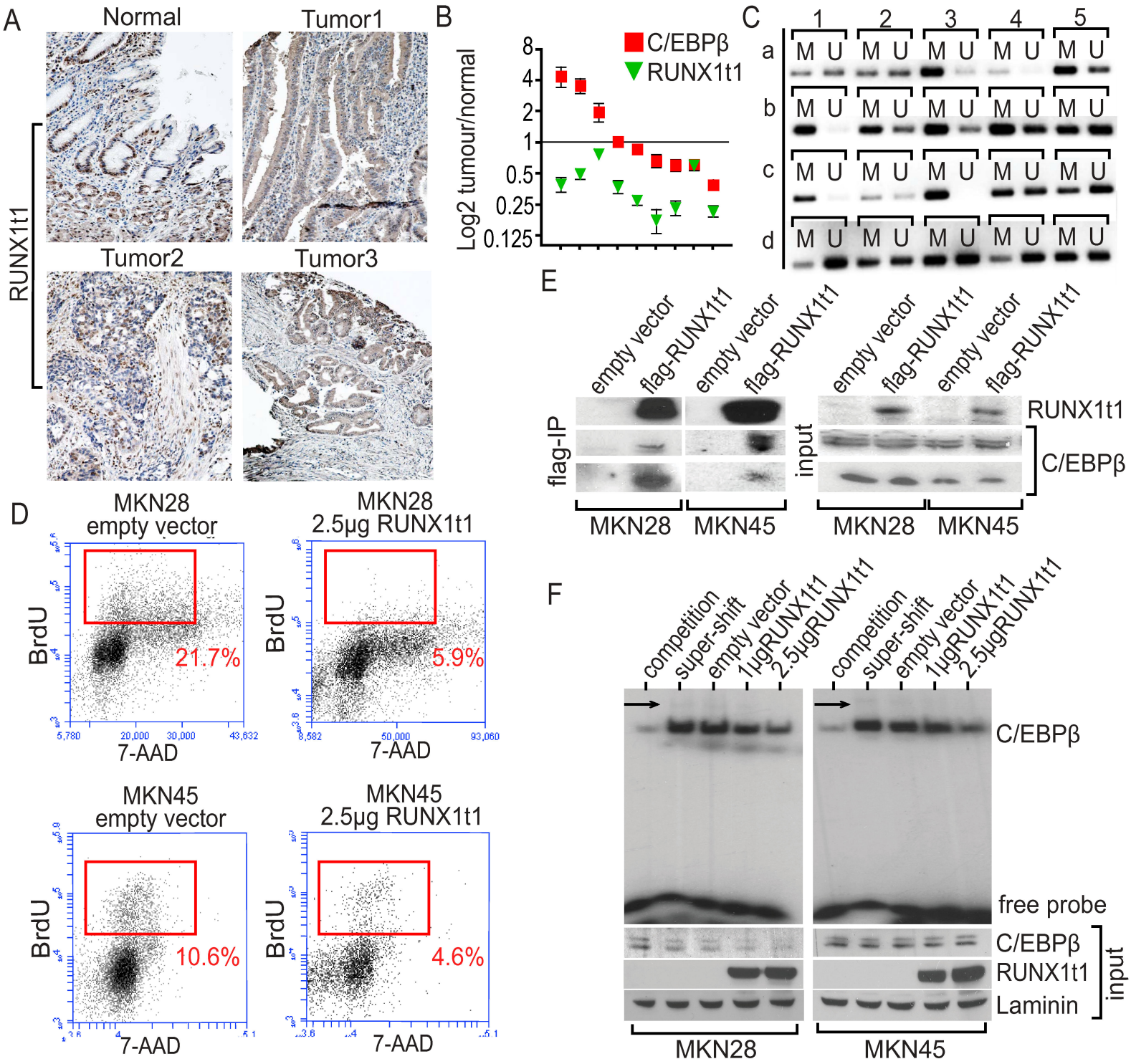


Figure 5, Regalo et al.

Additional files provided with this submission:

Additional file 1: Supplementary data.docx, 4115K
http://www.biomedcentral.com/imedia/1554313586152941/supp1.docx
Additional file 2: Table 1.docx, 18K
http://www.biomedcentral.com/imedia/8133459681529623/supp2.docx

Article III

Gastric Cancer
DOI 10.1007/s10120-014-0448-x

ORIGINAL ARTICLE

Interleukin-1B signalling leads to increased survival of gastric carcinoma cells through a CREB-C/EBPβ-associated mechanism

Carlos Resende · Gonçalo Regalo · Cecília Durães · Marta Teixeira Pinto · Xiaogang Wen · Ceu Figueiredo · Fátima Carneiro · José C. Machado

Received: 6 August 2014 / Accepted: 17 November 2014

Abstract
Background Polymorphisms in inflammation-related genes have been associated with a risk of gastric carcinoma (GC). However, the biological mechanisms underlying these associations are still elusive. Our objective was to determine whether chronic inflammation-associated IL1B signalling, as seen in the context of *Helicobacter pylori* infection, could be linked to gastric carcinogenesis by modulating the behaviour of gastric epithelial cells.
Methods The effect of IL1B was assessed by studying the expression and activation status of the IL1B-activated transcription factors C/EBPβ and CREB in GC cell lines. Interaction between CREB and C/EBPβ was explored through interference RNA, chromatin immunoprecipitation and chemical inhibition. CREB and C/EBPβ expression was analysed in 66 samples of primary GC and in normal gastric mucosa. GC cell growth was analysed in vitro by BrdU incorporation and in vivo employing a chicken embryo chorioallantoic membrane model.
Results We found that IL1B regulates the expression/activation status of both C/EBPβ and CREB in GC cells through an ERK1/2-dependent mechanism. Our results show that CREB is a direct transactivator of *CEBPB*, acting as an upstream effector in this regulatory mechanism. Furthermore, we found CREB to be overexpressed in 94 % of GC samples and significantly associated with C/EBPβ expression ($P < 0.05$). Finally, we demonstrated both in vitro and in vivo that CREB can mediate IL1B-induced GC cell proliferation.
Conclusions Our results support the hypothesis that the effect of chronic inflammation on gastric carcinogenesis, as seen in the context of genetically susceptible individuals infected with *Helicobacter pylori*, includes the modulation of signalling pathways that regulate survival mechanisms in epithelial cells.
Summary IL1B is able to increase the expression/activation status of CREB and its target gene C/EBPβ, which are mandatory for GC cell survival. Our results may help inform new strategies for the prevention and treatment of GC, including the control of chronic inflammation.

Keywords *Helicobacter pylori* · Gastric cancer · IL1B · Inflammation · Genetic susceptibility

C. Resende · C. Durães · M. T. Pinto · C. Figueiredo · F. Carneiro · J. C. Machado
Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

C. Resende · G. Regalo · C. Durães · M. T. Pinto · C. Figueiredo · F. Carneiro · J. C. Machado (✉)
Institute of Molecular Pathology and Immunology (Ipatimup), University of Porto, Porto, Portugal
e-mail: josem@ipatimup.pt

C. Resende
Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto, Portugal

X. Wen
Serviço de Anatomia Patológica, Centro Hospitalar Vila Nova de Gaia/Espinho, Gaia, Portugal

C. Figueiredo · F. Carneiro · J. C. Machado
Faculty of Medicine, University of Porto, Porto, Portugal

Introduction

Gastric carcinoma (GC) is the second most common cause of cancer-related death in the world. The main risk factor for the onset of GC is life-time infection with *Helicobacter pylori* (*H. pylori*), a stomach-colonising bacterium [1].

Published online: 05 March 2015

Infection with *H. pylori* leads to chronic gastritis that may progress to gastric atrophy, intestinal metaplasia, dysplasia and finally GC [2, 3].

The risk of developing GC depends both on environmental factors and host-related factors [4, 5]. In this model, gene polymorphisms that increase the production of pro-inflammatory mediators lead to an enhanced chronic inflammatory response to *H. pylori* infection and to an increased risk of progression towards GC [5, 6]. There are numerous studies demonstrating that polymorphisms in genes such as *IL1B*, *IL1RN*, *TNFA* and *IFNGR1* are associated with a risk of developing GC [7–11]. Moreover, these polymorphisms have been shown to be associated with increased gene expression, both in vitro and in vivo [12]. Perhaps the most striking evidence favouring this model comes from a transgenic mouse model showing that overexpression of the *IL1B* gene in gastric mucosa leads to an increased risk of developing gastric disease, including dysplasia and GC, even in the absence of *H. pylori* infection [13].

According to the prevailing model, the link between enhanced chronic inflammation and GC depends essentially on the "destructive" effects of inflammation over the gastric epithelium, resulting in atrophy of the gastric mucosa and increased cell turnover [14, 15]. However, it is well demonstrated that inflammatory mediators, and other growth factors secreted by inflammatory cells, can act directly on other cell types, such as epithelial cells. Therefore, in addition to the mucosal destruction and repair effect, enhanced chronic inflammation could also play a role in gastric carcinogenesis by providing gastric epithelial cells with a survival stimulus through the secretion of growth factors [16, 17]. Coupled with mutagenic events, this could ultimately lead to an increased risk of cell transformation and GC development.

In this regard, IL1B is particularly interesting since polymorphisms in its promoter region have been shown to be associated with an increased risk of GC [7–9, 18]. IL1B is a powerful pro-inflammatory cytokine that activates different transcription factors [19], some of which are also activated by *H. pylori* infection [20, 21]. One of the IL1B-activated transcription factors is CCAAT/enhancer-binding protein beta (C/EBPβ) [22]. We previously reported that C/EBPβ is overexpressed in pre-malignant lesions and in GC, suggesting that this protein may facilitate gastric carcinogenesis by inducing the expression of COX-2 [23]. Furthermore, C/EBPβ expression in GC was significantly associated with loss of expression of the putative gastric tumour-suppressor TFF1 [24, 25].

Another important IL1B-activated transcription factor is cAMP response element-binding protein (CREB), which has been described as a major player in inflammation [19, 26]. In non-small cell lung cancer, IL1B induces the activation of CREB through ERK1/2 signalling, resulting in the expression of a set of pro-angiogenic cytokines that are crucial factors in tumour progression [27]. Furthermore, CREB was recently described to play an important pro-oncogenic role in both cancer development and progression; it was found to be overexpressed in several cancer types [28–31]. It has been demonstrated, both in hepatocytes [32] and in pre-adipocytes [33], that CREB is able to regulate the transcription of the *CEBPB* gene by directly interacting with its promoter.

The main objective of this study was to determine whether chronic inflammation-associated IL1B signalling,

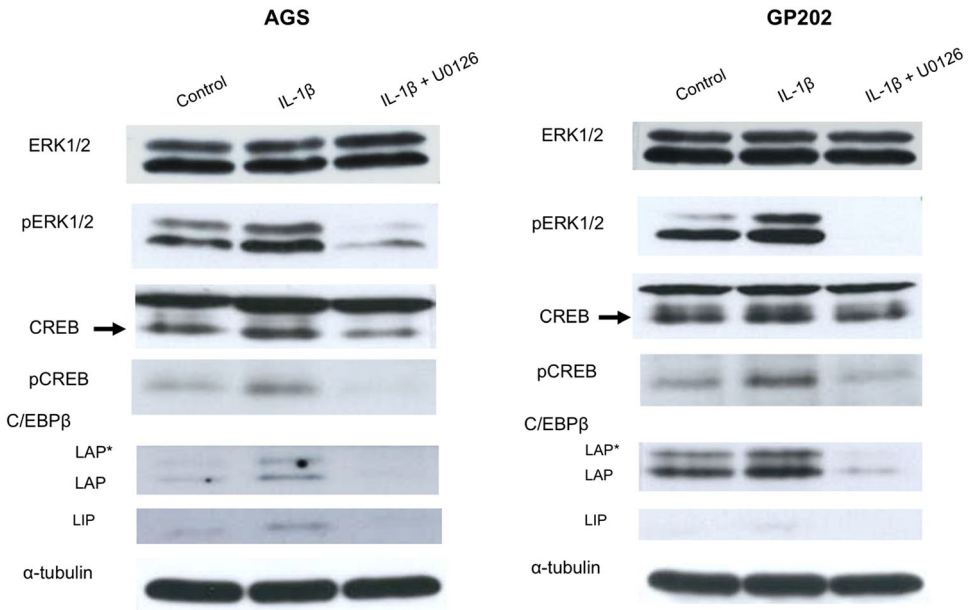


Fig. 1 Effects of IL1B stimulation and ERK1/2 inhibition on CREB, pCREB, and C/EBPβ protein levels. Both AGS and GP202 cells, when treated with 10 ng/mL of IL1B for 24 h, exhibited an increase in activated ERK1/2 (pERK1/2). In parallel, the expression levels of CREB, pCREB and C/EBPβ also increased. The ERK1/2 chemical inhibitor U0126 (25 μM) reverted the effect of IL1B on CREB, pCREB and C/EBPβ protein levels

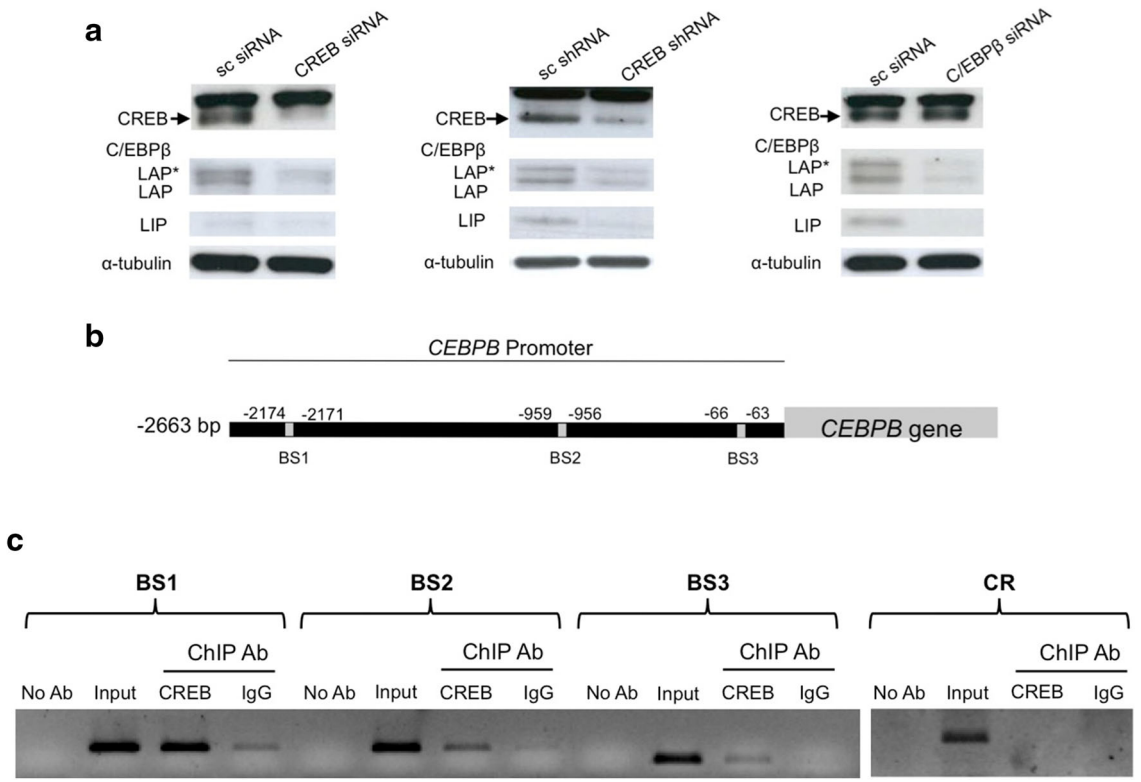


Fig. 2a–c Effect of CREB downregulation on C/EBPβ protein levels. **a** CREB silencing (CREB-specific bands are indicated by *arrows*; the *upper bands on the CREB blots* represent unspecific binding of the CREB antibody) was followed by a downregulation of C/EBPβ protein levels, whereas the silencing of C/EBPβ had no impact on CREB protein. **b** Schematic representation of the *CEBPB* promoter (2663 bp) showing the three CRE-binding sites (BS1, BS2 and BS3) and a control region (CR) located at the 3′ end of *CEBPB*. **c** CREB interacts with all three CRE-binding motifs present on the *CEBPB* promoter. *No Ab* no antibody used, *Input* 1/100 of the sheared initial chromatin, *CREB* chromatin immunoprecipitated using an anti-CREB antibody, *IgG* chromatin immunoprecipitated with an unspecific antibody of the same family as the anti-CREB antibody

as seen in the context of *H. pylori* infection, could be linked to gastric carcinogenesis by modulating the behaviour of gastric epithelial cells. We addressed this objective by showing that CREB and C/EBPβ transcription factors can be activated by IL1B signalling in the GC context. We also demonstrated that CREB acts upstream of C/EBPβ in GC cell lines. Finally, we showed in vitro and in vivo that this signalling mechanism promotes GC cell survival.

