UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Bioquímica y Biología Molecular II

TESIS DOCTORAL

Papel de C3G y p38α en la regulación de la migración, invasión y crecimiento tumoral en carcinoma de colon. Función de fibulina 3

Role of C3G and p38α in the regulation of migration, invasion and tumoral growth in colon carcinoma. Function of fibulin 3

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Neibla Priego Bendeck

Directora

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Madrid, 2016

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ABREVIATIONS

5A2dC: 5-Aza-2´-deoxicytidine
ADAM: α-disintegrin and metalloproteinase
Akt: AK strain Transforming; v-Akt murine thymoma viral oncogene homolog 1
APC: Adenomatous Polyposis Coli protein
ASK1: Apoptosis Signal Regulating Kinase 1
AT: Anaphylatoxin
ATF-1/2/6: Activating Transcription Factor 1/2/6
Bax: BCL2-Associated X protein
Bcr: Breakpoint Cluster Region protein
BiP: Binding immunoglobulin Protein
C/EBP: CCAAT-enhancer-binding proteins
C3G: Crk SH3-domain-binding Guanine-nucleotide-releasing factor
c-Abl: Belson murine leukemia viral oncogene homolog (mammal)
CAFs: Cancer Associated Fibroblasts
CASP5: Caspase 5, Apoptosis-Related Cysteine Peptidase
cbEGF: Calcium-binding EGF (Epidermal Growth Factor)
CCP: Complement Control Protein
Cdc42: Cell division cycle 42-related protein kinase
c-DNA: Complementary DNA
CHOP: C/EBP homologous protein
CML Chronic Myeloid Leukemia
CO_2: Carbon dioxide
COX-2: Cyclooxigenase-2
CpG: Cytosine-phosphate-Guanine
CRC: Colorectal Cancer
CSAIDs: pyridinyl imidazole anti-inflammatory drugs
CT: Threshold Cycle
CTNNB1: Catenin (cadherin-associated protein), beta 1
CXCL12: Chemokine (C-X-C motif) Ligand 12
CXCR4: Chemokine (C-X-C motif) Receptor 4
Dab2: Disabled-2
DCC: Deleted in Colorectal Carcinoma protein
DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DNMTs: DNA Methyl Transferases
DNRap1: Dominant negative Rap1
DTCs: Disseminated Tumour Cells
DTT: Dithiothreitol
ECM Extracellular Matrix
EDTA: Ethylenediaminetetraacetic acid
eEF2: Eukaryotic Elongation Factor 2
EFEMP-1: EGF-containing fibulin-like extracellular matrix protein 1
EGF: Epidermal Growth Factor
EGFR: Epithelial Growth Factor (EGF) receptor
EGTA: Ethylene Glycol-bis (2-aminoethylether)-N,N,N',N' tetraacetic acid
EMT: Epithelial-Mesenchymal Transition
ERKs: Extracellular Signals Regulated Kinases

ES: Embryonic Stem
FA: Focal Adhesion
FAK: Focal Adhesion Kinase
FAP: Familial Adenomatous Polyposis
FBS: Fetal Bovine Serum
FC-domain: Fibulin-like domain
FGF: Fibroblast Growth Factor
FoxC2: Forkhead Box C2
FoxO: Forkhead Box O
GADD153: Growth arrest and DNA damage inducible 153 protein
GAPs: GTPase-Activating Proteins
GC-rich regions: Guanine-Cytosine-rich regions
GEF: Guanine Nucleotide Exchange Factor
GH: Growth Hormone
GK: Guanylate Kinase
GLUT4: Glucose Transporter Type 4
Gly: Glycine
Grb2: Growth Factor Receptor-bound Protein 2
GSK3β: Glycogen Synthase Kinase 3β
H_2O_2: Hydrogen peroxide
HBP1 High mobility group-box protein 1
Hck: hemopoietic cell kinase
HGF: Hepatocyte Growth Factor
HIF-1: Hypoxia Inducible Factor
HNPCC: Hereditary Nonpolyposis Colorectal Cancer
Hsp27: Heat-shock protein 27
HuR: Human antigen R
ID4: Inhibitor of DNA binding 4
IGF-1/2: Insulin-like Growth Factor 1/2
IL-1/IL-3/ IL-4/ IL-6/IL-8: Interleukin-1/3/4/6/8
IRF8: Interferon Regulatory Factor 8
JNK: c-Jun N-terminal Kinases
KDa: Kilo Dalton
LPS: Lipopolysaccharide
LSC: Lung Stem Cells
MAP: MUTYH-Associated Polyposis
MAPK: Mitogen Activated Protein Kinases
MBD: Methyl-CpG-Binding Domain
MEF2: Myocyte Enhance Factor 2
MEFs: Mouse Embryonic Fibroblasts
Met: Met Tyrosine Kinase Receptor
MET: Mesenchymal–Epithelial Transition
MITF1: Microphthalmia-Associated Transcription Factor 1
MK2/MAPKAP-K2: MAPK-Activated Protein Kinases 2
MKK: MAP Kinase Kinase
MKKK: MAPK Kinase Kinase
MKP: MAP Kinase Phosphatases
MLK: Mixed Lineage Kinase

MMPs: Matrix Metalloproteases
MNK1: MAP Kinase Interaction Protein Kinase
mRNA: Messenger Ribonucleotic Acid
MSCs: Mesenchymal Stem Cells
MSK: Mitogen and Stress activated Kinase
mTORC1: Mammalian Target of Rapamycin Complex 1
NFAT: Nuclear Factor of Activated T-cells
NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NP40: Nonidet P-40
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PDGF: Platelet-Derived Growth Factor
PDXs: Patient Derived Xenografts
PDZ: Post Synaptic Density protein (PSD95), Drosophila Disc Large tumor suppressor (Dlg1) and Zonula Occludens-1 protein (ZO-1)
PFA: Paraformaldehyde
PI3K: Phosphatidylinositol 3-Kinase
PMSF: Phenylmethanesulfonylfluoride
PP2A/C: Protein Phosphatase 2A/C
PSD95: Postsynaptic Density Protein 95
PTHLH: Parathyroid Hormone-Like Hormone
PTP: Protein Tyrosine Phosphatase
Rac: Ras-Related C3 botulinum toxin
Rap1: Ras-related protein 1
Ras: Rat Sarcoma virus
REM: Ras-Exchange-Motif
Rheb: Ras Homolog Enriched in Brain
Rho: Ras Homolog
Rit: Ras-like protein in tissues
ROS: Reactive Oxygen Species
RT: Room Temperature
RT-qPCR: Quantitative PCR
SAP-1, SAP-90, SAP-95, SAP-97: Secreted Aspartyl Proteinase-1, -90, -95, -97
SDF-1: Stromal Cell-Derived Factor 1
SDS: Sodium Docecyl Sulphate
S.E.M.: Standard Error of the Mean
Ser: Serine
SFRP: Secreted Frizzled-Related Protein
SH2/3: Src Homology 2/3
shRNA: short hairpin RNA
SIP1: Smad Interacting Protein 1
SOD: Superoxide Dismutase
TAB-1: TAK1-binding protein 1
TAK-1: Transforming growth factor beta-activated protein kinase 1
TBS: Tris Buffered Saline
TCR: T-Cell Receptor
TGFβ: Tumour Growth Factor beta
Thr: Threonine

TIMPs: Inhibitors of Metalloproteases
TNFα: Tumour Necrosis Factor alpha
TRAF6: TNF receptor-associated factor 6, E3 ubiquitin protein ligase
TWEAK: Tumor necrosis factor-like weak inducer of apoptosis
Tyr: Tyrosine
WASP: The Wiskott–Aldrich Syndrome *protein*
Wip1: WASP-Interacting Protein 1
ZEB 1/2: Zinc finger E-box binding homeobox 1/2
ZO-1/2/3: Zona Occuldens proteins -1/2/3

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RESULTS

p38α MAPK down-regulates fibulin 3 expression through methylation of DNA regulatory sequences. Role in migration, invasion and tumor growth

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DISCUSSION

INTRODUCTION

1. COLORECTAL CANCER

Cancer is a class of complex diseases that includes more than 100 types and it is characterized by an uncontrolled cell growth and survival**.** Tumorigenesis in humans involves several alterations in cell physiology that drive to malignant transformation, which is characterized by self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and metastatic tissue invasion (Hanahan and Weinberg 2000).

Colorectal cancer (CRC) represents the third most common cancer type and the second leading cause of cancer-related death in the western world. It remains a major cause of cancer mortality in the developed world, due largely to its propensity to metastasize.

CRC results from the accumulation of both acquired genetic and epigenetic changes that transform normal glandular epithelium into adenocarcinoma (Lao and Grady 2011). It includes a broad spectrum of forms according to the stage, going from non-neoplastic to neoplastic polyps (adenomatous polyps and adenomas) and invasive CRC. The most common type is the adenocarcinoma which accounts for 95% of cases, although other rarer types such as lymphoma and squamous cell carcinoma can also develop. They are predominantly epithelial-derived tumors (Sameer 2013).

Sporadic colorectal carcinomas, devoid of any familial or inherited predisposition, account for approximately 70% of colorectal cancers and are generated probably as a result of dietary and environmental factors as well as normal aging (Sameer 2013).