Results

IL1B increases C/EBPβ and CREB expression in an ERK1/2-dependent manner

To evaluate the effect of IL1B on the expression and activation status of C/EBPβ and CREB, we incubated GC cell lines AGS and GP202 with IL1B for 24 h. In both AGS and GP202 cells, incubation with IL1B led to an increase in the expression of all isoforms (LAP*, LAP and LIP) of C/EBPβ (Fig. 1). Regarding CREB, we observed an increase in both expression and phosphorylation levels in both cell lines (Fig. 1).

Since ERK1/2 has been previously implicated in the regulation of C/EBPβ and CREB, we investigated whether it could mediate the effect of IL1B over those two transcription factors. Incubation of AGS and GP202 cells with the ERK1/2 inhibitor U0126 led to a decrease in the expression of C/EBPβ and CREB and to a decrease in phosphorylation levels of CREB (Fig. 1). The level of ERK1/2 phosphorylation in both cell lines was measured as a control for the efficacy of inhibition with U0126 (Fig. 1). These results demonstrate that IL1B is able to regulate the expression/activation status of both C/EBPβ and CREB in GC cells.

CREB is a transcriptional regulator of C/EBPβ in GC cells

Knowing that CREB is a transcriptional regulator of the *CEBPB* gene in other cell types, we decided to investigate whether the same regulatory mechanism could be at work

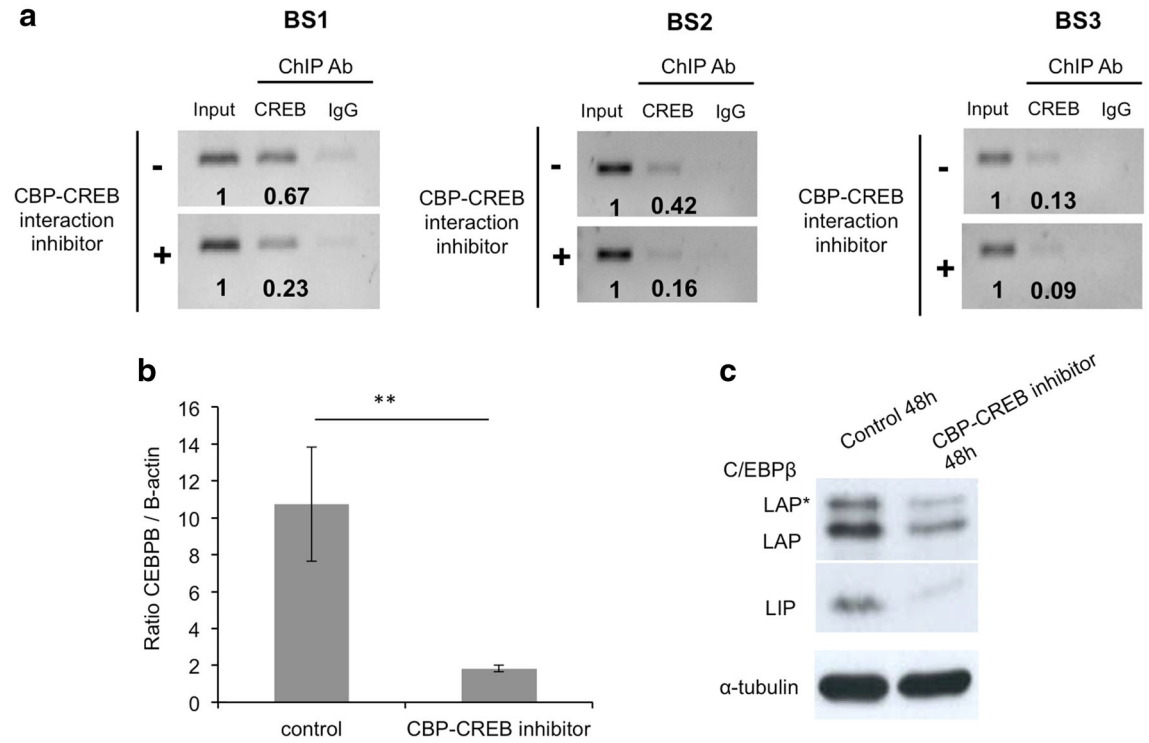


Fig. 3a–d Effects of CBP–CREB interaction inhibition on CREB activity and C/EBPβ. **a** ChIP performed on AGS cells after treatment with CBP–CREB interaction inhibitor (+) revealed a decrease in the amount of CREB linked to the *CEBPB* promoter compared to untreated cells (−). AGS cells treated with CREB–CBP interaction inhibitor (25 μM) for 48 h showed a significant decrease in C/EBPβ **b** transcript levels and **c** protein expression. Real-time PCR results represent the mean ± S.D. of three independent experiments. Significance: *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$)

in GC cells. Since both AGS and GP202 cells yielded the same results and the IL1B-stimulatory effect was more pronounced in AGS cells, we decided to perform the next set of experiments only in the AGS cells. Using both small interfering RNA (siRNA) and short-hairpin RNA (shRNA), we found that knocking down CREB results in down-regulation of C/EBPβ expression (Fig. 2a). Conversely, silencing C/EBPβ using siRNA had no impact on CREB expression (Fig. 2a). These results show that CREB acts upstream of C/EBPβ in this regulatory mechanism.

To check whether CREB acts directly on the *CEBPB* gene, we analysed the *CEBPB* promoter (2663 base pairs) in order to find putative cAMP response element (CRE)-binding motifs. We employed a previously described [32] nucleotide position numbering scheme. The analysis revealed the presence of three CRE-binding sites, ranging from nucleotides −2174 to −2171 (BS1), from −959 to −956 (BS2), and from −66 to −63 (BS3) (Fig. 2b). Using chromatin immunoprecipitation (ChIP), we observed that CREB binds all three CRE-binding sites on the *CEBPB* promoter (Fig. 2c).

These results were validated by showing that 24-h chemical inhibition of the interaction between CREB and its co-activator CREB binding protein (CBP) led to a reduced binding of CREB to the CRE-binding sites on the *CEBPB* gene promoter (Fig. 3a). To confirm that the decrease in CREB binding to the *CEBPB* promoter actually leads to downregulation of transcription and protein synthesis, we evaluated the relative amount of C/EBPβ mRNA and protein after 48 h of treatment with the CBP–CREB interaction inhibitor. This experiment resulted in a significant reduction in both C/EBPβ transcript levels (Fig. 3b) and C/EBPβ protein levels (Fig. 3c).

CREB and C/EBPβ proteins are co-expressed in normal gastric mucosa and in GC

To determine whether there is an association between the expression of CREB and C/EBPβ, we analysed the immunohistochemical (IHC) expression of these two proteins in a series of 66 cases of GC. In normal gastric mucosa, we found that both C/EBPβ and CREB are expressed in the nuclei of epithelial cells in the proliferative isthmus/neck zone (Fig. 4a, b). To confirm that CREB and C/EBPβ are expressed in the same cells, we performed double immunofluorescence in a tissue fragment of normal gastric mucosa (Fig. 4g, j). In GC, we observed that CREB and C/EBPβ were expressed in cell nuclei in 94 and 73 % of

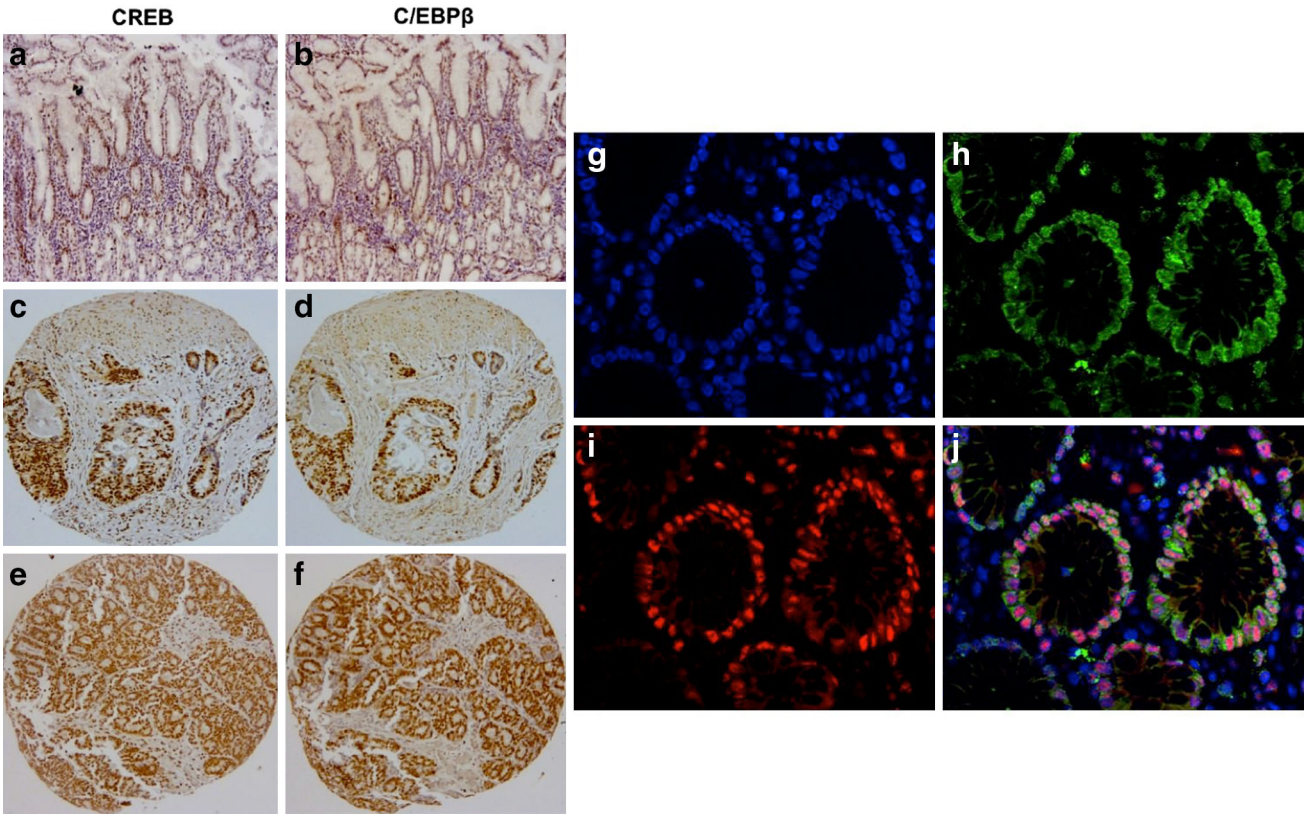


Fig. 4a–j Immunohistochemical expression of CREB and C/EBPβ in normal gastric mucosa and in GC cases. **a** CREB is expressed in epithelial cells located in the neck/isthmus region of normal gastric glands. **b** C/EBPβ is also expressed in neck/isthmus normal epithelial cells. **c**, **e** Examples of CREB-positive GC cases (scoring 3); **d**, **f** C/EBPβ is overexpressed in the same GC cases that are positive for CREB. Magnification 100×. **g–j** Double immunofluorescence staining for CREB and C/EBPβ in normal gastric mucosa, showing co-expression of the two proteins in gastric epithelial cells: **g** DAPI nuclear staining, **h** C/EBPβ staining, **i** CREB staining, **j** merged image for DAPI, C/EBPβ and CREB staining. Magnification 400×

the cases, respectively. A comparison of the IHC results indicates that there is a statistically significant association ($P = 0.04$) between CREB and C/EBPβ expression in our series of GC. We also observed that GC cases with higher CREB expression scores were significantly associated with intestinal and mixed histological subtypes ($P = 0.003$) (Table 1). No associations were detected between the expression of CREB and other clinicopathological characteristics of the tumours.

CREB modulates IL1B-induced proliferation of GC cells

To determine whether IL1B is able to increase the survival of GC cells, we measured BrdU incorporation and performed TUNEL assays in the AGS GC cell line after incubating cells with IL1B. In parallel, we determined whether any of the effects of IL1B are mediated by CREB. Our control experiments showed that CREB expression is downregulated by the shRNA used (Fig. 5a). Figure 5b shows that IL1B is able to significantly increase cellular proliferation, and that this effect can be reverted by downregulating CREB levels. IL1B had no significant effect on the rate of apoptosis (data not shown).

To explore the role of CREB in GC cell proliferation, we evaluated the expression of the cell-cycle regulator cyclin D1. After performing a dose–response experiment to determine the effect of the CBP–CREB interaction inhibitor on cell proliferation, we selected a concentration of 25 μM. In the AGS cell line, CREB inhibition had a significant inhibitory effect on cell proliferation (Fig. 5c). The effect was more pronounced after 48 h of treatment. Moreover, this effect was also confirmed by measuring the expression level of cyclin D1 after CREB inhibition after 24 and 48 h of treatment (Fig. 5f). The effect was also seen in the GP202 GC cell line (Fig. 5d, g) and in the intestinal-type GC cell line MKN28 (Fig. 5e, h). Overall, these results further support the notion that CREB plays an important role in GC cell survival, in both diffuse and intestinal histological types, by regulating the proliferation of GC cells.

Table 1 Relationship between the clinicopathological features of gastric cancer and CREB expression score

	No of cases (%)	CREB score (%)				*P* value
		0	1	2	3	
C/EBPβ score						
0	18 (27.3)	4 (22.2)	3 (16.7)	5 (27.8)	6 (33.3)	0.04
1	23 (34.8)	0 (0)	4 (17.4)	9 (39.1)	10 (43.5)	
2	12 (18.2)	0 (0)	1 (8.3)	4 (33.3)	7 (58.3)	
3	13 (19.7)	0 (0)	0 (0)	3 (23.1)	10 (76.9)	
Histological type						
Intestinal	25 (37.9)	1 (4.0)	2 (8.0)	7 (28)	15 (60.0)	0.003
Diffuse	11 (16.7)	0 (0)	3 (27.3)	7 (63.6)	1 (9.1)	
Mixed	20 (30.3)	0 (0)	3 (15.0)	4 (20.0)	13 (65.0)	
Unclassified	10 (15.1)	3 (30.0)	0 (0)	3 (30.0)	4 (40.0)	
Venous invasion						
Present	48 (72.7)	3 (6.2)	6 (12.5)	13 (27.1)	26 (54.2)	NS
Absent	18 (27.3)	1 (5.6)	2 (11.1)	8 (44.4)	7 (38.9)	
Perineural invasion						
Present	42 (63.6)	3 (7.1)	6 (14.3)	12 (28.6)	21 (50.0)	NS
Absent	24 (36.4)	1 (4.2)	2 (8.3)	9 (37.5)	12 (50.0)	
Tumour extent						
T1	2 (3.1)	0 (0)	0 (0)	2 (100.0)	0 (0)	NS
T2	32 (48.5)	1 (3.1)	4 (12.5)	11 (34.4)	16 (50.0)	
T3	23 (34.8)	2 (8.7)	3 (13.0)	4 (17.4)	14 (60.9)	
T4	9 (13.6)	1 (11.1)	1 (11.1)	3 (33.3)	4 (44.4)	
Lymph node invasion						
N0	8 (12.1)	0 (0)	0 (0)	5 (62.5)	3 (37.5)	NS
N1	23 (34.8)	2 (8.7)	1 (4.3)	7 (30.4)	13 (56.5)	
N2	21 (31.8)	2 (9.5)	4 (19.0)	5 (23.8)	10 (47.6)	
N3	14 (21.2)	0 (0)	3 (21.4)	4 (28.6)	7 (50.0)	

Guide to scores: 0, IHC positivity in <5 % of tumour cells; 1, IHC positivity in 6–50 % of tumour cells; 2, IHC positivity in 51–75 % of tumour cells; 3, IHC positivity in >75 % of tumour cells

CREB inhibition decreases GC cell growth in vivo

To evaluate the effect of CREB on tumour growth, we used the chicken embryo chorioallantoic membrane (CAM) model. The CAM effectively supports the growth of inoculated human cancer cells due to the immunodeficiency of the chick during its early developmental stages. Before CAM inoculation, we confirmed the knockdown of CREB protein on AGS cells transfected with anti-CREB shRNA (Fig. 6a). To avoid inter-animal differences in the results, scrambled shRNA and anti-CREB shRNA transfected AGS cells were inoculated in distinct areas of the CAM of the same egg and allowed to proliferate for 6 days. At this end point, the tumour area was quantified. As can be seen in Fig. 6b, inhibition of CREB led to reduced growth of the inoculated cells. Figure 6c shows that, on average, the tumour growth area was significantly smaller in cells with CREB inhibition. These results demonstrate that CREB-mediated signalling is important for GC cell growth in vivo.

Discussion

Our results demonstrate that IL1B is able to activate CREB and C/EBPβ in GC cells. This process is mediated by ERK1/2, since its inhibition by U0126 reverted the effects induced by IL1B. This is in agreement with previously reported results showing that IL1B is able to induce GC cell proliferation in an ERK1/2-dependent manner [16]. We also demonstrated that CREB is able to transactivate C/EBPβ in GC cells.

Our in vitro observations were reinforced by the analysis of the expression of CREB and C/EBPβ in a series of GC samples and normal gastric mucosa. We showed that in normal gastric mucosa, CREB is expressed in the proliferative neck/isthmus region of the gastric glands; in GC, it is overexpressed in the majority of tumour samples. These results are in accordance with those published by Chen et al. [34], showing that CREB mRNA levels are upregulated in GC samples when compared with adjacent normal mucosa.

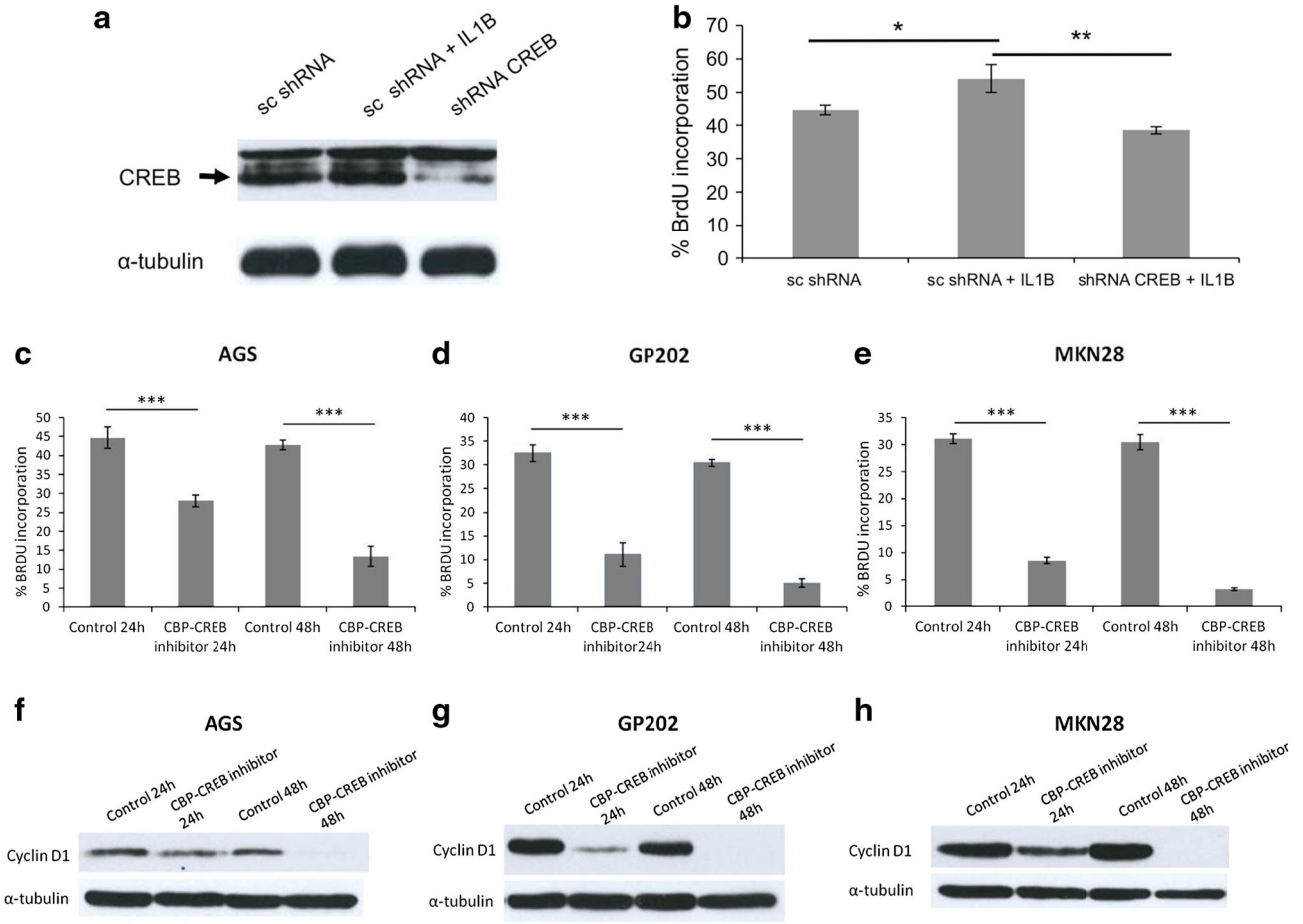


Fig. 5a–h CREB modulates both IL1B-induced and basal cell proliferation. **a** Downregulation of CREB expression using shRNA. **b** AGS cells expressing normal levels of CREB (transfected with scrambled shRNA) showed an increase in cell proliferation after 24 h of treatment with 10 ng/mL IL1B, while CREB downregulation (transfected with anti-CREB shRNA) was responsible for a significant decrease in IL1B-induced cell proliferation. *sc shRNA* control scrambled shRNA, *shRNA CREB* anti-CREB shRNA. **c** AGS, **d** GP202, and **e** MKN28 cells were treated for 24 and 48 h to assess the time-dependent impact of treatment on GC cell proliferation; the protein levels of cyclin D1 were checked in **f** AGS, **g** GP202 and **h** MKN28 cells after 24 and 48 h of CBP–CREB interaction inhibitor treatment. BrdU results represent the mean ± S.D. of three independent experiments. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

CREB expression was also significantly associated with the expression of C/EBPβ in GC. The observation that in normal gastric mucosa CREB and C/EBPβ are expressed in a cellular compartment that includes progenitor cells suggests that CREB and C/EBPβ may be involved in maintaining a proliferative phenotype in gastric epithelial cells. This would be in accordance with the observed pattern of overexpression of both proteins in the majority of the GC cases included in the present study. These results are also in keeping with our previous demonstration that C/EBPβ is overexpressed in pre-malignant lesions and in GC, suggesting that this protein might facilitate the transformation of gastric epithelial cells by inducing the expression of COX-2 [23] and by inhibiting the expression of the putative gastric tumor suppressor gene *TFF1* [24, 25].

In order to complement the aforementioned observations with a biological readout, we evaluated the role of CREB in mediating IL1B-induced changes in cell proliferation and apoptosis. Although no significant effect was observed in relation to apoptosis, our results show that CREB is an effector of IL1B-induced cell proliferation, since downregulation of CREB impairs the pro-mitogenic action of IL1B on GC cells in vitro. These results were further supported by the CAM assays showing that inhibition of CREB reduces the ability of GC cells to survive in this in vivo model.

Infection with *H. pylori* leads to chronic inflammation and an increased risk of developing GC. Our results support the hypothesis that the effect of chronic inflammation on tumourigenesis includes modulation of critical signalling pathways that regulate survival in epithelial cells.

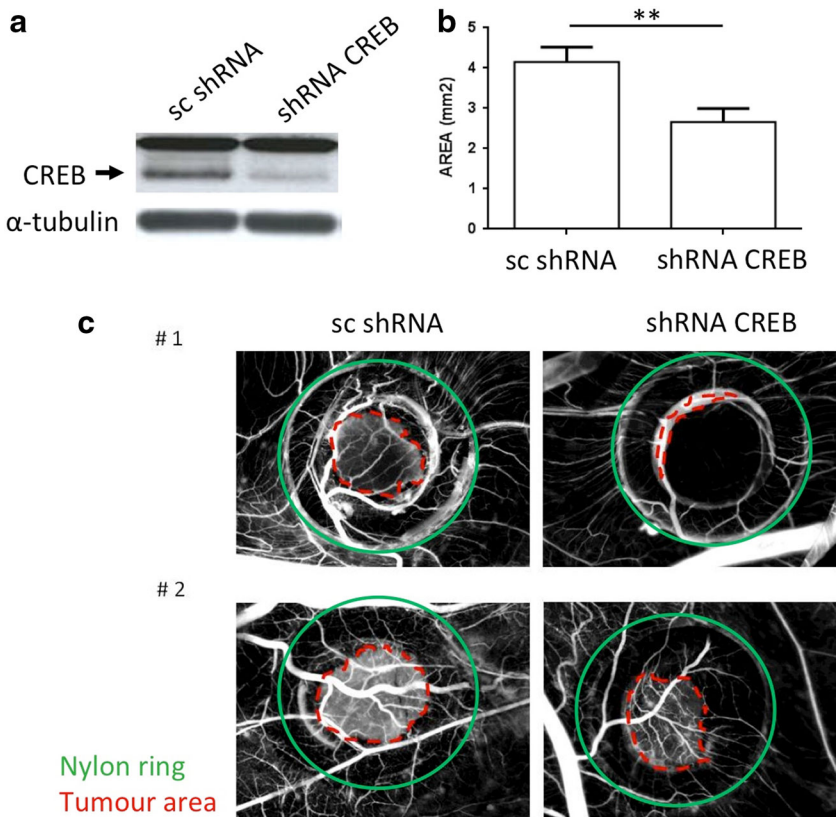


Fig. 6a–c CREB inhibition reduces in vivo cell growth. **a** Before inoculating AGS cells in the chorioallantoic membrane (CAM), CREB protein knockdown was checked by western blotting. **b** AGS cells transfected with shRNA against CREB give rise to small-sized tumours compared with scramble-transfected cells; **c** Images representing the different sizes (delimited by *red dashed lines*) of scramble and shRNA CREB tumours in two paired CAM experiments (#1 and #2). Values of $P < 0.05$ were considered to be statistically significant

In this scenario, *H. pylori* infection leads to overexpression of IL1B which, in turn, activates CREB and C/EBPβ. This effect may be more pronounced in individuals that carry genetic susceptibility polymorphisms that have been demonstrated to be associated with enhanced chronic inflammation, such as those in the *IL1B* gene promoter. Coupling cell survival with an increased likelihood of accumulating genetic mutations may help explain why individuals with pro-inflammatory genetic polymorphisms have an increased risk of developing GC.

Materials and methods

Tissue material

Surgical specimens from 66 GCs were resected and diagnosed at Hospital S. João, Porto, Portugal. Tissue fragments were fixed in 10 % formaldehyde followed by paraffin embedding. Tumour-representative areas of each GC were selected to create a tissue microarray (TMA) block. Serial sections of 3 μm were obtained from the TMA block and used for routine staining with haematoxylin and eosin and immunohistochemistry. This study was performed in accordance with institutional ethical standards. All of the samples enrolled in this study were unidentified.

Immunohistochemistry and immunofluorescence

Tissue sections from formalin-fixed paraffin-embedded (FFPE) tissues were first deparaffinised, hydrated, and then treated with 1× citrate buffer (pH 6.0) (Thermo Scientific, Waltham, MA, USA) for 45 min at 100 °C. All of the following steps were performed at room temperature (RT). Unspecific endogenous peroxidase activity was eliminated with 3 % hydrogen peroxidase in methanol for 15 min. To reduce nonspecific background staining, slides were blocked with Ultra V Block (Thermo Scientific) for 10 min. Slides were rinsed in PBS–0.1 % Tween20 and incubated for 1 h with the antibody anti-CREB [E306] (Abcam, Cambridge, UK) diluted 1:1000 and then overnight (ON) with anti-C/EBPβ (Abcam) diluted 1:1000 in UltraAB Diluent (Thermo Scientific). Slides were then incubated with Dako Real EnVision HRP Rabbit/Mouse solution (Dako, Glostrup, Denmark) for 30 min. Slides were washed, developed for 1–3 min with 2 % Dako REAL™ DAB+ Chromogen solution (Dako), counterstained with haematoxylin, dehydrated, and mounted with mounting medium (Thermo Scientific). All washing steps were performed in PBS–0.1 % Tween20 buffer. Normal gastric mucosa was used as a control, and negative controls were obtained by substituting the primary antibody with immunoglobulins of the same class and concentration. Slides were reviewed by a pathologist, and the percentage

of positive cells was semiquantitatively scored as either 0 (corresponding to positivity in <5 % of the tumour cells), 1 (corresponding to positivity in >5 % and <50 % of the tumour cells), 2 (corresponding to positivity in >50 % and <75 % of the tumour cells), or 4 (corresponding to positivity in >75 % of the tumour cells).

For immunofluorescence, antigen retrieval was performed with 1× citrate buffer (pH 6,0). After unspecific protein blocking with Ultra V Block (Thermo Scientific) for 10 min, slides were incubated with the antibody anti-CREB [E306] diluted 1:1000 for 1 h, followed by the antibody anti-C/EBPβ diluted 1:1000 ON. After washing twice with PBS–Tween 0.021 % for 10 min, the slides were incubated with a mixture of two secondary antibodies raised in different species (with Texas Red conjugated against rabbit and FITC conjugated against mouse) for 45 min at room temperature and protected from light. To counterstain cell nuclei, slides were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Finally, slides were visualised and images were captured (via ApoTome) under a fluorescence microscope (Zeiss, Oberkochen, Germany).