Inherited colorectal carcinomas represent approximately 5 to 10% of colorectal cancers. The most common ones are familial adenomatous polyposis (FAP), an autosomal dominant syndrome characterized by more than 100 colorectal adenomas. FAP is due to a pathogenic mutation in Apc*.* Another inherited polyposis syndrome is MUTYH-associated polyposis (MAP), an autosomal recessive cause of multiple colorectal adenomas originated by mutations in the DNA glycosylase *MUTYH*, which is involved in the repair of mispaired bases. MUTYH protects against mutations in different genes such as Apc and K-ras*,* critical in colorectal cancer. Other type of inherited CRC is the hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch syndrome, which is caused by mutations in DNA repair genes, *MLH1* and *MSH2* (Haggar and Boushey 2009, Worthley and Leggett 2010, Sameer 2013). The least understood CRC is known as familial colorectal carcinomas and accounts up to 25% of colorectal cancers (Sameer 2013).

There are several risk factors for developing CRC, for example, a low fiber and high fat diet, obesity or heavy alcohol consumption. Inflammatory intestinal conditions such as those occurring in Chrohn's disease and inflammatory bowel disease favor CRC development (Haggar and Boushey 2009).

Morson described the polyp-cancer sequence in which adenocarcinomas arise from pre-existing adenomatous polyps (Morson 1974). Then, Vogelstein proposed a model whereby colorectal cancer proceeds through a series of morphological steps due to specific genetic alterations, as shown in figure 1. The premises of this model are that the mutational activation of oncogenes and/or the inactivation of tumor suppressor genes result in colorectal carcinogenesis and at least four or five genes of a cell must undergo somatic mutations so as to get malignantly transformed. The model emphasizes the central role of the adenomatous polyp as the precursor lesion and provides evidence that in the majority of colorectal cancers the primary event is the aberrant activation of the APC/*β*-catenin pathway, followed by ras/raf mutations and loss of p53 function at later stages. However, nowadays only 7% of CRCs have been shown to bear mutations in all of these three genes. The genetic changes that occur in the origin of the tumor result in uninhibited cell growth, proliferation and clonal tumor development. The additive and accumulative effect of these somatic mutations has been shown to be the cause of sporadic colorectal cancer. The model also states that the characteristics of the tumor are dependent upon the accumulation of multiple genetic mutations rather than the sequence of mutations of the genes involved (Pancione et al. 2012, Sameer 2013).

Three different pathogenic pathways have been implicated in the development of colorectal cancer. And recently an alternative serrated pathway has been proposed.

The traditional models for these tumors are:

1-Predominant chromosomal instability pathway: Approximately 70%-85% of CRCs develop through chromosomal instability pathway. It results from the accumulation of numerical or structural chromosomal abnormalities by loss of heterozygosity, homozygous deletion, chromosomal rearrangements and aneuploidy (Hanahan and Weinberg 2000, Worthley and Leggett 2010, Lao and Grady 2011, Sameer 2013). It generally occurs within inherited tumors, such as familial adenomatous polyposis (FAP), but it has also been associated with the majority of sporadic CRCs (Lao and Grady 2011).

The chromosomal instability pathway is associated with the sequential deregulation of tumor suppressor genes and oncogenes such as Apc, K-ras, dcc/Smad4 and p53 (Pancione, Remo et al. 2012). Inactivation of Apc leads to activation of the Wingless/Wnt pathway, a common mechanism for initiating the polyp to cancer progression sequence (Worthley and Leggett 2010, Lao and Grady 2011). Other frequent mutations involve K-ras or B-raf, which occurs in approximately 55–60% of CRCs, leading to aberrant ERKs activation that induces proliferation and survival. p53 gene (TP53) is also frequently mutated or lost, being a late event in the transition adenoma to adenocarcinoma. Mutations in genes that regulate transforming growth factor β (TGFβ) signaling pathway are also important (Worthley and Leggett 2010, Lao and Grady 2011).

2-Pure microsatellite instability pathway: Microsatellite instability is responsible for the Lynch syndrome and 15–20% of sporadic tumors and it also occurs in patients with ulcerative colitis (Worthley and Leggett 2010, Pancione et al. 2012, Sameer 2013).

Microsatellites are short nucleotide repeat sequences scattered throughout the genome. DNA polymerase is particularly susceptible to making errors when copying these sequences leading to microsatellite instability. It is mainly caused by inactivation of DNA mismatch repair genes: *hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1 and 2* (Worthley and Leggett 2010, Lao and Grady 2011). Therefore, there is a dramatic increase in genetic errors in genes implicated in colorectal carcinogenesis such as tgfbr2, bax, casp5, ctnnb1, Apc, igf2, and e2f4 (Worthley and Leggett 2010).

3-The CpG island methylator phenotype pathway: The subtype of colorectal cancer called CpG island methylator phenotype is associated with a high frequency of methylated genes (Lao and Grady 2011). It accounts for approximately 15% of sporadic cases and they are usually characterized by an advanced pathology and a poor clinical outcome (Worthley and Leggett 2010, Lao and Grady 2011). It includes sporadic microsatellite instability cancers that occur sporadically in the context of *MLH*1 promoter methylation and its consequent transcriptional silencing. Such cancers exhibit both CpG island methylator phenotype and microsatellite instability pathways, and are usually considered as part of the CpG island methylator phenotype pathway (Worthley and Leggett 2010).

Epigenetic alterations involve global DNA hypomethylation (related with aging), as well as hypermethylation of classic tumor suppressor genes leading to their transcriptional repression. These epigenetic changes would promote CRC initiation (from adenoma to cancer), although some methylation changes might also contribute to CRC progression. So, at initial stages, apc, cxcl12/sdf-1 or vim are methylated and silenced, while timp3, Id4 and Irf8 are likely more methylated in metastatic CRC than in adenomas (Worthley and Leggett 2010, Lao and Grady 2011).

The CRC development model was revised by Issa (Issa 2008), who proposed a new route, the serrated pathway. According to it, distinct multiple pathways would be possible with different molecular mechanisms and variable prognosis, instead of a linear progression of single events. Thus, an alternative mechanism associated with a worse prognosis was proposed, where CRC would originate mainly from villous, but also from serrated adenomas and would be very heterogeneous. Serrated adenomas are characterized by a CpG island methylator phenotype low phenotype, predominant K-ras and occasional B-raf mutations, but without chromosomal instability (Lao and Grady 2011). Moreover, changes in the patterns of mRNAs and noncoding RNAs expression, as well as the consequent effect on protein expression and posttranslational modifications could play a role in CRC tumorigenesis (Sameer 2013).

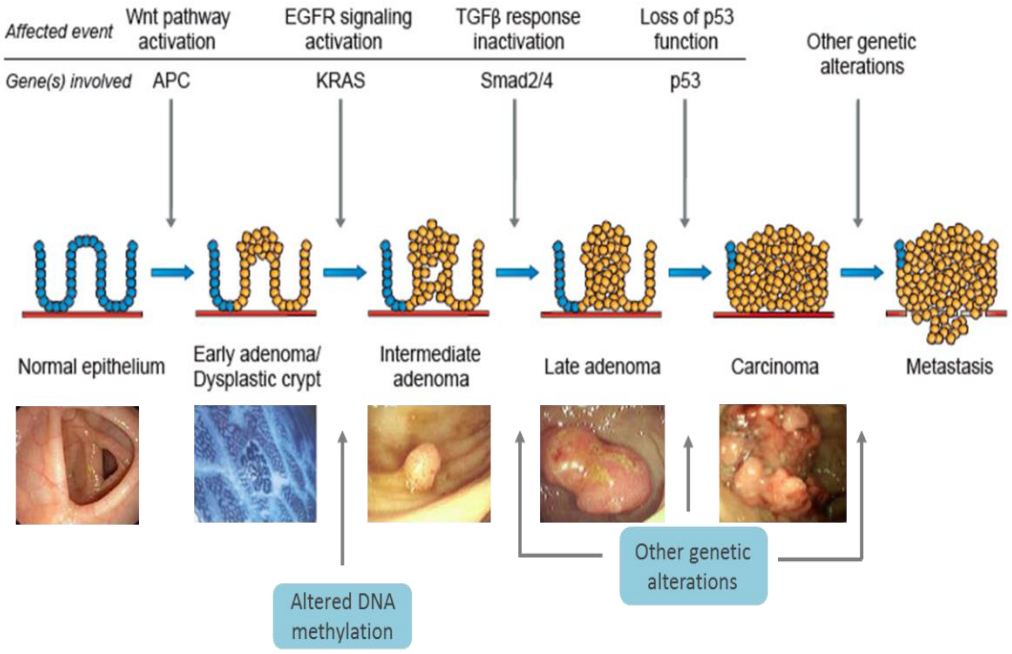

Figure 1-Progression of colorectal cancer. Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulation of alterations in particular genes (i.e. Apc, K-ras, Smad2/4, p53), which affects different signaling pathways. Adapted from Takayama et al. 1998 and Davies et al. 2005.

2. p38 MAPKs

2.1 GENERALITIES

p38 MAPKs belong to the superfamily of MAPKs. MAPK pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Dhillon et al. 2007).