Cell culture, chemical treatments and transfections

AGS, MKN28 and GP202 cell lines were maintained in RPMI medium (Gibco, Waltham, MA, USA), supplemented with 10 % foetal bovine serum (FBS) (PAA, Pasching, Austria), and 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco), in a humidified incubator under an atmosphere of 5 % CO_2 at 37 °C. Cells were grown until 60–80 % confluence and treated with 10 ng/mL of IL1B (Sigma, St. Louis, MO, USA) and incubated for 24 h.

For ERK1/2 inhibition, cells were treated with 25 μM U0126 (Cayman, Ann Arbor, MI, USA) together with IL1B for 24 h. As U0126 was diluted in DMSO, an appropriate volume of DMSO was added to the cells as the control condition. For CBP–CREB interaction inhibition, cells were treated with 25 μM of the specific inhibitor (Merck–Millipore, Darmstadt, Germany) or DMSO vehicle (Sigma) for 24 and 48 h.

For silencing experiments, AGS cells grown until 60–80 % confluence were transfected with 1.0 μg of anti-CREB shRNA expression vector or 1.0 μg scrambled shRNA (Origene, Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), according to the manufacturer's specifications. At 48 h post-transfection, cells were selected by adding 1.0 μg/mL of puromycin (Sigma) to the culture medium. Individual puromycin-resistant colonies were isolated after 2 weeks of selection and expanded in the presence of puromycin (1.0 μg/mL). For the transient silencing of CREB, AGS cells were transfected either with 100 nM of siRNA against CREB (Qiagen, Hilden, Germany) or with 100 nM of siRNA control (Qiagen), using Lipofectamine 2000 (Invitrogen) as a vehicle. In parallel, C/EBPβ silencing was performed by transfecting AGS cells with 150nM of siRNA against *CEBPB* or siRNA control (Qiagen). Protein downregulation after gene silencing was evaluated by western blotting following 72 h of culture.

Western blotting (immunoblotting)

Cells were washed with 1× PBS (pH 7.4) and lysed in NP-40 buffer supplemented with phosphatase (Sigma) and protease inhibitors (Roche, Penzberg, Germany). After Bradford protein quantification, 40 μg of total protein were loaded into 12.5 % acrylamide gels, separated by SDS-PAGE under denaturing conditions and electrotransferred to a Hybond ECL Nitrocellulose Membrane (GE Healthcare, Little Chalfont, UK). After blocking, membranes were incubated for 1.5 h with the primary antibodies anti-ERK1/2 #9102 (Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000, anti-pERK1/2 #9106 (Cell Signaling Technology) diluted 1:1000, anti-CREB [E306] diluted 1:500, anti-pCREB [E113] (Abcam) diluted 1:1000, anti-C/EBPβ [H-7] (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500, anti-cyclin D1 (Santa Cruz Biotechnology) diluted 1:500, and anti-α-tubulin (Sigma) diluted 1:10000 in PBS-0.5 % Tween20 plus 5 % non-fat dried milk or 4 % BSA (bovine serum albumin). The blots were then washed with PBS–0.5 % Tween20 and incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:10000 in PBS–0.5 % Tween20 plus 0.5 % non-fat dried milk. Chemiluminescent bands were visualised using Western Blot ECL (GE Healthcare).

BrdU incorporation assay

Cells were allowed to reach 60–80 % confluence and 1× BrdU was added to the culture medium for 1 h. Cells were then washed with 1× PBS and fixed in freshly prepared 4 % (v/v) paraformaldehyde at RT for 30 min. The glass slides were removed from the 6-well plate, transferred to individual wells in a 12-well plate, and washed with 1xPBS. In order to denature the DNA and permeabilize cells, hydrochloric acid (HCl) 2 M was added to each slide for 20 min, followed by washing steps with PBS–0.5 % Tween20 plus 0.05 % BSA. Cells in glass slides were incubated for 1 h with mouse primary antibody against BrdU (Dako) diluted 1:10, washed twice with PBS–0.5 % Tween20 plus 0.05 % BSA, and incubated for 30 min with anti-mouse secondary antibody marked with Alexa Fluor 594 (Invitrogen) diluted 1:100. Glass slides were rinsed in PBS–0.5 % Tween20 plus 0.05 % BSA twice, mounted

with Vectashield Mounting Medium with DAPI (Vector Laboratories), and finally visualised under fluorescent microscopy. For each experiment, the BrdU technique was performed in triplicate. In each assay, at least 1000 cells were counted and BrdU incorporation was expressed as the ratio of DAPI to BrdU-positive cells.

Promoter analysis and chromatin immunoprecipitation (ChIP)

The nucleotide sequence of human *CEBPB* promoter was obtained from the UCSC Genome Browser (http://genome.ucsc.edu/). The putative CRE-binding sites present on *CEBPB* promoter were identified using the Genomatix MatInspector software (http://www.genomatix.de/solutions/genomatix-genome-analyzer.html).

ChIP assay was performed using a Magna ChIP G Kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol instructions. Briefly, 1×10^7 of AGS cells were crosslinked with 1 % formaldehyde for 10 min at RT, and the reaction was stopped by 1× glycine solution for 5 min. Cells were rinsed with 1× PBS, lysed in order to isolate nuclei, and then sonicated in Nuclear Lysis to shear the chromatin to sizes of 200–500 bp. Then, 50 µl of the supernatant were immunoprecipitated by adding 2 µg of rabbit antibody anti-CREB (Abcam) or 2 µg of control rabbit polyclonal anti-IgG antibody (Abcam), and the mixture was placed on a rotator at 4 °C ON in the presence of magnetic G beads. DNA–protein crosslinks were reversed by heating samples at 62 °C for 2 h on a shaking platform. To elute DNA, a series of wash steps followed by elution (50 µl) were performed in spin columns. PCR conditions: 95 °C for 15 min, 35 times (95 °C for 1 min, 58–60 °C for 1 min, 72 °C for 1 min). The reactions were carried out with HotStarTaq DNA Polymerase (Qiagen) as described by the manufacturer, using 2 µl of DNA template. The PCR products were analysed by electrophoresis on a 2 % agarose gel. PCR primer pairs flanking CREB-binding sites and for the control region (CR) were designed using the Primer 3 software.

RNA isolation, cDNA synthesis and quantification of CEBPB mRNA transcript

Total RNA was isolated from AGS cells grown in 6-well culture plates using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using a SuperScript II Reverse Transcriptase Kit (Invitrogen) with 1000 µg of total RNA in a 20-µl volume reaction, after treatment with DNase I (Invitrogen).

To determine the relative amount of *CEBPB* transcript, we performed quantitative target amplification, using cDNA as the template, with the SYBR Green PCR Kit (Qiagen), according to the manufacturer's protocol. As an internal control, we quantified the expression levels of beta-actin transcript.

Analysis of in vivo tumour growth

The chicken embryo CAM model was used to evaluate the growth capability of AGS cells transfected with scramble or shCREB RNA ($n = 16$). Briefly, fertilised chick (*Gallus gallus*) eggs were incubated horizontally at 37.8 °C in a humidified atmosphere. On embryonic day 3 (E3), a square window was opened on the shell after removing 1.5–2 mL of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. Cells, re-suspended in 10 µl of complete medium, were placed on top of E10 growing CAM, and 2×10^6 cells per embryo from each cell line (scrambled shRNA vs CREB shRNA) were placed into a 3-mm nylon ring under sterile conditions. The eggs were re-sealed and returned to the incubator for an additional 5 days. After removing the ring, the CAM was excised from the embryos, photographed ex ovo under a stereoscope at 20× magnification (SZX16 coupled with a DP71 camera, Olympus, Tokyo, Japan). The area of CAM tumour was determined using the Cell A program (Olympus).

Statistical analysis

The clinicopathological features of GC cases were compared using the χ^2 test. When two conditions were compared, Student's t test was used, whereas ANOVA was employed when the comparison involved more than two conditions. The paired t test was used for tumour area comparisons. In order to accurately access putative differences in tumour areas between the two cell lines, only eggs bearing two tumours with areas ≥ 1 mm^2 ($n = 16$) were considered, independently of the cell group. Values of $P < 0.05$ were considered to be statistically significant.

Acknowledgments This work was supported by Fundação para a Ciência e Tecnologia (SFRH/BD/46504/2008; ERA-PTG/0001/2010), FEDER, National Funds through the FCT—Foundation for Science and Technology, under the projects "PEst-C/SAU/LA0003/2013" and Programa Operacional Factores de Competitividade—COMPETE. IPATIMUP integrates the i3S Research Unit, which is partially supported by FCT.

Conflict of interest None.

References

1. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
2. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society Award Lecture

on cancer epidemiology and prevention. Cancer Res. 1992;52: 6735–40.
3. Crew KD, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol. 2006;12:354–62.
4. Milne AN, Carneiro F, O'Morain C, et al. Nature meets nurture: molecular genetics of gastric cancer. Hum Genet. 2009;126: 615–28.
5. Resende C, Ristimäki A, Machado JC. Genetic and epigenetic alteration in gastric carcinogenesis. Helicobacter. 2010;15:34–9.
6. Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. Gastroenterology. 2008;134:306–23.
7. El-Omar EM, Carrington M, Chow W-H, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature. 2000;404:398–402.
8. Machado JC, Pharoah P, Sousa S, et al. Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. Gastroenterology. 2001;121:823–9.
9. Figueiredo C, Machado JC, Pharoah P, et al. *Helicobacter pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst. 2002;94: 1680–7.
10. Machado JC, Figueiredo C, Canedo P, et al. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology. 2003;125:364–71.
11. Canedo P, Corso G, Pereira F, et al. The interferon gamma receptor 1 (IFNGR1) −56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. Gut. 2008;57: 1504–8.
12. Hwang I-R, Kodama T, Kikuchi S, et al. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1β production in *Helicobacter pylori* infection. Gastroenterology. 2002;123: 1793–803.
13. Tu S, Bhagat G, Cui G, et al. Overexpression of interleukin-1β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell. 2008;14:408–19.
14. Beales ILP, Calam J. Interleukin 1β and tumour necrosis factor α inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. Gut. 1998;42:227–34.
15. Konturek P, Kania J, Konturek J, et al. *H. pylori* infection, atrophic gastritis, cytokines, gastrin, COX-2, PPAR gamma and impaired apoptosis in gastric carcinogenesis. Med Sci Monit 2003;9:SR53–66.
16. Beales I. Effect of interleukin-1beta on proliferation of gastric epithelial cells in culture. BMC Gastroenterol. 2002;2:7.
17. Yasunaga Y, Shinomura Y, Kanayama S, et al. Increased production of interleukin 1 beta and hepatocyte growth factor may contribute to foveolar hyperplasia in enlarged fold gastritis. Gut. 1996;39:787–94.
18. El-Omar EM, Rabkin CS, Gammon MD, et al. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology. 2003;124: 1193–201.
19. Newton K, Dixit VM. Signaling in innate immunity and inflammation. Cold Spring Harbor Perspect Biol. 2012;4:3.
20. Naumann M, Crabtree JE. *Helicobacter pylori*-induced epithelial cell signalling in gastric carcinogenesis. Trends Microbiol. 2004;12:29–36.
21. Kudo T, Lu H, Wu JY, et al. Pattern of transcription factor activation in *Helicobacter pylori*—infected Mongolian gerbils. Gastroenterology. 2007;132:1024–38.
22. Giltiay NV, Karakashian AA, Alimov AP, et al. Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin-1β in hepatocytes. J Lipid Res. 2005;46:2497–505.
23. Regalo G, Canedo P, Suriano G, et al. C/EBPbeta is over-expressed in gastric carcinogenesis and is associated with COX-2 expression. J Pathol. 2006;210:398–404.
24. Sankpal NV, Mayo MW, Powell SM. Transcriptional repression of TFF1 in gastric epithelial cells by CCAAT/enhancer binding protein-β. Biochim Biophys Acta 2005;1728:1–10.
25. Sankpal NV, Moskaluk CA, Hampton GM, et al. Overexpression of CEBPbeta correlates with decreased TFF1 in gastric cancer. Oncogene. 2006;25:643–9.
26. Zhao D. Protein kinase Cδ-mediated CREB activation regulates ghrelin-induced cyclooxygenase-2 expression and prostaglandin E2 production in human colonic epithelial cells. J Cell Biochem. 2007;102:1245–55.
27. Sun H, Chung WC, Ryu SH, et al. Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis. Cancer Prev Res (Phila). 2008;1:316–28.
28. Shankar DB, Cheng JC, Kinjo K, et al. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell. 2005;7:351–62.
29. Seo HS, Liu DD, Bekele BN, et al. Cyclic AMP response element-binding protein overexpression: a feature associated with negative prognosis in never smokers with non-small cell lung cancer. Cancer Res. 2008;68:6065–73.
30. Aggarwal S, Kim SW, Ryu SH, et al. Growth suppression of lung cancer cells by targeting cyclic AMP response element-binding protein. Cancer Res. 2008;68:981–8.
31. Chhabra A, Fernando H, Watkins G, et al. Expression of transcription factor CREB1 in human breast cancer and its correlation with prognosis. Oncol Rep. 2007;18:953–8.
32. Niehof M, Manns MP, Trautwein C. CREB controls LAP/C/EBP beta transcription. Mol Cell Biol. 1997;17:3600–13.
33. Zhang JW, Klemm DJ, Vinson C, et al. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. J Biol Chem. 2004;279:4471–8.
34. Chen L, Yang Q, Kong WQ, et al. MicroRNA-181b targets cAMP responsive element binding protein 1 in gastric adenocarcinomas. IUBMB Life. 2012;64:628–35.

Article IV

Helicobacter

Helicobacter ISSN 1523-5378

Genetic and Epigenetic Alteration in Gastric Carcinogenesis

Carlos Resende,[*,†,‡] Ari Ristimäki[§] and José C. Machado[*,†]

*IPATIMUP - Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal, [†]Faculty of Medicine, University of Porto, Porto, Portugal, [‡]Instituto de Ciências Biomédicas da Universidade do Porto (ICBAS), Porto, Portugal, [§]Department of Pathology, HUSLAB and Haartman Institute, and Genome-Scale Biology Research Program, University of Helsinki, Helsinki, Finland

Keywords
Single nucleotide polymorphism, molecular alterations, experimental models.

Reprint requests to: José Carlos Machado, IPATIMUP, Rua Dr. Roberto Frias, s/n, 4200–465 Porto, Portugal.
E-mail: josem@ipatimup.pt

Abstract

Gastric cancer (GC) is an important cause of morbidity and mortality worldwide. In addition to environmental factors, genetic factors also play an important role in GC etiology, as demonstrated by the fact that only a small proportion of individuals exposed to the known environmental risk factors develop GC. Molecular studies have provided evidence that GC arises not only from the combined effects of environmental factors and susceptible genetic variants but also from the accumulation of genetic and epigenetic alterations that play crucial roles in the process of cellular immortalization and tumorigenesis. This review is intended to focus on the recently described basic aspects that play key roles in the process of gastric carcinogenesis. Genetic variation in the genes *DNMT3A*, *PSCA*, *VEGF*, and *XRCC1* has been reported to modify the risk of developing gastric carcinoma. Several genes have been newly associated with gastric carcinogenesis, both through oncogenic activation (*MYC*, *SEMA5A*, *BCL2L12*, *RBP2* and *BUBR1*) and tumor suppressor gene inactivation mechanisms (*KLF6*, *RELN*, *PTCH1A*, *CLDN11*, and *SFRP5*). At the level of gastric carcinoma treatment, the HER-2 tyrosine kinase receptor has been demonstrated to be a molecular target of therapy.

Gastric cancer (GC) is an important cause of morbidity and mortality worldwide [1]. The etiology of GC has a significant environmental component characteristic of the geographically varied incidence in the disease distribution [1–4]. Several environmental factors, including *Helicobacter pylori* infection, consumption of salted and nitrated foods, and cigarette smoking, have been found to be associated with the risk of developing GC [2–5]. In addition to environmental factors, genetic factors also play an important role in GC etiology, as demonstrated by the fact that only a small proportion of individuals exposed to the known environmental risk factors develop GC [4,6–8].

Molecular studies have provided evidence that GC arises not only from the combined effects of environmental factors and susceptible genetic variants but also from the accumulation of genetic and epigenetic alterations that play crucial roles in the process of cellular immortalization and tumorigenesis [2,3,9].

This review is intended to focus on the recently described basic aspects that play key roles in the process of gastric carcinogenesis. New advances in the fields of the individual's genetic susceptibility for gastric carcinogenesis, deregulation of gene expression, genetic profile present in tumors with microsatellite instability (MSI), and new options for treatment of GC will be discussed.

Genetic Susceptibility

In recent years, molecular epidemiological studies have described some relatively common genetic variants as biomarkers for genetic susceptibility to GC development, namely single-nucleotide polymorphisms (SNPs) [4–7,10]. These genetic variants may modulate the effects of environmental factors by regulating multiple biologic pathways in response to the exposure during gastric carcinogenesis, thus exerting an effect on population attributable risks. One major advantage of SNPs as prognostic markers is that they can be determined independently from the availability and quality of tumor material as they can be easily evaluated from a blood sample from individual patients. For example, Fan et al. [11] described that the *DNMT3A*-448A>G polymorphism is involved in the genetic susceptibility

to GC. DNA methyltransferase-3A (DNMT3A) is essential for mammalian development and is responsible for the generation of genomic methylation patterns [12]. *De novo* DNMT3A expression was reported as playing a role in gastric carcinogenesis [13]. In another study, Ju et al. [14] reported that the *PTPRCAP* −309G>T polymorphism is associated with increased susceptibility to diffuse-type GC by increasing *PTPRCAP* expression. The protein tyrosine phosphatase receptor type C-associated protein (PTPRCAP) is involved in the activation of the Src family kinases (SFKs) [15], and it is known that overexpression of SFK is involved in the disruption of the epithelial cell–cell adhesion by inducing impairment in the membrane localization of E-cadherin [16].

Another gene that has been reported as having a role in gastric carcinogenesis is the *PSCA* [17]. Interestingly, *PSCA* was found to be expressed in differentiating gastric epithelial cells, where it exerts a cell-proliferation inhibitory activity in vitro, and it is frequently found silenced in GC cells. Lu et al. [18] reported that two polymorphisms (rs 2976392 and rs 2294008) in PSCA gene may contribute to the etiology of gastric carcinogenesis, at least in a Chinese population.

Also, vascular endothelial growth factor (*VEGF*) gene has been the focus of many associative studies. *VEGF*, the key mediator of angiogenesis, plays an important role in the development of different tumors, including GC [19], where it plays a critical role in the invasive process of cancer cells [20]. Guan et al. [21] described that the VEGF −634G>C polymorphism is associated with the risk to develop GC. They showed that the heterozygous −634CG and the combined −634CG+CC carriers had an increased risk of developing GC when compared with the −634GG genotype. In another study, Tahara et al. [22] reported that the polymorphism 1612G>A in the 3′-UTR of VEGF was associated with an increased risk of GC. They suggest that the nucleotide polymorphism in the 3′-UTR, such as SNPs and triplet nucleotide repeat, are associated with the deregulation of affected genes.

The integrity and maintenance of the DNA nucleotide composition are vital for cell's normal function. X-ray repair cross-complementing group 1 (XRCC1) is one of the proteins involved in the base excision repair pathway, which functions in the repair of single-strand breaks caused by exposure to ionizing radiation, alkylating agents, and metabolic toxins [4,23]. It is known that the presence of the *XRCC1*-77T>C promoter polymorphism is associated with human cancer, namely, with non-small cell lung cancer [24]. Corso et al. [25] reported an association between the presence of the *XRCC1*-77T>C polymorphism and the increased risk of gastric cardia carcinoma, so the referred polymorphism was considered by the authors as a relevant host susceptibility factor for GC.

Various other articles were published last year establishing an association between genetic polymorphisms and the risk of GC. Host genetic factors are emerging as key elements in the risk for the development of cancer, and the interaction of numerous polymorphisms on a countless genes products, combined with environmental triggers may provide crucial clues explaining diverse risks in various populations.

Molecular Alterations in Gastric Cancer

Understanding the molecular mechanisms and alterations behind the initiation and progression of gastric tumorigenesis is crucial for the early detection of the disease and to identify novel therapeutic and clinical targets for GC. A number of molecular abnormalities have been identified in GC, namely gene overexpression and gene silencing, and MSI-associated gene mutations. Nevertheless, the molecular pathogenesis of GC is still incompletely understood.

Gene Overexpression

Over the last decade, a vast amount of articles referring to the overexpression of various genes in GC was published. Some of those genes were classified as activated oncogenes, like *Her-2/neu* [26] and *c-Myc* [27], playing roles in the induction of cell proliferation. Following the search for other deregulated genes that are involved in cell proliferation, Pan et al. reported the overexpression of SEMA5A (Semaphorin 5A) in GC [28]. With in vitro models, and using siRNA-mediated semaphorin 5A knockdown, those authors concluded that semaphorin 5A may be involved in gastric carcinogenesis by promoting cell proliferation and inhibiting apoptosis. In another study, Florou et al. [29] described how *BCL2L12*, a member of the BCL2 family that could function as an anti-apoptotic factor, was overexpressed in early stages of GC compared to normal mucosa.

The histone-modifying enzymes are responsible for acetylation, phosphorylation, and methylation of histone proteins, playing a key role in the regulation of gene transcription by mediating chromatin reconfiguration [30]. Zeng et al. [31] described the overexpression of histone demethylase RBP2 in GC, and they observed that *RBP2* depletion triggers the senescence of malignant cells at least partially by derepressing CDKIs.

It is known that GC shows a high frequency of DNA aneuploidy [32], and it was recently described that knockdown or overexpression of spindle assembly checkpoint molecules resulted in ploidy errors and

carcinogenesis in mice [33]. Knowing that, Ando et al. [34] assessed the expression of *BUBR1* kinase, one of the key molecules in the spindle assembly checkpoint, in GC samples. These authors observed a high expression of BUBR1 in GCs that were aneuploid, establishing a relation between BUBR1 expression and induction of aneuploidy. To confirm that association, they enforced expression of BUBR1 in cell lines and, as a result, they observed changes in the ploidy of the cells.

Gene Silencing

Gene silencing in GC can occur mainly because of the point mutations, loss of heterozygosity, and promoter hypermethylation [2,3]. Genetic alterations were reported by Sangodkar et al. [35] as the cause of *KLF6* downregulation in GC, being this gene-silencing critical to abrogate the repressive effect on proliferation of wild-type KLF6.

Transcriptional inactivation of specific genes via aberrant promoter hypermethylation of CpG islands, causing permanent gene silencing, is a major epigenetic event in carcinogenesis. Reported genes whose expression was downregulated in GC by promoter hypermethylation are *CDH1* [9], *RELN* [36], *PTCH1a* [37], *HLA* class I [38], *CLDN11* [39], *SFRP5* [40], and probably *CEBPA*, because it does not harbor gene mutations that could explain its downregulation in about 30% of GCs [41].

Microsatellite Instability

MSI is defined as the presence of replication errors in simple repetitive microsatellite sequences because of the defects in mismatch repair genes [10,42]. Many cancer-associated genes have been found to harbor mutations at mono- or dinucleotide repeats in the coding sequences in cancers with MSI [42], and GC is no exception [43]. Recently, Velho et al. [44] reported that in MSI gastric tumors, *MLK3*, a gene that codifies a kinase involved in MAP kinase pathway, is frequently found mutated. Noteworthy, they found that the missense mutations found in *MLK3* harbor transforming and tumorigenic potential, in vitro and in vivo.

Autophagy-related genes ATG2B, ATG5, ATG9B, and ATG12 were also reported as harboring mutations in MSI tumors, contributing to cancer development by deregulation of the autophagy process [45].

Gastric Cancer Treatment

Despite recent advances in perioperative and adjuvant chemotherapy, most patients with advanced disease have a median survival of less than a year. The best prognostic parameters of the disease are TNM-staging (invasion depth and metastasis to lymph nodes or to distant sites) and complete surgical removal of the neoplastic tissue. However, these traditional prognostic clinicopathological characteristics provide limited information about predictive measures of the disease. So far, genome-wide screens have provided no clinically applicable predictive value in GC, and partly owing to this, it has been more promising to focus on specific targeted cancer treatment modalities and methods to identify their molecular targets. Several of these novel treatment options and their putative predictive markers have not yet been proven to show clinical value in GC (for example cyclooxygenase-2 and nonsteroidal anti-inflammatory drugs or antibodies and small molecular inhibitors of epidermal growth factor receptor and amplification or mutations of the receptor). However, HER-2 has been recently demonstrated to be a molecular target in GC.

HER-2 and Carcinogenesis

Cell proliferation is tightly regulated through cellular signal transduction pathways, and growth factors and their receptors play an important role in regulation of these intracellular responses. One central family of growth factor receptors are the four related proteins named HER/ErbB receptors [46,47]. Each of these receptors are transmembrane proteins, and HER-1, HER-2, and HER-4 have an intracellular domain with tyrosine kinase activity. HER-1 (also called epidermal growth factor receptor), HER-3, and HER-4 can bind ligands with their extracellular domain, and the bound ligand induces either homo- or heterodimerization of the receptors and generates autophosphorylation, which can lead to multiple intracellular signals. HER-2 does not bind to any known ligand, but it can heterodimerize with other members of the family. This is especially evident, when HER-2 is overexpressed or activated through either amplification or mutation of the gene. HER receptors have been shown to activate Ras-Raf-MAPK, PI3K-AKT, and STAT pathways that can inhibit apoptosis and promote proliferation, migration, angiogenesis, invasion, and metastasis. Thus, HER receptors are a rational target for cancer treatment. Indeed, work using in vitro and in vivo models of carcinogenesis have shown that inhibition of HER-1 and HER-2 suppresses cancer cell growth and survival. Finally, both monoclonal antibodies against HER-1 (cetuximab, panitumab) and HER-2 (trastuzumab) are currently used to treat patients with metastasized colorectal cancer and breast cancer, respectively. Predictive

marker for anti-HER-1 treatment is wild-type KRAS oncogene and for anti-HER-2 amplification of the HER-2 gene. In addition, small molecular tyrosine kinase inhibitors against HER-1 receptor (gefitinib, erlotinib) and a dual HER-1/2 inhibitor (lapatinib) have been approved for certain carcinoma treatments.

HER-2 in Experimental Gastric Cancer Models

Growth of human GC cells in vitro and in xenograft models in vivo has been shown to be inhibited by the anti-HER-2 monoclonal antibody tratuzumab. This effect, which seems to require HER-2 overexpression, and combination of trastuzumab with chemotherapy were more effective than either treatment alone [48,49]. More recently it was shown that both HER-2-targeted transient transfection of siRNA molecules and stable lentiviral-mediated shRNA expression decreased GC cell viability, and the latter treatment was also shown to suppress xenograft tumor growth of upper gastrointestinal adenocarcinoma cell lines [50,51]. Combination of 5-fluorouracil and HER-2-targeting agents, trastuzumab or lapatinib (the dual HER-1/HER-2 tyrosine kinase inhibitor), synergistically inhibited the proliferation and enhanced the apoptosis in GC cells with HER-2 amplification (but not in those without it), which may depend on downregulation of thymidylate synthase expression, which is the target of 5-fluorouracil [52]. In addition, lapatinib sensitized GC cells to SN-38, the active metabolite of irinotecan [53]. Finally, lapatinib acted in a synergistic manner with trastuzumad as an anticancer agent both in in vitro and in vivo conditions [54]. These data support the hypothesis that anti-HER-2 treatment could be effective in patients with GC at least in HER-2 amplified tumors and in combination with cytostatic drugs.

HER-2 in Clinical Gastric Cancer Trials

Overexpression of membranous HER-2 protein positivity has been detected by immunohistochemistry in 8–53% of gastric adenocarcinomas [48,49]. This relatively wide range of positivity most likely is dependent on differences in study materials (distribution of different histologic types and location of the tumor) and attributable to variable staining protocols and scoring criteria. When Hofmann et al. [55] pooled data from 16 different studies (3264 tumor samples), a mean value of 18% of HER-2 immunopositivity was obtained, and nine studies (from 1232 tumors) showed a mean value of 19% of HER-2 amplified cases using either fluorescence or chromogen in situ hybridization (HER-2/CEN-17≥2). These values are well in the range reported for HER-2 amplification in breast cancer (15–25%). In several studies, intestinal-type GCs were shown to express HER-2 more frequently (16–34%) than the diffuse-type tumors (2–7%). Probably because of this association with intestinal type histology, HER-2 expression is higher in gastroesophageal junction carcinomas when compared to conventional (corpus and antrum) GC (24–32% vs 10–18%), because the intestinal type is more frequent in the proximal location. The role of HER-2 as a prognostic factor in GC is somewhat controversial, because several studies have failed to show any role in prognosis, while others have indicated that HER-2 is an independent prognostic factor in GC [48,49,56–59].

A randomized multicenter phase III trial (ToGa study) has shown that first-line treatment with trastuzumab in combination with either cisplatin and 5-fluorouracil or cabecitapin is effective against metastatic gastric adenocarcinoma [60]. Median survival was improved (from 11.1 to 13.8 months; n = 584) in patients receiving trastuzumab in combination with cytostatic drugs, which was even more impressive in the subgroup of the HER-2 immunohistochemistry 3+ and 2+ with amplification positivity (median survival 11.8 vs. 16.0 months; n = 446). No major safety issues were reported between the two treatment arms. Consistent with earlier data, HER-2 was more frequently positive in intestinal (32%) than in diffuse-type tumors (6%), and in gastroesophageal junctional cancers (33%) when compared to those in the stomach (21%). Overall rate of HER-2 positivity was 22% (immunohistochemistry 3+ or amplification positive) [61]. Based on these data, trastuzumab has been approved by the EMEA for metastatic GC and adenocarcinoma of the gastroesophageal junction.

Assessment of HER-2 positivity in GC has become increasingly important because of the results of the ToGa study. Earlier studies have shown only modest concordance between HER-2 immunopositivity and amplification rates [48], but more recent studies have indicated that a much higher (over 90%) concordance between immunohistochemistry positivity and amplification can be obtained [55]. In the ToGa trial, a 87.5% concordance was reported [61]. This suggests that similarly to breast cancer also in GC the major mechanism for overexpression of the protein is the amplification of the gene. The major difference between breast cancer and GC, based on the ToGa trial, was the relatively high frequency of immunohistochemistry 0 or 1+ that were amplification positive. A modified scoring system of immunohistochemistry for GC has been proposed [55]. These modifications acknowledge incomplete basolateral (U-shaped)

membrane staining pattern of glandular cells as positive. Also, relatively high frequency of tumor heterogeneity (5%) was found in GC, and the 10% cutoff of area of positivity is restricted to surgical specimens, but no such cutoff was recommended to the biopsy samples.