MAPKs are activated upon dual phosphorylation of threonine and tyrosine residues in a conserved Thr-X-Tyr motif (where X is any amino acid) in the activation loop of kinase subdomain VIII by a MAP kinase kinase (MKK), which in turn is activated when phosphorylated by a MAPK kinase kinase (MKKK). MAPK phosphatases reverse the phosphorylation and return MAPKs to their inactive state (Chang and Karin 2001, Dhillon et al. 2007, Kyriakis and Avruch 2012)

In mammals, there are more than a dozen of MAPK genes codifying for different MAPKs. The best known subfamilies of MAPKs are ERKs (Extracellular signal-regulated kinases): ERK1/2, mainly activated by growth factors, and the stress MAPKs, which were initially described as kinases activated mainly by stress. These include JNKs (c-Jun N-terminal Kinases): JNK-1, JNK-2 and JNK-3, and p38 MAPKs: p38α, p38β, p38γ and p38δ. Other MAPKs are ERK3/4, ERK5 or ERK7/8, which belong to

the non-classical subfamily and have distinct regulation and functions (Dhillon et al. 2007, Raman et al. 2007). MAPKs are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/4/6 for p38 (detailed below), MKK4/7 (JNKK1/2) for JNKs, and MEK5 for ERK5. At the same time, each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling (Chang and Karin 2001, Kyriakis and Avruch 2012).

There are four p38 MAPKs isoforms in mammals: α, β, δ and γ, which are 60% identical in their amino acid sequence. The four p38 MAPKs are encoded by different genes and show specific and overlapping functions and substrate specificity *in vitro*. They can be differentially regulated by the three known p38 MAPK kinases and they have different patterns of tissue expression (Nebreda and Porras 2000). It therefore appears that the four p38 MAPKs are likely to be involved in specific cellular processes. Furthermore, p38 MAPK isoforms also differ in their sensibility to the inhibitors SB203580, SB202190 and BIRB796, and in their substrate specificity (Cuadrado and Nebreda 2010, Kyriakis and Avruch 2012).

The first member of the p38 MAPK family was independently identified by four groups. It was identified for the first time as a 38 KDa protein kinase that was tyrosine phosphorylated in response to LPS (lipopolysaccharide) stimulation. They showed that p38 and its yeast homolog HOG1 had sequences, adjacent to critical phosphorylation sites, that distinguish these proteins from most other MAP kinase family members (Han et al. 1994). Then, two other groups independently identified p38α as a MAPK kinase activated by stress and similar to HOG1 (Rouse et al. 1994), and as a kinase activated by IL-1 and inactivated by protein phosphatases (Freshney et al. 1994). p38α was also reported as a polypeptide receptor for a class of pyridinyl imidazole anti-inflammatory drugs (CSAIDs), related with cytokine production (Lee et al. 1994). In later years, three other p38 MAPK isoforms were described: p38β (Jiang et al. 1996), p38γ (Cuenda et al. 1997) and p38δ (Jiang et al. 1997, Muller et al. 2001).

Although all p38 isoforms are widely expressed, p38α is the best characterized and ubiquitously expressed at significant levels. p38β is also ubiquitous, but it is expressed at lower levels. p38γ is most significantly expressed in skeletal muscle and p38δ is mainly found in testis, pancreas, kidney and small intestine (Cuenda and Rousseau 2007).

The p38 MAPKs are strongly activated *in vivo* in response to extracellular stimuli such as environmental stresses (UV light, heat or osmotic shock), inflammatory cytokines (TNFα and IL-1), pathogen/damaged-associated molecular patterns from the immune response and less by serum and growth factors (Zarubin and Han 2005, Muslin 2008, Kyriakis and Avruch 2012).

The canonical activation of p38 MAPKs, outlined in figure 2, occurs via dual phosphorylation of their Thr-Gly-Tyr motif by three MKKs. Activation by a specific MKK depends on the stimuli, but also on the cell type, as its level of expression varies. MKKs are in turn activated by phosphorylation by a MAPK kinase kinase (MKKK) such as MLKs, ASK1, TAK1 and some members of the MEKK family. Upstream them there are small G proteins from the Rho subfamily such as Rac1, Cd242, Rho and Rit, and

heterotrimeric G proteins. MKK6 can phosphorylate the four p38 MAPK isoforms, whereas MKK3 activates p38α, p38δ and p38γ, but not p38β. In addition, p38α can be also phosphorylated by MKK4 in response to some stimuli such as ultraviolet radiation. This specificity of MKKs to activate individual MAPK isoforms is mediated, in part, by an interaction between the N-terminal region of MKKs and different docking sites of MAPKs, and also by the structure of the MAPK activation loop with the dual phosphorylation motif (Cuenda and Rousseau 2007, Cuadrado and Nebreda 2010).

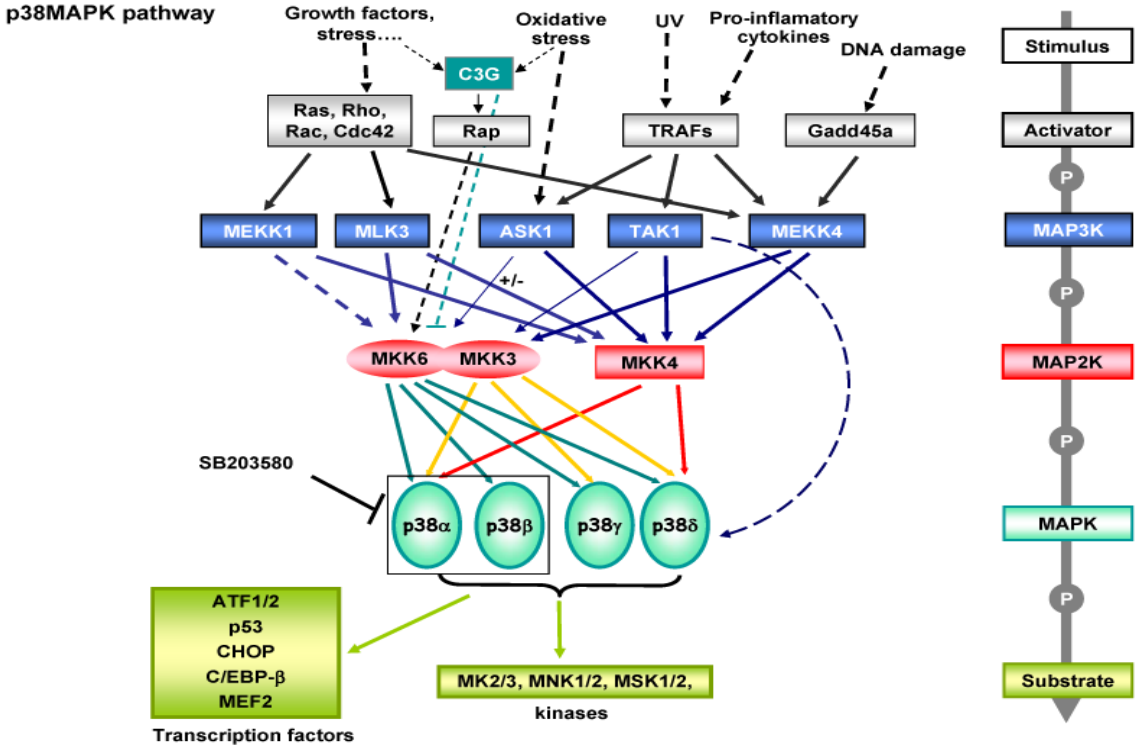

Figure 2-p38 MAPK signaling pathway. A variety of stimuli activate p38 MAPK through GTPases, receptor adapter proteins or cell cycle checkpoint proteins that transmit the stimulus to complex kinase cascades including a MAP3K that phosphorylates a MAPK2 that, in turn, phosphorylates p38 MAPKs. MKK6 is the major activator of all p38 MAPK isoforms, while MKK3 and MKK4 are more specific and can only activate some isoforms. Once activated, the different p38 MAPKs either phosphorylate cytoplasmic targets or translocate into the nucleus leading to the regulation of transcription factors (Porras and Guerrero 2010).

Non-canonical mechanisms for p38α (and probably p38β) activation have also been described. For example, in T cells upon T cell receptor (TCR) activation, p38 is phosphorylated in Tyr 323, which leads to autophosphorylation in Thr 180 and Tyr 182 (Salvador et al. 2005). Interaction of TAB-1 (Transforming growth factor-β-activated protein 1 (TAK1)-binding protein 1) with p38α also induces p38α auto-phosphorylation and activation (forming a TRAF6-TAB1-p38α complex) (Ge et al. 2002, Li et al. 2005).

The magnitude and duration of p38 MAPKs signal are critical determinants of their biological effects. Activation of p38 MAPK occurs within minutes in response to most stimuli and is transient, being down-regulated by dephosphorylation. Many dual-

specificity phosphatases from the MAPK phosphatase family (MKP, also named as DUSP, dual specificity phosphatase) can efficiently dephosphorylate p38α and p38β, but not p38γ or p38δ. In addition, other protein phosphatases, such as serine/threonine protein phosphotase type 2C (PP2C) and Tyr phosphatase (PTP), can inactivate all p38 MAPK isoforms (Zarubin and Han 2005, Cuenda and Rousseau 2007).