In conclusion, the best way to help the patients with GC would obviously be to prevent the disease altogether. However, especially in the Western world we are faced with the fact that most patients are diagnosed in advanced stage of the disease. Although combination chemotherapies have shown to be effective, new therapeutic strategies are clearly needed because of the relatively rapid progression of the disease despite the treatment. To this end, new molecular targets should be identified and personalized treatment offered. Mechanisms of resistance against trastuzumab treatment include mutation of the HER-2 receptor, masking of the receptor, activation of insulin-like growth factor-1 receptor or PTEN deficiency. These alterations may be overcome by novel antibodies against HER-2 or by small molecular inhibitors of the receptor or its downstream targets. Indeed, ongoing phase II and III trials test the use of lapatinib in patients with GC.

Conflict of Interest

The authors declare no conflict of interest.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics. *CA Cancer J Clin* 2002;55:74–108.

2 Vogiatzi P, Vindigni C, Roviello F, Renieri A, Giordano A. Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis. *J Cell Physiol* 2007;211:287–95.

3 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006;12:354–62.

4 Yin M, Hu Z, Tan D, Ajani JA, Wei Q. Molecular epidemiology of genetic susceptibility to gastric cancer: focus on single nucleotide polymorphisms in gastric carcinogenesis. *Am J Transl Res* 2009;1:44–54.

5 Shikata K, Doy Y, Yonemoto K, et al. Population-based prospective study of the combined influence of cigarette smoking and *Helicobacter pylori* infection on gastric cancer incidence: the Hisayama study. *Am J Epidemiol* 2008;12:1409–15.

6 Machado JC, Figueiredo C, Canedo P, et al. A proinflamatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* 2003;2:364–71.

7 Canedo P, Corso G, Pereira F, et al. The interferon gamma receptor 1 (IFNGR1) -56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. *Gut* 2008;11:1504–8.

8 Canedo P, Duraes C, Pereira F, et al. Tumour necrosis factor alpha extended haplotypes and risk of colorectal and gastric carcinoma. *Cancer Epidemiol Biomarkers Prev* 2008;17:2416–20.

9 Pinheiro H, Bordeira-Carriço R, Seixas S, et al. Allele-specific CDH1 downregulation and hereditary diffuse gastric cancer. *Hum Mol Genet* 2010;5:943–52.

10 Milne AN, Carneiro F, O'Morain C, Offerhaus GJ. Nature meets nurture: molecular genetics of gastric cancer. *Hum Genet* 2009;126:615–28.

11 Fan H, Liu D, Qiu X, et al. A functional polymorphism in the DNA methyltransferase-3A promoter modifies the susceptibility in gastric cancer but not in esophageal carcinoma. *BMC Med* 2010;8:12.

12 Samuel MS, Suzuki H, Buchert M, et al. Elevated Dnmt3a activity promotes polyposis in Apc Min mice by relaxing extracellular restrains on Wnt signaling. *Gastroenterology* 2009;137:902–13.

13 Ding WJ, Fang JY, Chen XY, Peng YS. The expression and clinical significance of DNA methyltransferase proteins in human gastric cancer. *Dig Dis Sci* 2008;53:2083–9.

14 Ju H, Lim B, Kim M, et al. A regulatory polymorphism at position -309 in PTPRCAP is associated with susceptibility to diffuse-type gastric cancer and gene expression. *Neoplasia* 2009;11:1340–7.

15 Motoya S, Kitamura K, Matsuda A, Maizel AL, Yamamoto H, Takeda A. Interaction between CD45-AP and protein-tyrosine kinases involved in T cell receptor signaling. *J Biol Chem* 1999;274:14392–9.

16 Avizienyte E, Wyke AW, Jones RJ, et al. SRC-induced de-regulation of E-cadherin in colon cancer cells requires integrin signaling. *Nat Cell Biol* 2002;4:632–8.

17 Sakamoto H, Yoshimura K, Saeki N, et al. Genetic variation in PSCA is associated with susceptibility to diffuse-type gastric cancer. *Nat Genet* 2008;40:730–40.

18 Lu Y, Chen J, Ding Y, et al. Genetic variation of PSCA gene is associated with the risk of both diffuse and intestinal-type gastric cancer in a Chinese population. *Int J Cancer* 2010;Feb 3. [Epub ahead of print].

19 Ke Q, Liang J, Wang LN, et al. Potentially functional polymorphisms of the vascular endothelial growth factor gene and risk of gastric cancer. *Mol Carcinog* 2008;47:647–51.

20 Zhao R, Liu XQ, Wu XP, et al. Vascular endothelial growth factor (VEGF) enhances gastric carcinoma invasiveness via integrin alpha (v) beta6. *Cancer Lett* 2010;287:150–6.

21 Guan X, Zhao H, Niu J, Tang D, Ajani JA, Wei Q. The VEGF -634G>C promoter polymorphism is associated with risk of gastric cancer. *BMC Gastroenterol* 2009;9:77.

22 Tahara T, Shibata T, Nakamura M, et al. Effect of polymorphisms in the 3′ untranslated region (3′-UTR) of vascular endothelial growth factor gene on gastric cancer and peptic ulcer diseases in Japan. *Mol Carcinog* 2009;48:1030–7.

23 Caldecott KW, Tucker JD, Stanker LH, Thompson LH. Characterization of the XRCC1-DNA ligase III complex in vitro and its absence from mutant hamster cells. *Nucleic Acids Res* 1995;23:4836–43.

24 Hao B, Miao X, Li Y, et al. A novel T-77C polymorphism in DNA repair gene XRCC1 contributes to diminished promoter activity and increased risk of non-small cell lung cancer. *Oncogene* 2006;25:3613–20.

25 Corso G, Marrelli D, Pedrazzani C, et al. Gastric cardia carcinoma is associated with the promoter -77T>C gene polymorphism of X-ray cross-complementing group 1 (XRCC1). *J Gastrointest Surg* 2009;13:2233–8.

26 Ross JS, McKenna BJ. The HER-2/neu oncogene in tumours of the gastrointestinal tract. *Cancer Invest* 2001;19:554–68.

27 Kozma L, Kiss I, Hajdu J, Szentkereszty Z, Szakall S, Ember I. C-myc amplification and cluster analysis in human gastric carcinoma. *Anticancer Res* 2001;21:707–10.
28 Pan G, Lv Hui, Ren H, et al. Elevated expression of semaphoring 5A in human gastric cancer and its implication in carcinogenesis. *Life Sci* 2010;86:139–44.
29 Florou D, Papadopoulos IN, Scorilas A. Molecular analysis and prognostic impact of the novel apoptotic gene BCL2L12 in gastric cancer. *Biochem Biophys Res Commun* 2010;391:214–8.
30 Berger SL. The complex language of chromatin regulation during transcription. *Nature* 2007;447:407–12.
31 Zeng J, Ge Z, Wang L, et al. The histone demethylase RBP2 is overexpressed in gastric cancer and its inhibition triggers senescence of cancer cells. *Gastroenterology* 2010;138:981–92.
32 Buffart TE, van Grieken NCT, Tijssen M, et al. High resolution analysis of DNA copy-number aberrations of chromosomes 8, 13, and 20 in gastric cancers. *Virchows Arch* 2009;455:213–23.
33 van Deursen JM. Rb loss causes cancer by driving mitosis mad. *Cancer Cell* 2007;11:1–3.
34 Ando K, Kakeji Y, Kitao H, et al. High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer. *Cancer Sci* 2010;101:639–45.
35 Sangodkar J, Shi J, DiFeo A, et al. Functional role of the KLF6 tumour suppressor gene in gastric cancer. *Eur J Cancer* 2009;45:666–76.
36 Dohi O, Takada H, Wakabayashi N, et al. Epigenetic silencing of RELN in gastric cancer. *Int J Oncol* 2010;36:85–92.
37 Du P, Ye HR, Gao J, et al. Methylation of PTCH1a gene in a subset of gastric cancers. *World J Gastroenterol* 2009;15:3799–806.
38 Ye Q, Shen Y, Wang X, et al. Hypermethylation of HLC class I is associated with HLA class I down-regulation in human gastric cancer. *Tissue Antigens* 2010;75:30–9.
39 Agarwal R, Mori Y, Cheng Y, et al. Silencing of claudin-11 is associated with increased invasiveness of gastric cancer cells. *PLoS ONE* 2009;4:e8002.
40 Zhao C, Bu X, Zhang N, Wang W. Downregulation of SFRP5 expression and its inverse correlation with those of MMP-7 and MTI-MMP in gastric cancer. *BMC Cancer* 2009;9:224.
41 Regalo G, Resende C, Wen X, et al. C/EBPalpha expression is associated with homeostasis of the gastric epithelium and with gastric carcinogenesis. *Lab Invest* 2010;Apr 12. [Epub ahead of print].
42 Rhyu MG, Park WS, Meltzer SJ. Microssatelite instability occurs frequently in human gastric carcinoma. *Oncogene* 1994;1:29–32.
43 Carneiro F, Oliveira C, Leite M, Seruca R. Molecular targets and biological modifiers in gastric cancer. *Semin Diagn Pathol* 2008;4:274–87.
44 Velho S, Oliveira C, Paredes J, et al. Mixed lineage kinase 3 gene mutations in mismatch repair deficient gastrointestinal tumours. *Hum Mol Genet* 2010;19:697–706.
45 Kang RM, Kim MS, Oh JE, et al. Frameshift mutations of autophagy-related genes ATG2B, ATG5, ATG9B and ATG12 in gastric and colorectal cancers with microsatellite instability. *J Pathol* 2009;217:702–6.
46 Lurje G, Lenz HJ. EGFR signaling and drug discovery. *Oncology* 2009;77:400–10.
47 Jones KL, Buzdar AU. Evolving novel anti-HER2 strategies. *Lancet Oncol* 2009;10:1179–87.
48 Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008;19:1523–9.
49 Jørgensen JT. Targeted HER2 treatment in advanced gastric cancer. *Oncology* 2010;78:26–33.
50 Arrington AK, Dahlberg PS, Davydova J, Vickers SM, Yamamoto M. ERBB2 suppression decreases cell growth via apoptosis in gastrointestinal adenocarcinomas. *Surgery* 2009;146:213–9.
51 Arrington AK, Davydova J, Vickers SM, Yamamoto M. Anti-ERBB2 sh-RNA suppress both cell growth and tumour growth in ERBB2-overexpressing upper gastrointestinal adenocarcinomas. *J Gastrointest Surg* 2009;13:1754–61.
52 Tanizaki J, Okamoto I, Takezawa K, et al. Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification. *Mol Cancer Ther* 2010;9:1198–207.
53 LaBonte MJ, Manegold PC, Wilson PM, et al. The dual EGFR/-HER-2 tyrosine kinase inhibitor lapatinib sensitizes colon and gastric cancer cells to the irinotecan active metabolite SN-38. *Int J Cancer* 2009;125:2957–69.
54 Wainberg ZA, Anghel A, Desai AJ, et al. Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo. *Clin Cancer Res* 2010;16:1509–19.
55 Hofmann M, Stoss O, Shi D, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008;52:797–805.
56 Kim JW, Hwang I, Kim MJ, Jang SJ. Clinicopathological characteristics and predictive markers of early gastric cancer with recurrence. *J Korean Med Sci* 2009;24:1158–64.
57 Barros-Silva JD, Leitão D, Afonso L, Vieira J, et al. Association of ERBB2 gene status with histopathological parameters and disease-specific survival in gastric carcinoma patients. *Br J Cancer* 2009;100:487–93.
58 Grabsch H, Sivakumar S, Gray S, Gabbert HE, Müller W. HER2 expression in gastric cancer: rare, heterogeneous and of no prognostic value - conclusions from 924 cases of two independent series. *Cell Oncol* 2010;32:57–65.
59 Marx AH, Tharun L, Muth J, et al. HER-2 amplification is highly homogenous in gastric cancer. *Hum Pathol* 2009;40:769–77.
60 Van Cutsem E, Kang Y, Chung H, et al. Efficacy results from the ToGA trial: a phase Ill study of trastuzumab added to standard chemotherapy in frist-line human epidermal growth factor receptor 2 positive advanced gastric cancer. *J Clin Oncol* 2009;27: (suppl; abstr LBA4509).
61 Bang Y, Chung H, Xu J, et al. Pathological features of advanced gastric cancer (GC): relationship to human epidermal growth factor receptor 2 (HER2) positivity in the global screening programme of the ToGA trial. *J Clin Oncol* 2009;27: (suppl; abstr 4556).

Article V

Helicobacter

Helicobacter ISSN 1523-5378

Gastric Cancer: Basic Aspects

Carlos Resende,*,†,‡ Alexandra Thiel,§,¶ José C. Machado*,† and Ari Ristimäki§,¶

*IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal, †FMUP, Faculty of Medicine, University of Porto, Porto, Portugal, ‡ICBAS, Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal, §Genome-Scale Biology, Research Program Unit, University of Helsinki, Helsinki, Finland, ¶Department of Pathology, HUSLAB and Haartman Institute, and Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland

Keywords
Gastric cancer, genetic susceptibility, gene overexpression, gene silencing, cyclooxygenase-2.

Reprint requests to: José Carlos Machado, IPATIMUP, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal.
E-mail: josem@ipatimup.pt

Abstract

Gastric cancer (GC) is a world health burden, ranging as the second cause of cancer death worldwide. Etiologically, GC arises not only from the combined effects of environmental factors and susceptible genetic variants but also from the accumulation of genetic and epigenetic alterations. In the last years, molecular oncobiology studies brought to light a number of genes that are implicated in gastric carcinogenesis. This review is intended to focus on the recently described basic aspects that play key roles in the process of gastric carcinogenesis. Genetic variants of the genes *IL-10*, *IL-17*, *MUC1*, *MUC6*, *DNMT3B*, *SMAD4*, and *SERPINE1* have been reported to modify the risk of developing GC. Several genes have been newly associated with gastric carcinogenesis, both through oncogenic activation (*GSK3β*, *CD133*, *DSC2*, *P-Cadherin*, *CDH17*, *CD168*, *CD44*, metalloproteinases *MMP7* and *MMP11*, and a subset of miRNAs) and through tumor suppressor gene inactivation mechanisms (*TFF1*, *PDX1*, *BCL2L10*, *XRCC*, *psiTPTE-HERV*, *HAI-2*, *GRIK2*, and *RUNX3*). It also addressed the role of the inflammatory mediator cyclooxygenase-2 (COX-2) in the process of gastric carcinogenesis and its importance as a potential molecular target for therapy.

Gastric cancer (GC) is the fourth most common cancer and the second cause of cancer mortality worldwide [1]. The etiology of GC has a significant environmental component characteristic of the geographically varied incidence in the disease distribution [1–3]. Several environmental factors, including *Helicobacter pylori* infection, consumption of salted and nitrated foods, and cigarette smoking, have been found to be associated with the risk of developing GC [2–4]. In addition to environmental factors, genetic factors also play an important role in GC etiology, as demonstrated by the fact that only a small proportion of individuals exposed to the known environmental risk factors develop GC [3,5–8].

Molecular studies have provided evidence that GC arises not only from the combined effects of environmental factors and susceptible genetic variants but also from the accumulation of genetic and epigenetic alterations that play crucial roles in the process of cellular immortalization and tumorigenesis [2,4].

The present review is intended to focus on the recently described basic aspects that play key roles in the process of gastric carcinogenesis. New advances in the fields of the individual's genetic susceptibility for gastric carcinogenesis and molecular alterations in GC will be discussed.