Although p38 MAPKs have overlapping substrate specificity, some substrates appear to be preferentially phosphorylated by one or more isoforms. The first p38α substrate identified was the MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2). Other well-known p38α and p38β substrates are the kinases MNK1, PRAK, MSK1 and transcription factors like activating transcription factor 1, 2 and 6 (ATF-1/2/6), SRF accessory protein (Sap1), CHOP (growth arrest and DNA damage inducible gene 153, or GADD153), p53, C/EBPβ, myocyte enhance factor 2C (MEF2C), MEF2A, MITF1, DDIT3, ELK1, NFAT and high mobility group-box protein 1 (HBP1). Others identified substrates are cytoskeletal proteins, translational machinery components and other proteins such as tau, keratin 8, metabolic enzymes, glycogen synthase or cytosolic phospholipase A2 (Zarubin and Han 2005).

p38γ and p38δ MAPK isoforms can phosphorylate typical p38 MAPK substrates such as the transcription factors ATF-2, Elk-1 or SAP-1, but they have specific ones such as α1-syntrophin, SAP90/PSD95 and SAP97/Hd1g for p38γ, and stathmin, tau and eEF2 (eukaryotic elongation factor 2) kinase for p38δ (Cuenda and Rousseau 2007, Cuadrado and Nebreda 2010).

2.2. FUNCTIONS

p38 MAPKs are involved in the regulation of several cellular functions such as proliferation, differentiation, survival or migration, among others.

To study p38 MAPKs physiological roles, the most important experimental approaches have been the use of chemical inhibitors and genetically modified mice. *In vitro* and *in vivo* assays have demonstrated that p38α and p38β are inhibited by the pyridinyl imidazole derived SB203580 and SB202190 compounds, but not p38γ and p38δ. BIRB796 can inhibit the four isoforms, although at different doses (Cuenda et al. 1997, Goedert et al. 1997, Kuma et al. 2005).

To identify specific roles played by the different isoforms, knock-out mice have been generated. p38α knock-out mouse was the first one. It is embryonic lethal, so mice dye at midgestation due a placental defect, which shows the essential role of p38α for embryonic development (Adams et al. 2000, Mudgett et al. 2000). Using cardiac-specific p38α knock-out mice, it has been also shown that p38α is a key negative regulator of cardiomyocyte proliferation (Engel et al. 2005). In contrast, p38β knock-out mice are viable (Beardmore et al. 2005) and only show a reduced bone mass phenotype (Greenblatt et al. 2010). Mice with combined deletion of p38α and p38β display diverse developmental defects at midgestation, including major cardiovascular defects and liver abnormalities. Using knock-in mice that express p38β under control of

the endogenous p38α promoter, it has also been demonstrated that heart development requires endogenous p38β expression (del Barco Barrantes et al. 2011). p38γ and p38δ and double p38γ/p38δ knock-out mice are viable and fertile and have no obvious disorders (Sabio et al. 2005).

Based on both *in vitro* and *in vivo* studies, many different functions played by p38 MAPKs have been identified and characterized so far, such as:

1. Inflammation: p38α plays essential roles in pro-inflammatory cytokines production (Kumar et al. 2003) and induction of the pro-inflammatory mediator COX-2 (Cyclooxygenase-2) (Xu and Shu 2007). p38γ and p38δ are also crucial regulators of inflammation in collagen-induced arthritis (Criado et al. 2014), colitis-associated colon cancer (Del Reino et al. 2014) and skin inflammation during squamous cell development (Zur et al. 2015). In contrast, p38β does not seem to be required for acute or chronic inflammatory response (O'Keefe et al. 2007).

2. Differentiation: p38α/β have been implicated in various cell differentiation processes such as 3T3-L1 fibroblasts adipocytic differentiation, PC12 cells neuronal differentiation, differentiation of C2C12 myoblasts to myotubes, ATDC5 cells chondrogenic differentiation or erythroid differentiation of SKT6 cells (Nebreda and Porras 2000). In this line, p38α has a fundamental role in muscle formation *in vitro* and *in vivo* (Perdiguero et al. 2007, Liu et al. 2012) and p38γ regulates muscle growth and regeneration (Gillespie et al. 2009).

3. Stemness: p38 signaling has also an important role in maintaining the functional microenvironment of stem cells to control lung homeostasis. Thus, the activation of p53 and the regulation of the miR-17-92 are involved in the response to extracellular signals in lung stem cell fate decision (Oeztuerk-Winder et al. 2012). In this context, activation of p38α in fibroblasts, is involved in the response to SDF-1 produced by lung stem cells (LSCs) and induces cytokine expression in a paracrine feedback loop, which is necessary for proper LSC differentiation (Ruiz et al. 2014). Furthermore, p38 upon activation by chondrogenic cytokines in mesenchymal stem cells, is an essential mediator of shape-induced terminal differentiation of skin stem cells (Oeztuerk-Winder and Ventura 2012).

4. Development: p38 MAPKs are involved in the regulation of various developmental processes in *Drosophila* (Nebreda and Porras 2000)**.** p38α is essential for mouse embryonic development (Adams et al. 2000, Mudgett et al. 2000), while other p38 MAPK isoforms are not (Beardmore et al. 2005, Sabio et al. 2005).

5. Proliferation: Depending on the cell type and stimulus, p38 MAPKs can have either a positive or a negative role in cell proliferation (Nebreda and Porras 2000). p38α can inhibit cell cycle progression, both at the G1/S, and G2/M transition acting through different mechanisms (Perdiguero et al. 2007, Gillespie et al. 2009). p38γ and p38δ play also a role in proliferation, so that p38γ and p38δ double knock-out MEFs growth slightly quicker than *wt* cells (Cerezo-Guisado et al. 2011). In contrast, it has been suggested an *in vivo* role for p38δ promoting cell proliferation (Schindler et al. 2009).

6. Cell death: p38α can play a dual role as a regulator of cell death, acting as a mediator of either cell survival or cell death through different mechanisms. The specific function of p38 MAPKs depends on the cell type, the stimuli and/or the activated isoform (Nebreda and Porras 2000, Wagner and Nebreda 2009). p38α sensitizes cardiomyocytes derived cell lines to apoptosis through up-regulation of pro-apoptotic proteins such as Fas and Bax and down-regulation of the activity of ERKs and Akt survival pathways (Porras et al. 2004, Zuluaga et al. 2007). p38α is also a negative regulator of embryonic stem (ES) cells survival (Guo and Yang 2006) and it is essential for cisplatin-induced apoptosis in human colon carcinoma HCT116 cell line (Bragado et al. 2007).

p38 MAPKs also have an anti-apoptotic role in DNA-damaged fibroblasts (Heron-Milhavet and LeRoith 2002), differentiating neurons (Okamoto et al. 2000), activated macrophages (Park et al. 2002) or pulmonary arterial endothelial cells (Zhang et al. 2003, Zhang et al. 2005).

In addition, p38 MAPKs are relevant in the coordination of cellular stress responses to reactive oxygen species (ROS), mediating cell death or survival. ROS-induced activation of p38α in the initial stages of tumor transformation promotes apoptosis (Dolado et al. 2007). In contrast, p38α can also mediate cell survival in response to oxidative stress through different mechanisms (Thornton et al. 2008, Gutierrez-Uzquiza et al. 2010, Joaquin et al. 2012). For instance, p38α up-regulates some anti-oxidant enzymes such as catalase (Sen et al. 2005) and SOD in response to low doses of H_2O_2 (Gutierrez-Uzquiza et al. 2010), as well as heme oxygenase-1 mRNA levels (Aggeli et al. 2006).

7. Adhesion: p38α negatively regulates cell adhesion in various cell lines such as ES cells (Guo and Yang 2006) and cardiomyocyte derived cell lines (Zuluaga et al. 2007). In chronic myeloid leukemia (CML) cells, although p38α inhibits the expression and/or phosphorylation of some focal adhesion (FA) proteins, it favors cell adhesion (Maia et al. 2013).

8. Migration and invasion: p38α mediates cell migration and invasion of several cell types, including tumor cells (Cuenda and Rousseau 2007, Wagner and Nebreda 2009), as it will be further explained later.

9. Senescence: p38 MAPKs have been described to play a role in senescence induction in response to telomere shortening (Haq et al. 2002, Bihani et al. 2004), oncogenes (Bulavin et al. 2003, Kwong et al. 2009, Kwong et al. 2013) or endothelial function (McMullen et al. 2005).

10. Cancer: p38α is involved in the regulation of different aspects of tumor progression and metastasis, either acting as a tumor promoter or suppressor. p38α role in cancer will be detail below.

2.3 p38 MAPK IN CANCER

p38α MAPK can act both, promoting or suppressing tumor growth playing different roles during tumor progression as shown in figure 3. In the initial stages of tumor development, p38α can act as a tumor suppressor. For instance, p38α plays a key role in lung tumorigenesis through inhibition of proliferation, differentiation induction and control of lung stem cells self-renewal (Ventura et al. 2007). Thus, p38α conditional deletion in adult mice favors not scheduled proliferation of progenitor cells and K-Ras G12V-induced tumorigenesis. p38α liver-specific deletion also enhances hepatocarcinoma development (Hui et al. 2007). In addition, p38α inhibits malignant transformation induced by oncogenic H-Ras through induction of apoptosis and/or, cell cycle arrest (Dolado et al. 2007, Grossi et al. 2014). In this line, inactivation or disruption of Wip1 phosphatase suppresses *in vitro* transformation and *in vivo* mammary carcinogenesis through activation of p38 MAPK-p53 pathway (Bulavin et al. 2004). Moreover, loss of Gadd45a accelerates Ras-driven mammary tumor formation through a decrease in JNK and p38 kinase activation, leading to a reduced apoptosis and Ras-induced senescence (Tront et al. 2006).

In contrast, other studies support a role for p38 MAPK as a tumor promoter. For example, activation of p38 MAPK has been correlated with bad prognosis in HER-2 negative breast cancer or hepatocellular carcinoma and p38 MAPK act as a tumor promoter in follicular lymphoma, lung, thyroid, colorectal, ovarian, breast, gliomas and head and neck squamous cell carcinomas (Bakin et al. 2002, Junttila et al. 2007, Kim et al. 2003, Hsieh et al. 2007, Matrone et al. 2010). In addition, p38 MAPK pathway may contribute to the survival or proliferation of cancer cell lines from breast, prostate or skin (Grossi et al. 2014). p38 MAPK also promotes migration and invasion processes, features associated with cancer aggressiveness. For example, it enhances metastasis in breast and hepatocellular carcinomas (Igea and Nebreda 2015), which will be explained more in detail later.

Other p38 MAPK isoforms have been involved in cancer regulation. For example, p38δ plays an essential role in skin carcinogenesis (Schindler et al. 2009) and both p38γ and δ isoforms are involved in the inflammatory response during squamous cell development (Zur et al. 2015). Moreover, p38γ/δ modulate inflammation and the procarcinogenic environment in colitis-associated colon cancer formation (Del Reino et al. 2014). In contrast, another study revealed a tumor suppressor role for p38γ and p38δ in mouse transformed fibroblasts (Cerezo-Guisado et al. 2011).

In head and neck cancer, p38α/β activation induces growth arrest through up-regulation of p27 and down-regulation of CDKs, leading to tumor dormancy, which favors survival of disseminated tumor cells (Aguirre-Ghiso 2007, Bragado et al. 2013). p38α/β also makes dormant HEp3 cells highly resistant to chemotherapy through upregulation of chaperone BiP/Grp78, which inhibits Bax activation (Ranganathan et al. 2006). Moreover, p38α allows survival of dormant tumor cells through activation of Rheb/mTOR pathway (Schewe and Aguirre-Ghiso 2008).

p38 signaling has also an important role in maintaining the functional microenvironment for tumor stem cells. For example, low p38 MAPK activity and PI3K-Pdk1 signaling lead to selection of more malignant cells, with a less differentiated stem-like profile in lung adenocarcinomas (Voisset et al. 2013).

There are several studies showing a relevant role for p38 MAPKs, especially p38α, in colorectal cancer development and progression.

p38 MAPK-mediates cell death in colorectal cancer cells in response to different pro-apoptotic signals (Fassetta et al. 2006). In particular, p38α plays a role in the regulation of oxidative stress, which results from the high proliferative and/or metabolic rate of colorectal cancer cells (Simone 2007). In addition, in the HCT116 human colorectal cancer cell line, cisplatin apoptotic response requires p53/ROS-mediated p38α MAPK activation (Bragado et al. 2007). p38 activation also induces cell death in K-ras-mutated human colon cancer cells by mechanisms involving the suppression of vitamin D receptor activity (Qi et al. 2004).

However, p38α can also act as a tumor promoter in colorectal cancer. p38α is required for colorectal cancer cell homeostasis. Hence, p38α inhibition or genetic inactivation causes cell cycle arrest, autophagy and finally, cell death, although initially represents a survival pathway (Comes et al. 2007). Accordingly, p38α regulates the expression and/or localization of proteins required for autophagosome formation, which leads to inhibition of autophagy and survival of colorectal cancer cells (Grossi et al. 2014).

p38α is also required to maintain colorectal cancer cell metabolism and its inhibition reduces tumor growth, both *in vitro* and *in vivo* (Chiacchiera et al. 2009, Chiacchiera et al. 2012).

Results derived from a mouse model of colitis associated colon-cancer indicate that p38α MAPK plays a dual role, suppressing tumor initiation, while contributing to cell proliferation and survival later on (Gupta et al. 2014). According to this, chemical inhibition of p38α MAPK with PH797804 decreases colorectal cancer tumor growth in patient derived xenografts (PDXs) bearing a K-Ras mutation (Gupta et al. 2015). This involves a reduction in cell proliferation and survival through a mechanism mediated by down-regulation of CXCL-1/2 and IL-6, which leads to JNK activation (Gupta et al. 2015).

On the other hand, recent data has shown that a reduced p38 MAPK signaling, along with a high ERK2 activation, favors the generation of colon cancer metastasis in the lung from previously established liver metastatic lesions (Urosevic et al. 2014). This is mediated by the increased expression of PTHLH cytokine, which contributes to colon cancer cell extravasation (Urosevic et al. 2014). Moreover, the expression of Wnt/β-catenin target genes in colorectal cancer cells induces epithelial-mesenchymal transition (EMT) and enhances cell migration and invasion in tissue culture by activating p38 MAPK pathway (Qi et al. 2015).

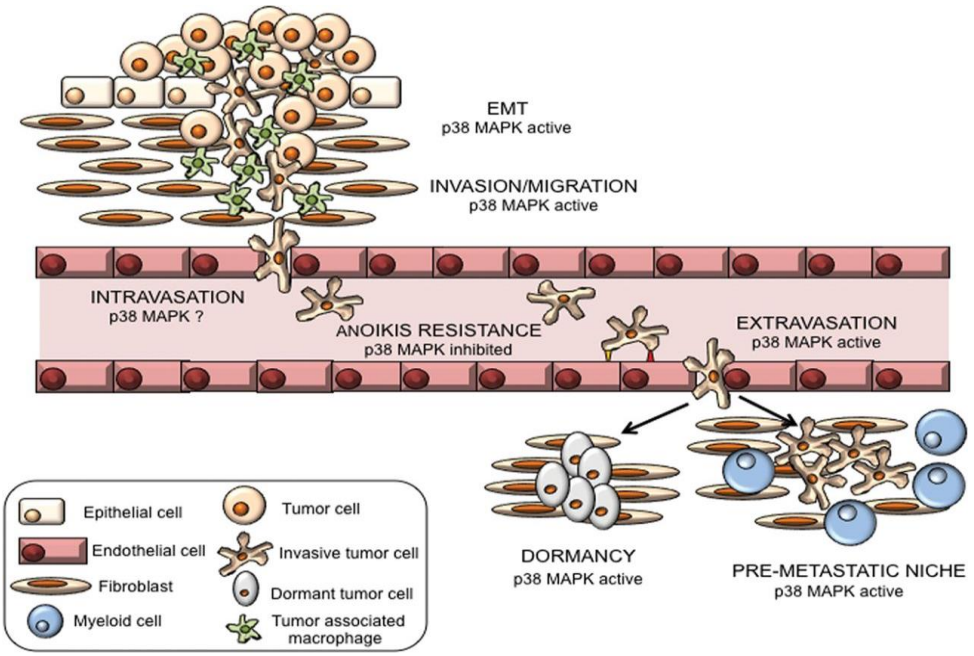

Figure 3-Roles of p38 MAPKs in tumor progression: p38 are involved in the EMT process of cells from the primary tumor, acquisition of invasive and migratory properties, resistance to anoikis, extravasation of migrating cells, pre-metastatic niche formation and tumor cell dormancy (del Barco Barrantes and Nebreda 2012)**.**

2.4 p38 MAPK CROSS-TALK WITH OTHER SIGNALING PATHWAYS

There are different examples of interplay between p38 MAPKs signaling and other pathways, resulting in a complex network that contributes to explain their involvement in several biological cell functions in a context-dependent manner (Cuadrado and Nebreda 2010). For example, p38α MAPK negatively regulates the activity of the ERKs pathway, as a consequence, in the absence of p38α ERKs activation is increased (Porras et al. 2004). This p38 MAPK-mediated inactivation of ERKs appears to be PP2A dependent (Junttila et al. 2008). In a tumor context, a reciprocal compensatory role of p38α and MEK/ERK pathways is also possible. Thus, in colorectal cancer p38α inhibition enhances the activation of other kinases cascades such as ERKs contributing to tumor growth. Therefore, inhibition of both p38α and MEK1 improves cancer therapy efficiency, reducing *in vivo* tumor growth (Grossi et al. 2014).

There is also a cross-talk between JNKs and p38 MAPKs pathways. In fact, although multiple stimuli can simultaneously activate both pathways, their activation has antagonistic effects in many cases. p38 MAPKs can negatively regulate JNK activity at different levels. For example, at the level of MAP3Ks, either by phosphorylating MLK3 or the TAK1 regulatory subunit TAB1 (Cuadrado and Nebreda 2010). In a different way, p38α controls myoblast proliferation by antagonizing the proliferation-promoting function of JNK (Perdiguero et al. 2007). p38α MAPK also down-regulates JNK activity by decreasing ROS in various tumor cell lines, including colorectal cancer cells (Pereira

et al. 2013). Therefore, either p38α/β inhibition or p38α knock-down leads to ROS upregulation, which in turn activates the JNK pathway via inactivation of phosphatases, sensitizing these cancer cells to cisplatin-induced apoptosis (Pereira et al. 2013).