Genetic Susceptibility

Molecular epidemiological studies have described some relatively common genetic variants as biomarkers for genetic susceptibility to GC development, namely single nucleotide polymorphisms (SNPs) [3–7,9]. These genetic variants may modulate the effects of exposure to environmental factors by regulating multiple biological pathways during gastric carcinogenesis.

Genetic variants in inflammation-related genes, especially cytokines and their receptors, are thought to play a role in tumor initiation and promotion [5,6,8]. In this perspective, the role of genetic polymorphisms in GC risk has motivated increasing interest in recent years. For example, a meta-analysis performed by Zhuang et al. [10] suggests that the interleukin 10 (*IL-10*) -592C>A promoter polymorphism may be associated with GC among Asians. Even regarding IL-10 polymorphisms, Won et al. [11] reported that the *IL-10*-1082A>G polymorphism influences the risk of GC in

populations from East Asia but not in Caucasians, supporting the idea that different mechanisms of selection may be operating on this gene region in Caucasians and East Asian populations. In another study, Wu et al. [12] found an association between the interleukin IL-17F A7488G coding variant and GC, especially with the intestinal-type GC. This association is interesting and relevant, because it was previously shown that IL-17F 7488 polymorphism is associated with increased inflammation in *H. pylori* infection context [13]. Recently, Persson et al. [14] performed a series of meta-analyses for a group of inflammation-related gene polymorphisms. The clearest results were found for the association between the *IL-1RN2* polymorphism and the risk for GC in non-Asian populations. In Asian populations, the C carriers for the *IL-1B*-31 polymorphism had a reduced overall risk of GC. According to Persson et al., the simultaneous analysis for multiple polymorphisms within genes with related functions results in a broader overview and allows for more detailed comparisons.

Genetic variants in noninflammation-related genes and their association with GC have also been described. For example, Saeki et al. [15] described that the A carriers for the mucin 1 (MUC1) rs4072037 polymorphism are at increased risk of developing GC, especially the diffuse type. These authors showed that rs4072037 has a role in transcriptional regulation and also in splicing site selection of MUC1. In another study, Kwon et al. [16] reported the association between a new minisatellite located in intron 26 of MUC6 (MUC6-MS5) and the susceptibility to develop GC. It is noteworthy to refer that mucins are glycosylated proteins that play important roles in the protection of epithelial cells from pathogens and have been implicated in the process of epithelial renewal and differentiation, and that both MUC1 and MUC6 are well-known stomach-secreted mucins that may have a role in GC development [17].

The DNA methylation process is a major epigenetic modification that involves the addition of a methyl group to specific dinucleotide sequences [18], and it is accepted that aberrant DNA methylation is one of the most relevant epigenetic changes observed in cancer [19]. In this matter, Hu et al. [20] studied the promoter of the enzyme DNA methyltransferase 3B (*DNMT3B*) gene, and they found that individuals with at least one −579G allele were at decreased risk of developing GC compared with those having a −599TT genotype. According to the authors, the results are significant at least in Chinese populations.

Transforming growth factor (TGF)-β signaling is one of the most important tumor suppressor pathways [21]. SMAD proteins are crucial components of TGF-β signaling, which negatively regulates cell growth and promotes apoptosis of epithelial cells [21]. Recently, loss of SMAD4, especially loss of nuclear SMAD4 expression, was described in GC progression [22]. Given the role of SMAD4 in gastric tumor suppression, Wu et al. [23] searched for genetic variants in the *SMAD4* gene that could be associated with the risk of GC. Of the five SNPs studied, the authors found an association between the allele C at position rs17663887 and the allele G at position rs12456284 with increased expression of SMAD4 protein and decreased risk of GC.

Proteolytic breakdown of the extracellular matrix is an essential event involved in tumor invasion, metastasis, and angiogenesis [24]. Serpin peptidase inhibitor, clade E, member 1 (SERPINE1), plays a key role in tumorigenesis, because it prevents excessive proteolysis, which is necessary for capillary morphogenesis, cell migration, and invasion [25]. According to Ju et al. [26] a polymorphism in intron 7 (c.1162 + 162C>T) of *SERPINE1* is strongly associated with susceptibility to diffuse-type GC. Using luciferase reporter assays, the authors detected an increase in gene expression associated with the risk haplotype when compared with non-risk haplotype. The results obtained are interesting, because expression levels of SERPINE1 are elevated in GC tissues compared with normal stomach tissue [27].

In the last year, numerous articles were published establishing an association between genetic polymorphisms and the risk of GC. It is becoming evident that host genetic factors are key agents in the risk for the development of cancer and that the interaction of different polymorphisms combined with environmental triggers may provide crucial clues to explain diverse risks in various populations.

Molecular Alterations in Gastric Cancer

Understanding the molecular mechanisms and alterations behind the initiation and progression of gastric tumorigenesis is crucial for the early detection of the disease and to identify novel therapeutic and clinical targets for GC. A number of molecular abnormalities have been identified in GC, namely gene overexpression and gene silencing. Nevertheless, it is of vital importance to decipher the mechanisms of gastric carcinogenesis, because the molecular pathogenesis of GC is still incompletely understood.

Gene Overexpression

In the last years, a vast amount of articles reporting the overexpression and/or amplification of various genes in GC were published. Recently, Zheng et al. [28] reported the overexpression of the inactive form of glycogen

synthase kinase (GSK)-3β and p-GSK3β-ser^9 in GC when compared with normal mucosa. Noteworthy, the authors addressed that the overexpression of p-GSK3β-ser^9 was positively correlated with a poor prognosis. Interestingly, Mishra et al. [29] described that p-GSK3β-ser^9 is gastrin induced and that inhibition of GSK3β leads to an increase in expression of Snail, nuclear translocation of β-catenin, and an increase in GC cell migration.

Many transmembrane proteins are described as playing a role in cancer [30], and they constitute an active target of research in the identification of novel biomarkers for cancer diagnosis and novel targets for treatment [31]. Zhao et al. [32] analyzed the expression of the transmembrane protein CD133 in GC, because it was described that CD133 is overexpressed in various solid tumors [33]. They found that CD133 was overexpressed in more than 55% of GC and has a positive correlation with the expression of Ki-67. In another study, Anami et al. [34] found an overexpression of the membrane protein desmocollin-2 (DSC2) in intestinal-type GC. Interestingly, they showed that expression of DSC2 was induced by CDX2, suggesting that expression of desmocollin-2 could be a key regulator for GC with intestinal phenotype. One transmembrane protein for which a new targeted compound is being studied in clinical trials on solid tumors is P-cadherin. Kim et al. [35] reported recently that P-cadherin is not expressed in normal gastric mucosa but is overexpressed in GC, especially in tumors of the intestinal type. The authors reported that the increased expression of P-cadherin in GC was found to be significantly correlated with promoter hypomethylation. Another member of the cadherin superfamily, CDH17, was also reported by Lee et al. [36] as a promising marker for early-stage gastric cancer. Also according to Lee et al., CDH17 expression was positively associated with a good prognosis.

Hyaluronic acid (HA) is a component of the extracellular matrix. In cancerous tissue, HA is greatly secreted from stromal fibroblasts in response to factors derived from tumor cells [37]. The two most well-known cell receptors for HA are CD168 and CD44 [38]. In a recent study, Ishigami et al. [39] reported the overexpression of CD168 in a panel of GC cases. According to these authors, CD168 positivity was significantly associated with the depth of invasion and metastasis of GC, an association that was previously reported for other types of cancer [40]. In a different study, da Cunha et al. [41] described the *de novo* expression of a CD44 variant (CD44v6) in GC. Noteworthy, they observed that CD44v6 was rarely expressed in normal gastric mucosa but was increasingly expressed in premalignant and malignant lesions. A recent study by Ishimoto et al. [42] sheds light about some roles of CD44 variants (CD44v) expression in gastrointestinal tumors. Ishimoto et al. found that CD44v controls the intracellular level of reduced glutathione (GSH), and cancer cells that express more CD44v showed an enhanced capacity for GSH synthesis and defence against reactive oxygen species, promoting tumor growth.

Matrix metalloproteinases (MMP), a family of zinc-dependent endopeptidases, are involved in various physiological and pathological processes, such as extracellular matrix degradation, tissue remodeling, inflammation, and tumor invasion and metastasis [43]. Owing to the roles of MMPs in disease, two independent reports arouse establishing a relation between the expression of MMPs and GC. Koskensalo et al. [44] analyzed the expression of MMP-7 , and Zhao et al. [45] described the expression of MMP-11. In both reports, the results were equivalent: overexpression of MMPs in a panel of GC cases, when compared with normal gastric mucosa, and a significant shorter survival for patients that overexpressed MMPs.

MicroRNAs (miRNAs) are a subset of noncoding RNA molecules (21–23 nucleotides in length) that are believed to regulate gene expression [46]. Altered expression of miRNAs has been associated with several diseases, particularly cancer [47]. Recently, Liu et al. [48] performed a genome-wide serum miRNA expression profile in patients with GC and controls, and they identified a set of five miRNAs (miR-1, miR-20a, miR-27a, miR-34, and miR-423-5p) whose overexpression was positively correlated with tumor stage. In a different study, Li et al. [49] identified a seven-miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, and miR-126) that associates with an increased risk of recurrence and decreased overall survival, even stratifying patients by stage or histology. These results indicate that miRNAs may play an important role in the carcinogenesis and prognosis of GC.

Gene Silencing

Gene silencing in GC can occur mainly because of point mutations, loss of heterozygosity, and promoter hypermethylation [2,3]. A putative gastric tumor suppressor gene whose expression is frequently downregulated in GC is trefoil factor 1 (*TFF1*) [50], especially by promoter hypermethylation [51]. Tomita et al. [52] reported recently that the peptide hormone gastrin exerts a suppressive effect in gastric carcinogenesis by suppressing *TFF1* promoter hypermethylation. Pancreatic duodenal homeobox-1 (*PDX1*) is another putative tumor suppressor gene whose expression is frequently downregulated in GC [53]. Ma et al. [54] described the mechanism

responsible for PDX1 loss of expression in GC as promoter hypermethylation. Many more articles were published last year reporting gene promoter hypermethylation as a cause of loss of protein expression in GC. As examples, loss of expression by promoter methylation was described for BCL2L10 [55], XRCC1 [56], the endogenous retrovirus-related gene psiTPTE-HERV [57], HAI-2 [58], and GRIK2 [59].

Nevertheless, it is crucial to understand that the loss of expression of one gene can occur by different mechanisms acting in that particular gene. As an example, *Runx3* is considered a gastric tumor-suppressor gene whose expression is frequently downregulated in GC by promoter hypermethylation [60]. However, Lai et al. [61] described recently that Runx3 expression can be negatively regulated at transcriptional level by the microRNA-130b. In another study, Tsang et al. [62] reported that *H. pylori* virulence factor CagA is able to bind to Runx3, inducing the ubiquitination and degradation of Runx3 by the proteasome machinery.

The Role of Cyclooxigenase-2 (COX-2) on GC

Cyclooxygenase-2 (COX-2) is the key regulatory enzyme in prostanoid synthesis and the primary target of nonsteroidal anti-inflammatory drugs (NSAIDs) in inflammatory and neoplastic conditions [63]. Activation of COX-2 has been shown to be involved in many processes leading to tumor progression such as angiogenesis, survival, proliferation, invasion, and immunosuppression [63]. An epidemiologic cohort and case–control studies have suggested that use of aspirin and other NSAIDs reduces mortality from GC [64,65]. As a result, COX-2 enzyme is considered a potential therapeutic target in cancer prevention and treatment. Further support for the role of COX-2 in gastric carcinogenesis is provided by data which suggest that certain variants of the gene make individuals susceptible to GC, especially in relation to *H. pylori* infection [66–69]. Furthermore, *H. pylori* infection associates with COX-2 expression in gastric mucosa with intestinal metaplasia and dysplasia [70], which are precursor lesions of GC. As *H. pylori* infection also associates with VEGF expression [71], and manipulation of COX-2 expression in GC cell lines leads to altered VEGF expression [70], it is possible that *H. pylori*-induced VEGF expression is at least partially regulated by COX-2-derived prostanoids.

In humans, COX-2 expression, but not that of COX-1, is elevated in GC tissues, and elevated level of COX-2 expression is an independent prognostic factor in patients with gastric cancer [72–74]. Furthermore, in an extended multivariate model with eight prognostic markers and clinicopathological factors, COX-2 expression is an independent prognostic factor alongside with p53, stage, and intent of surgery [74]. It is important to note that chemoprevention of GC is not recommended in general population by using NSAIDs or COX-2-selective drugs, because they increase the risk for cardiovascular events [75]. However, it may be possible to recognize high-risk patients by screening for genetic polymorphisms, and use these drugs to treat patients with cancer [75]. Thus, these data should encourage further prospective clinical trials aiming at clinical use of COX-2 inhibitors as a part of combination chemotherapy.

The mechanism of COX-2 overexpression in GC cells has been widely studied, and signal transduction pathways that induce COX-2 expression include PI3K/Akt/GSK-3β pathway, mitogen-activated protein kinases (MEK 1/2, p38, and JNK), Notch1 signal pathway, and nuclear factor-κB. Recently, microRNAs (miRNAs) were shown to regulat COX-2 expression. When miRNA-101 was overexpressed in GC cells lines, the mRNA level of COX-2 was decreased [76]. Furthermore, miRNA-101 overexpression resulted in inhibition of proliferation, migration, and invasion in these cells, and overexpression of miRNA-101 in GC cells leads to reduced tumor growth in nude mice [76]. In other mouse models, COX-2 has been shown to be involved with tumor growth, which has been demonstrated by genetic manipulation. Recently, it was shown that in mice, where transgenic overexpression of Cox-2 and mPGES-1 is combined with activation of Wnt pathway (K19-Wnt1/C2mE) to drive adenocarcinoma development, a COX-2-selective drug celecoxib and ZD1839, an EGFR inhibitor, decreased the tumor volume by 90% and 76%, respectively, and combination of both drugs led to a complete regression of the tumors [77]. Additionally, ligands for EGFR and metalloproteinases (that shed the ectodomains of EGFR ligands and thus activate them) were upregulated directly and indirectly by the COX-2-derived PGE_2 [77]. The activation of the EGFR pathway by PGE_2 signaling might be responsible for tumor cell proliferation in this model, as both Cox-2 and EGFR inhibition decreased the number of Ki-67-positive cells.

Concluding Remarks

Gastric cancer is a complex disease that arises by the combined interaction of different major players. The lifestyle and alimentary habits of individuals, combined with genetic susceptible variants and molecular alterations acquired during lifetime, are at the base of the carcinogenic process of GC. Much work has been

carried out to find molecular markers for GC. However, the true mechanisms are barely known and much more work is needed to understand the causes of GC and the best clinical approaches to assure a correct diagnosis and efficient treatment.