The interaction between p38α MAPK and Akt has been widely described. Akt can inhibit the activation of p38α MAPK through phosphorylation of the upstream regulator, ASK1, on Ser 83 (Yuan et al. 2003). On the other hand, p38α negatively modulates Akt activity through activation of PP2A in the caveola, which is favored by the interaction between caveolin-1 and PP2A (Zuluaga et al. 2007). At a different level, there is also a connection between PI3K/Akt and p38 MAPK pathways. Thus, p38 MAPK was identified as an upstream regulator of mTORC1 activity in *Drosophila melanogaster* and in a transformed human cell line in response to different stimuli (Cully et al. 2010). Moreover, in quiescent tumor cells, activation of ATF6 via p38α up-regulates mTORC1 activation, leading to survival of dormant cells (Schewe and Aguirre-Ghiso 2008).

A link between p38 MAPK and Wnt/β-catenin pathways has also been reported, so that, p38α MAPK inactivates GSK3β by phosphorylation, leading to β-catenin accumulation (Thornton et al. 2008).

There are also different connections between p38 MAPKs and small GTPases. For instance, a functional cross-talk between Rac and p38 MAPK has been largely reported. p38 can act as either an effector or activator of Rac, as well as an inhibitor. For example, in embryonic cardiomyocytes derived cell lines, p38α acts as either a positive or a negative regulator of Rac1 depending on the presence of growth factors (Zuluaga et al. 2007). On the other hand, to control cell motility, Rac induces p38 activation in some cellular systems (Nobes and Hall 1995).

There is a regulation of p38 MAPK by Rap1, which could be either positive or negative. Rap1 can inhibit Ras-induced p38 MAPK activation in a thymoma cell line (Stork and Dillon 2005), while FGF2 (Fibroblast growth factor 2)-induced p38 MAPK activation in endothelial cells is mediated by Rap1 (McDermott and O'Neill 2002). On the other hand, the combined actions of p38 MAPK and Rap1 are required for LPS (lipopolysaccharide)-induced chemotaxis breakdown (Yi et al. 2012). Moreover, our group has recently described a new functional interaction between the Rap1 GEF, C3G (RapGEF1), and p38α MAPK involved in the regulation of apoptosis in MEFs (Gutierrez-Uzquiza et al. 2010) and in the K562 chronic myeloid leukemia cell line (Maia et al. 2009). This C3G/p38 crosstalk also regulates adhesion in K562 cells (Maia et al. 2013), which will be further explained later on.

An interplay between p38 MAPKs and Rho GTPase signaling also exists in some inflammatory and hyperpermeability responses. For example, upon stimulation by gram positive pathogens, there is an endothelial pulmonary dysfunction induced by different pattern of crosstalk between Rho, p38 MAPK and NFκB signaling (Wu et al. 2013).

3. C3G

3.1 GENERALITIES

C3G (Crk SH3-domain-binding guanine-nucleotide-releasing factor) is a guanine nucleotide exchange factor (GEF) for some members of Ras and Rho families of small GTPases, mainly the Ras family members: Rap1 (Gotoh et al. 1995) and R-Ras (Gotoh et al. 1997). In addition, C3G can activate other Ras family members such as TC21 and the Rho family member TC10, leading to MAPK activation (Ehrhardt et al. 2002, Radha et al. 2011). Alternate names of C3G are RapGEF1, GRF2, DKFZ p781p1719 or CRK SH3-BINDING GNRP.

C3G can also act through GEF independent mechanisms. For instance, to suppress transformation induced by different oncogenes (Guerrero et al. 1998, Guerrero et al. 2004) or to activate apoptosis through interaction with Hck (Shivakrupa et al. 2003).

C3G was the first Rap GEF identified, originally isolated as an interacting partner of Crk and Grb2/ASH in the upstream pathway of Ras (Tanaka et al. 1994). The nucleotide sequence of the 4.1 kb C3G c-DNA contains a 3.2 kb open reading frame encoding a 121 kDa protein. Antibodies against C3G recognize a 130-140 kDa protein (Tanaka et al. 1994). Human C3G protein is encoded by rapgef1 gene, which is located on chromosome 9q34.13, comprising 24 exons and spanning 163 kb (Takai et al. 1994).

C3G is ubiquitously expressed. However, some tissue-specific differences in expression have been detected. In humans, adult skeletal muscle, placenta, fetal heart and brain present higher C3G levels than other tissues such as liver (Tanaka et al. 1994). In mouse, C3G expression is high in brain, heart, liver and muscle and low in adipose tissue, kidney and spleen (Radha et al. 2011).

Sequence comparison revealed 88% identity in the nucleotide sequence between mouse and human C3G cDNA (Zhai et al. 2001). In humans, two predominant C3G isoforms have been found, a and b, which arise from alternative splicing and differ in their N-terminus, where three amino acids of isoform a are replaced by 21 amino acids in isoform b (Radha et al. 2011). A truncated C3G isoform, named p87C3G, which lacks 305 residues from N-terminal region and interacts with Bcr-Abl, has been shown to be abundantly expressed in chronic myeloid leukemia (CML) cells and primary cells from CML patients (Gutierrez-Berzal et al. 2006). The expression levels of this isoform correlates with the stage of the disease as it decreases upon treatment-induced remission, suggesting a role for C3G in CML (Gutierrez-Berzal et al. 2006). In mice, two transcripts with or without a 114 bp insertion in 5´ region are expressed, which lead to the expression of two C3G isoforms in most tissues (Zhai et al. 2001).

C3G protein can be divided into three modular domains clearly differentiated, both structurally and functionally, as it is outlined in figure 4. In the C-terminal region, there is a CDC25 homologous domain that, together with the REM (Ras-Exchange-Motif) domain, are responsible for its GEF activity. The central region contains five proline-rich sequences that bind to Src Homology-3 (SH3) domains of Crk, p130Cas, Grb2, c-Abl and Hck (Radha et al. 2011). The N-terminal region negatively regulates C3G GEF activity, since its deletion results in constitutive catalytic activity (Ichiba et al. 1999). In

this N-terminal region (from amino acids 144 to 230) there is a binding site for the cytoplasmic domain of E-cadherin, which is involved in the interaction of E-cadherin and C3G during cell-cell contacts formation (Hogan et al. 2004).

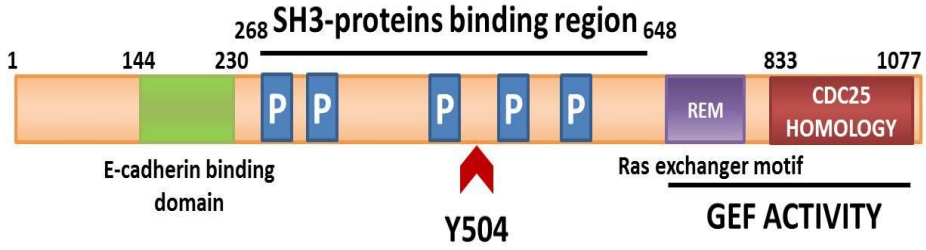

Figure 4-C3G structure. Schematic diagram showing the domain organization of C3G protein. The C-terminal catalytic region of C3G contains a domain homologous to CDC25, that together with the REM domain are responsible for its GEF activity. The N-terminal region has a domain that interacts with E-cadherin. The central region has a protein interaction domain (also known as Crk-binding region, CBR) containing multiple proline-rich sequences that bind SH3 domains of Crk, Cas or c-Abl. The non-catalytic sequences negatively regulate the catalytic activity of C3G (Radha et al. 2011).

The mechanisms regulating C3G expression and activation are still poorly understood. It is known that several stimuli such as T cell receptor stimulation (Reedquist et al. 1996), hepatocyte growth factor (HGF) (Sakkab et al. 2000), growth hormone (Ling et al. 2003), platelet-derived growth factor (PDGF) (Yokote et al. 1998), nerve growth factor (NGF) (York et al. 1998), interferon-γ (Alsayed et al. 2000), integrins (Buensuceso and O'Toole 2000) or interleukin-3 (Arai et al. 2001) engage C3G-mediated signaling. In addition, other GEFs could be involved in the regulation of C3G. For example, the Rac GEF DOCK-180 might down-regulate C3G, as its knock-down leads to an increased C3G expression (Wang et al. 2010). Furthermore, based on bioinformatic studies from our group (unpublished data) C3G expression might be regulated by different hematopoietic transcription factors, mainly some members of GATA family. However, this needs to be validated. There is an example of C3G regulation by the novo methylation of C3G regulatory sequences, which would lead to a decreased expression in cervical squamous carcinoma (Okino et al. 2006).

The major C3G activating mechanism in response to the above mentioned stimuli is the multimolecular complex formation. It primarily occurs through membrane recruitment of Crk-C3G complex, which facilitates its interaction with the substrate Rap1 (Gotoh et al. 1995, Van den Bergh et al. 1997). The SH2 domain in Crk enables translocation of the Crk-C3G complex to tyrosine-phosphorylated molecules (Ichiba et al. 1997), such as receptor tyrosine kinases (Yokote et al. 1998). Interaction between C3G and Crk is influenced by Crk phosphorylation, which can be regulated by the tyrosine phosphatase PTP1B (Radha et al. 2011). Moreover, C3G activation has been shown to be mediated by tyrosine phosphorylation at Y504 and membrane targeting, enabled by its interaction with Crk in response to some stimuli. In this way, the negative regulatory action of N-terminal domain is impaired (Ichiba et al. 1999, Radha et al. 2004). c-Src, Hck, Fyn and c-Abl are kinases known to phosphorylate C3G at Y504. In addition, to Y504, C3G can be phosphorylated in other tyrosine residues, which function remains unknown (Radha, et al. 2011).

3.2 FUNCTIONS

C3G is involved in different signaling pathways, regulating many cellular functions (figure 5). Some of its actions are mediated by its GEF activity, while others depend on protein-protein interactions such as its suppressor activity against oncogenic-induced transformation in fibroblasts (Guerrero et al. 1998, Shivakrupa et al. 2003).

C3G-/- homozygous mice died before embryonic day 7.5 due to a defect in integrin-mediated cellular adhesion (Ohba et al. 2001). Moreover, studies using C3G knock-out mouse embryonic fibroblasts has revealed that C3G-dependent activation of Rap1 is required for adhesion and spreading. Other reports also support that C3G plays a relevant role in cell adhesion, spreading, migration and cell-cell junction assembly and disassembly (Radha et al. 2011), as it would be explained more in detail later.

C3G is also important for regulation of proliferation in different cell types such as neuroblastoma cells (Radha et al. 2008). In agreement with this, C3G-deficient mice show an over-proliferation of the cortical neuroepithelium (Voss et al. 2006), having defects in cortex development. In addition, C3G is involved in the regulation of some differentiation processes such as adipocyte differentiation (Jin et al. 2000) or skeletal muscle differentiation (Sasi Kumar et al. 2015).

C3G regulates vesicle traffic, too. For instance, C3G-mediated TC10 activation promotes GLUT4 (glucose transporter type 4) membrane translocation in response to insulin in muscle and adipose tissue through a mechanism dependent on cytoskeletal rearrangement (Chiang et al. 2001).

C3G is also a regulator of cell death. It can induce apoptosis acting through Hck (Shivakrupa et al. 2003) or c-Abl (Radha et al. 2008). Moreover, C3G through the negative regulation of p38α activity mediates, either cell survival or death, depending on the stimulus and cell type (Maia et al. 2009, Gutierrez-Uzquiza et al. 2010).

The function of C3G in cancer is quite controversial as it can act as either a tumor suppressor or promoter. As a tumor suppressor, C3G prevents malignant transformation induced by several oncogenes (Guerrero et al. 1998, Guerrero et al. 2004). According to this, C3G expression was shown to be reduced in cervical squamous cell carcinoma (Okino et al. 2006). In contrast, an increased C3G expression was found in human non-small-cell lung cancer in comparison to the corresponding non-cancerous lung tissues (Hirata et al. 2004). In agreement with this, Crk-C3G-Rap1 pathway, downstream RET, has been involved in the process of transformation produced in papillary thyroid carcinoma (De Falco et al. 2007). The expression of p87C3G isoform in CML cells is also associated with the development of this type of cancer (Gutierrez-Berzal et al. 2006). Moreover, recent data suggest that C3G acting through Rap1, might promote migration and invasion of epithelial ovarian cancer cells (Che et al. 2015), which indicates that C3G could regulate key events in later stages of cancer progression.

Figure 5-C3G interacting partners and functions. Members of different signaling pathways that interact with the proline-rich Crk-binding region of C3G through their SH3 domain and implication in the regulation of cellular functions (Radha et al. 2011)**.**

4. FIBULINS

4.1 GENERALITIES

Fibulins are a family of extracellular matrix (ECM) secreted glycoproteins in the 50-200 KDa range. In 1989, Argraves described Fibulin-1 for the first time as a protein that interacts with the Fibronectin Receptor β (Argraves et al. 1989) and later its main characteristics were reported (Argraves et al. 1990). Since then, six more family members have been identified in mammals (de Vega et al. 2009).

The structure of fibulins is organized in three domains (figure 6), which have been implicated in mediating a number of protein-protein interactions. All fibulins share a globular C-terminal domain referred to as “fibulin-like” or “FC domain” (domain III).

Domain II represents the central portion and contains a variable number of EGF-like modules in a tandem array. Most of the EGF-like modules contain a consensus sequence for calcium binding and are known as calcium-binding EGF (cbEGF)-like modules. Domain I represents the N-terminus and its sequence vary among the family members (Obaya et al. 2012).

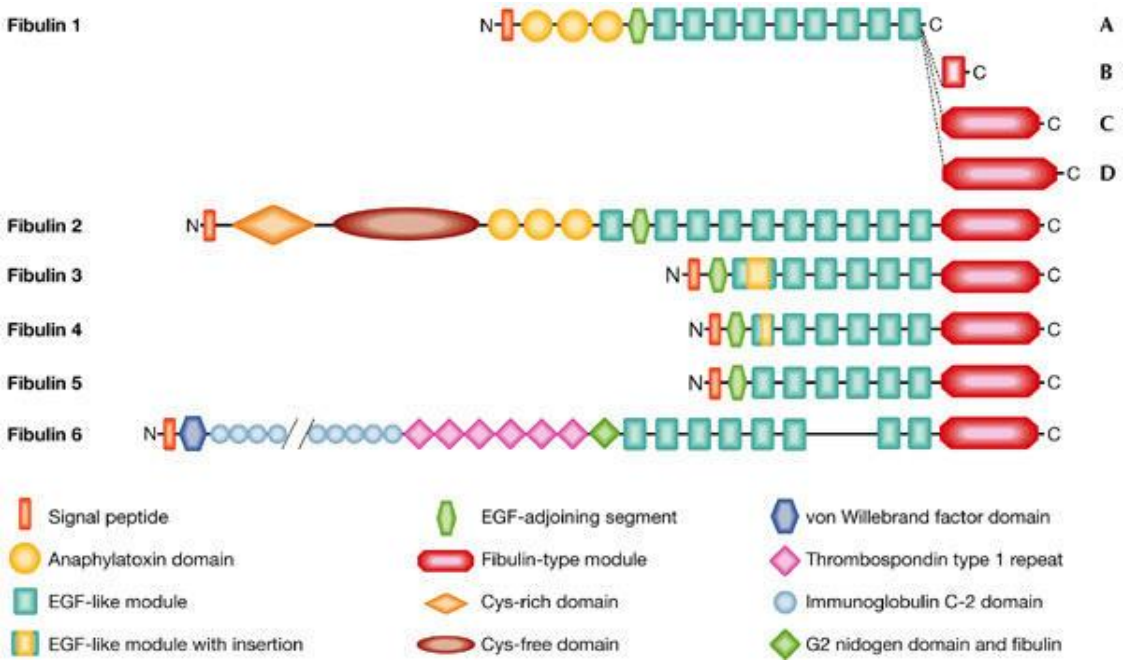

Figure 6-Fibulins structure. Scheme showing the different domains of fibulins: the "fibulin-like" C-terminal domain, the central domain containing EGF-like modules in tandem and the variable N-terminus domain. Alternative splice variants of Fibulin 1 (A, B, C and D). Only 9 of the 48 immunoglobulin domains in Fibulin 6 are present in the scheme (Argraves et al. 2003).

Fibulin family members have been classified into two subgroups according to their domains organization. The first subgroup includes Fibulin 1 (100kDa) and Fibulin 2 (195kDa), which are larger than the other members. They have an extra domain with three anaphylatoxin (AT) modules in domain I and more EGF-like modules in the central portion. The second subgroup includes the rest of the members, from Fibulin 3 to Fibulin 7. They are smaller proteins ranging 50-60 kDa, except Fibulin 6, which is the largest member of the family (615 kDa) due to the presence of 44 immunoglobulin C-2 domains in tandem and 6 thrombospondins type-I modules within domain I. Fibulin 7 has a unique N-terminal domain I, bearing a sushi domain, also known as complement control protein (CCP) domain (de Vega et al. 2009).

The different fibulins have a distinct tissue distribution; although some of them are expressed in the same places, being important their presence in blood vessels. The mechanisms that regulate fibulins expression are still poorly understood (Argraves et al. 2003). Evidence indicates that steroids regulate the expression of Fibulins 1, 2 and 3 (Ishibashi et al. 2002, Hayashi et al. 2003, Okada et al. 2003).

To study the functional relevance of fibulins, different mouse models deficient in a specific fibulin gene have been generated and show specific phenotypes. Mice lacking Fibulin 1 do not survive long after birth, as they undergo severe bleeding in muscle, skin and perineural tissue (Kostka et al. 2001). In contrast, mice deficient in Fibulin 2 are viable, fertile and show no apparent defects (Sicot et al. 2008). Fibulin 3 knock-out mice evidence alterations associated with premature aging and reduced lifespan, reproductive defects, loss of body mass and generalized atrophy in some tissues such as fat or muscle (McLaughlin et al. 2007). Fibulin 4 deficient mice exhibit lung and vascular disorders, dying perinatally (McLaughlin et al. 2006). Finally, mice lacking Fibulin 5 display highly disorganized elastic fibers (Yanagisawa et al. 2002).

Fibulins exhibit a wide number of protein-protein interactions, particularly, with other extracellular matrix proteins. Indeed, fibulins might act as intramolecular bridges within the ECM, connecting various supramolecular structures in order to mediate certain cell signaling events (Gallagher et al. 2005). Fibulins have been shown to modulate a number of cellular functions such as cell morphology, growth, adhesion and motility. The dysregulation of particular fibulins occurs in a number of human disorders. For example, Fibulin 2 is increased in streptozotocin-induced diabetic glomerulosclerosis (Wada et al. 2001) or Fibulin 5 is increased in elastase-induced emphysema in mice (Kuang et al. 2003). In addition, Fibulin 4 might have a role in response to sepsis (Heine et al. 1999). Furthermore, different fibulins have been found to have a function in cancer due to their role in the regulation of adhesion, migration, invasion or other processes. So far, different studies conclude that both tumor suppressive functions and oncogenic activities can be elicited by fibulins (Obaya et al. 2012).