Conflicts of Interest

The authors have declared no conflicts of interest.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74–108.
2 Vogiatzi P, Vindigni C, Roviello F, Renieri A, Giordano A. Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis. *J Cell Physiol* 2007;211:287–95.
3 Yin M, Hu Z, Tan D, Ajani JA, Wei Q. Molecular epidemiology of genetic susceptibility to gastric cancer: focus on single nucleotide polimorphisms in gastric carcinogenesis. *Am J Transl Res* 2009;1:44–54.
4 Shikata K, Doy Y, Yonemoto K, Arima H, Ninomiya T, Kubo M, et al. Population-based prospective study of the combined influence of cigarette smoking and Helicobacter pylori infection on gastric cancer incidence: the Hisayama study. *Am J Epidemiol* 2008;12:1409–15.
5 Machado JC, Figueiredo C, Canedo P, Pharoah P, Carvalho R, Nabais S, et al. A Proinflamatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* 2003;2:364–71.
6 Canedo P, Corso G, Pereira F, Lunet N, Suriano G, Figueiredo C, et al. The interferon gamma receptor 1 (IFNGR1) -56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. *Gut* 2008;11:1504–8.
7 Canedo P, Duraes C, Pereira F, Regalo G, Lunet N, Barros H, et al. Tumor necrosis factor alpha extended haplotypes and risk of colorectal and castric carcinoma. *Cancer Epidemiol Biomarkers Prev* 2008;17:2416–20.
8 González CA, Sala N, Capellá G. Genetic susceptibility and gastric cáncer risk. *Int J Cancer* 2002;100:249–60.
9 Milne AN, Carneiro F, O'Morain C, Offerhaus GJ. Nature meets nurture: molecular genetics of gastric cancer. *Hum Genet* 2009;126:615–28.
10 Zhuang W, Wu XT, Zhou Y, Liu L, Liu GJ, Wu TX, et al. Interleukin10 -592 promoter polymorphism associated with gastric cancer among Asians: a meta-analysis of epidemiologic studies. *Dig Dis Sci* 2010;55:1525–32.
11 Won HH, Kim JW, Kim MJ, Kim S, Park JH, Lee KA. Interleukin 10 polymorphisms differentially influence the risk of gastric cancer in East Asians and Caucasians. *Cytokine* 2010;51:73–7.
12 Wu X, Zeng Z, Chen B, Yu J, Xue L, Hao Y, et al. Association between polymorphisms in interleukin-17A and interleukin-17F genes and risks of gastric cancer. *Int J Cancer* 2010;127:86–92.
13 Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, et al. Genetic polymorphisms of molecules associated with inflammation and immune response in Japanese subjects with functional dyspepsia. *Int J Mol Med* 2007;20:717–23.
14 Persson C, Canedo P, Machado JC, El-Omar EM, Forman D. Polymorphisms in inflammatory response genes and their association with gastric cancer: a HuGE systematic review and meta-analyses. *Am J Epidemiol* 2011;173:259–70.
15 Saeki N, Saito A, Choi IJ, Matsuo K, Ohnami S, Totsuka H, et al. A functional single nucleotide polymorphism in mucin 1, at chromosome 1q22, determines susceptibility to diffuse-type gastric cancer. *Gastroenterology* 2011;140:892–902.
16 Kwon JA, Lee SY, Ahn EK, Seol SY, Kim MC, Kim SJ, et al. Short rare muc6 minisatellites-5 alleles influence susceptibility to gastric carcinoma by regulating gene expression. *Hum Mutat* 2010;31:942–9.
17 Kufe DW. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer* 2009;9:874–85.
18 Bheemanaik S, Reddy YV, Rao DN. Structure, function and mechanism of exocyclic DNA methyltransferases. *Biochem J* 2006;399:177–90.
19 Fang JY, Xiao SD. Alteration of DNA methylation in gastrointestinal carcinogenesis. *J Gastroenterol Hepatol* 2001;16:960–8.
20 Hu J, Fan H, Liu D, Zhang S, Zhang F, Xy H. DNMT3B promoter polymorphism and risk of gastric cancer. *Dig Dis Sci* 2010;55:1011–6.
21 Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998;95:737–40.
22 Wang LH, Kim SH, Lee YL, Choi YL, Kim YC, Park TS, et al. Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression. *Clin Cancer Res* 2007;13:102–10.
23 Wu DM, Zhu HX, Zhao QH, Zhang ZZ, Wang SZ, Wang ML, et al. Genetic variations in the SMAD4 gene and gastric cancer susceptibility. *World J Gastroenterol* 2010;16:5635–6341.
24 Bajou K, Maillard C, Jost M, Lijnen RH, Declerck P, Carmeliet P, et al. Hos-derived plasminogen activator-1 (PAI-1) concentration is critical for *in vivo* tumoral angiogenesis and growth. *Oncogene* 2004;23:6986–90.
25 Devy L, Blacher S, Grignet-Debrus C, Bajou K, Masson V, Gerard RD, et al. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J* 2002;16:147–54.
26 Ju H, Lim B, Kim M, Noh SM, Kim WH, Ihm C, et al. SERPINE1 intron polymorphisms affecting gene expression are associated with diffuse-type gastric cancer susceptibility. *Cancer* 2010;116:4248–55.
27 Cho JY, Chung HC, Noh SH, Roh JK, Min JS, Kim BS. High level of urokinase-type plasminogen activator is a new prognostic marker in patients with gastric carcinoma. *Cancer* 1997;79:878–83.
28 Zheng HC, Xu XY, Xia P, Yu M, Takahashi H, Takano Y. Involvement of inactive GSK3β overexpression in tumorigenesis and progression of gastric carcinomas. *Hum Pathol* 2010;41:1255–64.
29 Mishra P, Senthivinayagam S, Rana A, Rana B. Glycoden synthase kinase-3beta regulates Snail and beta-catenin during gastrin-induced migration of gastric cancer cells. *J Mol Signal* 2010;5:1–10.
30 Yan B, Yau EX, Omar SS, Ong CW, Pang B, Yeoh KG, et al. A study of HER2 gene amplification and protein expression in gastric cancer. *J Clin Pathol* 2010;63:839–42.
31 Buckhaults P, Rago C, St CroixB, Romans KE, Saha S, Zhang L, et al. Secreted and cell surface genes expressed in benign and malignant colorectal tumors. *Cancer Res* 2001;61:6996–7001.
32 Zhao P, Li Y, Lu Y. Aberrant expression of CD133 protein correlates with Ki-67 expression and is a prognostic marker in gastric adenocarcinoma. *BMC Cancer* 2010;10:218.
33 Hilbe W, Dirnhofer S, Oberwasserlechner F, Schmid T, Gunsilius E, Hilbe G, et al. CD133 positive endothelial progenitor

cells contribute to the tumour vasculature in non-small cell lung cancer. *J Clin Pathol* 2004;57:965–9.
34 Anami K, Oue N, Nogushi T, Sakamoto N, Sentani K, Hayashi T, et al. Search for transmembrane protein in gastric cancer by the Escherichia coli ampicillin secretion trap: expression of DSC2 in gastric cancer with intestinal phenotype. *J Pathol* 2010;221:275–84.
35 Kim MA, Jung EJ, Lee HS, Lee HE, Yang HK, Bang YJ, et al. P-cadherin expression in gastric carcinoma: ist regulation mechanism and prognostic significance. *Hum Pathol* 2010;41:877–85.
36 Lee HJ, Nam KT, Park HS, Kim MA, Lafleur BJ, Aburatani H, et al. Gene expression profiling of metastatic lineages identifies CDH17 as a prognostic marker in early stage gastric cancer. *Gastroenterology* 2010;139:213–25.
37 Yeo TK, Nagy JA, Yeo KT, Dvorak HF, Toole BP. Increased hyaluronan at sites of attachment to mesentery by CD44-positive mouse ovarian and breast tumor cells. *Am J Pathol* 1996;148:1733–40.
38 Sironen RK, Tammi M, Tammi R, Auvinen PK, Anttila M, Kosma VM. Hyaluronan in human malignancies. *Exp Cell Res* 2011;317:283–391.
39 Ishigami S, Ueno S, Nishizono Y, Matsumoto M, Kurahara H, Arigami T, et al. Prognostic impact of CD168 in gastric cancer. *BMC Cancer* 2011;11:106.
40 Assmann V, Gillett CE, Poulsom R, Ryder K, Hart IR, Hanby AM. The pattern of expression of the microtubule-binding protein RHAMM/HABP in mammary carcinoma suggests a role in the invasive behaviour of tumour cells. *J Pathol* 2001;195:191–6.
41 da Cunha CB, Oliveira C, Wen X, Gomes B, Sousa S, Suriano G, et al. De novo expression of CD44 variants in sporadic and hereditary gastric cancer. *Lab Invest* 2010;90:1604–14.
42 Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc and thereby promotes tumor growth. *Cancer Cell* 2011;19:387–400.
43 Shim KN, Jung SA, Joo YH, Yoo K. Clinical significance of tissue levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in gastric cancer. *J Gastroenterol* 2007;42:120–8.
44 Koskensalo S, Mrena J, Wiksten JP, Nordling S, Kokkola A, Hagstrom J, et al. MMP-7 overexpression is an independent prognostic marker in gastric cancer. *Tumor Biol* 2010;31:149–55.
45 Zhao ZS, Chu YQ, Ye ZY, Wang YY, Tao HQ. Overexpression of matrix metalloproteinase 11 in human gastric carcinoma and its clinicopathologic significance. *Hum Pathol* 2010;41:686–96.
46 Bartel BP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
47 Jay C, Nemunaitis J, Chen P, Fulgham P, Tong AW. miRNA profiling for diagnosis and prognosis of human cancer. *DNA Cell Biol* 2007;26:293–300.
48 Liu R, Zhang C, Hu Z, Li G, Wang C, Yang C, et al. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as fingerprint for gastric cancer diagnosis. *Eur J Cancer* 2011;47:784–91.
49 Li X, Zhang Y, Zhang Y, Ding J, Wu K, Fan D. Survival prediction of gastric cancer by a seven-microRNA signature. *Gut* 2010;59:579–85.
50 Machado JC, Carneiro F, Ribeiro P, Blin N, Sobrinho-Simões M. pS2 protein expression in gastric carcinoma. Na immunohistochemical and immunoradiometric study. *Eur J Cancer* 1996;32:1585–90.
51 Fujimoto J, Yasui W, Tahara H, Tahara E, Kudo Y, Yokazaki H, et al. DNA hypermethylation at the pS2 promoter region is associated with early stage of carcnogenesis. *Cancer Lett* 2000;149:125–34.
52 Tomita H, Takaishi S, Menheniott TR, Yang X, Shibata W, Jin G, et al. Inhibition of gastric carcinogenesis by the hormone gastrin is mediated by suppressor of TFF1 epigenetic silencing. *Gastroenterology* 2011;140:879–91.
53 Ma J, Chen M, Wang J, Xia HH, Zhu S, Liang Y, et al. Pancreatic duodenal homeobox-1 (PDX1) functions as a tumor suppressor in gastric cancer. *Carcinogenesis* 2008;29:1327–33.
54 Ma J, Wang JD, Zhang WJ, Zou B, Chen WJ, Lam CS, et al. Promoter hypermethylation and histone hypoacetylation contribute to pancreatic-duodenal homeobox 1 silencing in gastric cancer. *Carcinogenesis* 2010;31:1552–60.
55 Xu J, Cao XX, Long ZW, Liu XP, Furuya T, Xu JW, et al. BCL2L10 protein regulates apoptosis/proliferation trough differential pathways in gastric cancer cells. *J Pathol* 2011;223:400–9.
56 Wang P, Tang JT, Peng YS, Chen XY, Zhang YJ, Fang JY. XRCC1 downregulated through promoter hypermethylation is involved in human gastric carcinogenesis. *J Dig Dis* 2010;11:343–51.
57 Liang Q, Ding Xu R, Xu Z, Zheng S. The novel human endogenous retrovirus-related gene, psiTPTE22-HERV, is silenced by DNA methylation in cancers. *Int J Cancer* 2010;127:1833–43.
58 Dong W, Chen X, Xie J, Sun P, Wu Y. Epigenetic inactivation and tumor suppressor activity of HAI-2/SPINT2 in gastric cancer. *Int J Cancer* 2010;127:1526–34.
59 Wu CS, Lu YJ, Li HP, Hsueh C, Lu CY, Leu YW, et al. Glutamate receptor, ionotropic, kainite 2 silencing by DNA hypermethylation possesses tumor suppressor function in gastric cancer. *Int J Cancer* 2010;126:2542–52.
60 Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* 2002;109:113–24.
61 Lai KW, Koh KX, Loh M, Tada K, Subramaniam MM, Lim XY, et al. MicroRNA-130b regulates the tumour suppressor RUNX3 in gastric cancer. *Eur J Cancer* 2010;46:1456–63.
62 Tsang YH, Lamb A, Romero-Gallo J, Huang B, Ito K, Peek RM Jr, et al. Helicobacter pylori CagA targets gastric tumor suppressor RUNX3 for proteasome-mediated degradation. *Oncogene* 2010;29:5643–50.
63 Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010;10:181–93.
64 Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31–41.
65 Tian W, Zhao Y, Liu S, Li X. Meta-analysis on the relationship between nonsteroidal anti-inflammatory drug use and gastric cancer. *Eur J Cancer Prev* 2010;19:288–98.
66 Dong J, Dai J, Zhang M, Hu Z, Shen H. Potentially functional COX-2-1195G>A polymorphism increases the risk of digestive system cancers: a meta-analysis. *J Gastroenterol Hepatol* 2010;25:1042–50.
67 Ke-Xiang Z, Yu-Min L, Xun L, Wen-Ce Z, Yong S, Tao L. Study on the association of COX-2 genetic polymorphisms with risk of gastric cancer in high incidence Hexi area of Gansu province in China. *Mol Biol Rep* 2011;38:649–55.
68 Li Y, He W, Liu T, Zhang Q. A new cyclo-oxygenase-2 gene variant in the Han Chinese population is associated with an

increased risk of gastric carcinoma. *Mol Diagn Ther* 2010;14:351–5.
69 Liu JL, Liang Y, Wang ZN, Zhou X, Xing LL. Cyclooxygenase-2 polymorphisms and susceptibility to gastric carcinoma: a meta-analysis. *World J Gastroenterol* 2010;16:5510–7.
70 Yao L, Liu F, Hong L, Sun L, Liang S, Wu K, et al. The function and mechanism of COX-2 in angiogenesis of gastric cancer cells. *J Exp Clin Cancer Res* 2011;30:13.
71 Liu D, He Q, Liu C. Correlations among Helicobacter pylori infection and the expression of cyclooxygenase-2 and vascular endothelial growth factor in gastric mucosa with intestinal metaplasia or dysplasia. *J Gastroenterol Hepatol* 2010;25:795–9.
72 Gou HF, Chen XC, Zhu J, Jiang M, Yang Y, Cao D, et al. Expressions of COX-2 and VEGF-C in gastric cancer: correlations with lymphangiogenesis and prognostic implications. *J Exp Clin Cancer Res* 2011;30:14.
73 Li Y, Tan BB, Fan LQ, Zhao Q, Liu Y, Wang D. Expression of COX-2, survivin in regional lymph node metastases of gastric carcinoma and the correlation with prognosis. *Hepatogastroenterology* 2010;57:1435–41.
74 Mrena J, Wiksten JP, Kokkola A, Nordling S, Ristimaki A, Haglund C. COX-2 is associated with proliferation and apoptosis markers and serves as an independent prognostic factor in gastric cancer. *Tumour Biol* 2010;31:1–7.
75 Menter DG, Schilsky RL, DuBois RN. Cyclooxygenase-2 and cancer treatment: Understanding the risk should be worth the reward. *Clin Cancer Res* 2010;16:1384–90.
76 Wang HJ, Ruan HJ, He XJ, Ma YY, Jiang XT, Xia YJ, et al. MicroRNA-101 is down-regulated in gastric cancer and involved in cell migration and invasion. *Eur J Cancer* 2010;46:2295–303.
77 Oshima H, Popivanova BK, Oguma K, Kong D, Ishikawa TO, Oshima M. Activation of epidermal growth factor receptor signaling by the prostaglandin E(2) receptor EP4 pathway during gastric tumorigenesis. *Cancer Sci* 2011;102:713–9.