Fibulin1 is down-regulated in colorectal cancer through promoter methylation (Xu et al. 2015), which is associated with poor prognosis (Zhu et al. 2015). Fibulin 5 is also downregulated in lung cancer, where it acts as a tumor suppressor inhibiting MMP7 (Yue et al. 2009) as well as in urothelial carcinoma (Hu et al. 2011) or hepatocellular carcinoma (Tu et al. 2014). It plays also an important role in the tumor microenviroment, inhibiting MMP9 production and invasion (Moller et al. 2011). Fibulin 5 can also act as a tumor promoter in gastric cancer (Shi et al. 2014) or pancreatic tumors (Schluterman et al. 2010). Moreover, Fibulin 5 has been involved in the regulation of TGFβ-induced EMT in cervical cancer metastasis (Xiao et al. 2013) and in mammary epithelial cells (Lee et al. 2008). Fibulin 3 expression is also associated with cancer, as it will be shown below.

4.2 FIBULIN 3

Fibulin 3, also known as S1-5 or EFEMP-1, was isolated by subtractive screening of a c-DNA library derived from mRNA of senescent human diploid fibroblasts (HDF) established from a patient with Werner syndrome of premature aging (Lecka-Czernik et al. 1995).

Fibulin 3 is expressed in bone and cartilage structures during development and is also present at high levels in retina and epithelial and endothelial cells throughout the body (de Vega et al. 2009). In adults, Fibulin 3 expression is prominent in some capillaries

and it is overexpressed in Werner syndrome and in senescent normal HDFs. Growth arrest in young normal cells also induces its expression, while high serum significantly decreases it (Lecka-Czernik et al. 1995). In addition to changes in Fibulin 3 levels occurring in some pathologies, the R345W mutation appears to cause age-related macular degeneration and is associated with Malattia Leventinese (Argraves et al. 2003, Marmorstein et al. 2007).

Concerning cancer, Fibulin 3 has both anti- and pro- oncogenic effects. Fibulin 3 is simultaneously overexpressed with MMP2, MMP9 and ADAMTS-5 in glioma cells promoting tumor invasion and survival (Hu et al. 2009). Fibulin 3 plays also a pro-angiogenic role in gliomas through the activation of Notch signaling (Nandhu et al. 2014). Moreover, Fibulin 3 has been associated with progression of pancreatic adenocarcinomas through interaction with epithelial growth factor (EGF) receptor, which leads to Akt activation (Camaj et al. 2009).

Nevertheless, many studies have described an anti-tumoral role of Fibulin 3. For example, in non-small cell lung cancer, Fibulin 3 down-regulates MMP2 and MMP7 and the subsequent decrease in its invasiveness (Kim et al. 2012). Down-regulation of Fibulin 3 also contributes to lung cancer invasion and metastasis through activation of Wnt/β-catenin pathway (Chen et al. 2014). Moreover, Fibulin 3 levels decrease in nasopharyngeal carcinomas, where it has the ability to suppress cell migration and invasion by decreasing Akt activity (Hwang et al. 2010). This Fibulin 3 down-regulation observed in different types of cancer, including lung cancer (Kim et al. 2012), hepatocellular carcinoma (Nomoto et al. 2010) or colorectal cancer (Tong et al. 2011), correlates with promoter methylation.

Fibulin 3 is also linked with the EMT process related to cancer progression. For instance, Fibulin 3 suppresses both EMT and self-renewal of lung cancer stem cells by modulating the IGF1R/PI3K/Akt/GSK3β pathway (Kim et al. 2014). During breast cancer progression, Fibulin-3 expression decreases, which impairs its inhibitory effect on TGFβ signaling in cancer and endothelial cells microenvironment, promoting EMT, migration, invasion and endothelial permeability (Tian et al. 2015).

5. CELL ADHESION, MIGRATION AND INVASION. GENERALITIES

Cell adhesion plays an important role during embryonic development and in several physiological and pathological processes in the adult, including cancer. It contributes to cellular organization in tissues, as well as to cell proliferation and survival.

Cells in tissues interact both with the extracellular matrix and neighboring cells. Rather than simply playing a passive role in maintaining attachment and resisting external forces, it is becoming increasingly evident that cell-extracellular matrix and cell-cell adhesions are also sites of transmission of active signals (Maruthamuthu et al. 2011).

Epithelial cell-cell adhesions are maintained by three basic structures connecting adjacent cells: tight junctions (zonula occludens), adherent junctions (zonula adherens) and desmosomes (macula adherens) (Le Bras et al. 2012).

Adherent junctions are made up by calcium-dependent homophilic binding of the extracellular cadherin domains, nectins and afadins. Adherent junctions are highly dynamic structures, which are remodeled by endocytosis and recycling of their components. Endocytic pathways are often misregulated in cancer. There are different endocytic pathways, non-clathrin and clathrin-mediated. The last one involves adaptor proteins such as Numb and Disabled-2 (Dab2) and participates in the regulation of members of adherens junctions during EMT (Le Bras et al. 2012).

Nascent adhesions can either turn over rapidly, in ~60 seconds, or mature to larger focal complexes (approximately 1 μm in diameter) that reside in the lamellipodium-lamellum interface and persist for several minutes. These complexes can continue to mature into larger focal adhesions that reside in large actin bundles or stress fibers (2 μm wide and 3-10 μm long). As traction forces move the cell forwards, focal adhesions assemble and disassemble. For productive advance of the cell body, contraction must be coupled to de-adhesion (Gardel et al. 2010, Parsons et al. 2010). These adhesions contain integrins, talin, vinculin, α-actinin, paxillin and FAK, among other proteins, and are enriched in phosphotyrosine because they form active signaling complexes (Parsons et al. 2010).

Cell migration plays a key role in the development and maintenance of multicellular organisms under physiological and pathological conditions such as metastasis in cancer. For example, migration is required for skin and intestine cell renewal, so that new epithelial cells migrate from the basal layer or the crypts, respectively, to the top layer. Likewise, migration plays an essential role in tissue repair and immune response (Ridley et al. 2003).

Under pathological conditions, migration is also involved in several diseases, like vascular diseases, osteoporosis, chronic inflammatory diseases or cancer, where it leads to invasion and the generation of metastasis (Franz et al. 2002, Ridley et al. 2003). Metastasis is a complex process, encompassing local infiltration of tumor cells from the initial tumor into adjacent tissues, intravasation (transendothelial migration of cancer cells) into vessels, survival in the circulatory system and extravasation, leading to colonization and formation of the secondary tumor (van Zijl et al. 2011).

There are different types of cell migration. Cells can migrate as individual cells by a mesenchymal movement, which involves directional migration with proteolysis at the leading edge or by an amoeboid movement with lack of direction and polarity (Le Bras et al. 2012). In addition, they can migrate in solid cell strand, sheets, files or clusters, which is called “collective” migration (Friedl and Wolf 2003, Rorth 2009).

Cell migration results from a continuous cycle of coordinated and interdependent steps that involves the cytoskeletal machinery (Friedl and Brocker 2000). Movement of the cell body is dependent on contractility generated by actin and myosin II filaments. Migrating cells are polarized, and at the front, extensions (filopodia) of lamellipodia are driven by dynamic polymerization of actin (Franz et al. 2002).

The actin polymerization in the lamellipodium is regulated by the Rho GTPases, Rac and Cdc42 (Parsons et al. 2010). Maturation of focal complexes into focal adhesions involves activation of Rho, perhaps through the recruitment of a Rho GEF to adhesions, and Rho might inhibit Rac (Sanz-Morenoet al. 2008, Tomar and Schlaepfer 2009). Rho activity promotes the assembly of contractile actomyosin structures that regulate traction and tension-mediated changes (Gardel et al. 2010). In addition to Rho GTPases, other molecules such as FAK, Src, paxillin, talin or p130Cas are involved in the regulation of cell protrusion, adhesion and contraction, controlling the interplay between the F-actin cytoskeleton and integrin-mediated adhesion (Gardel et al. 2010).

5.1 EXTRACELLULAR MATRIX AND MATRIX METALLOPROTEASES (MMPs)

Cell migration *in vivo* is dependent on the modification and degradation of extracellular matrix components by the actions of secreted proteases such as MMPs (Sternlicht and Werb 2001). The ECM is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents, but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis. It mediates protection by a buffering action and water retention and directs essential morphological organization and responses (Frantz et al. 2010). In cancer, ECM also determines the preferred metastatic organs. For example, based on the deposition of fibronectin or the expression of tissue inhibitors of metalloproteinases (TIMPs) (Tse and Kalluri 2007).

ECM is composed of a large collection of biochemically distinct components including collagens, laminins, fibronectin, elastin, fibrillins, tenascin, nidogen, entactin, fibulins, fibrinogen, thrombospondins, proteoglycans or hyaluronans. Different isoforms and/or family members of these components can be present in different tissues. ECM provides mechanical support for cells through its binding to integrins (Gout and Huot 2008, Halper and Kjaer 2014). The basal lamina, a specialized sheet that underlies epithelial cell sheets and tubes, is a particular type of ECM that is produced through cooperation of epithelial and stromal cells and plays a significant role in cancer (Gout and Huot 2008).

During cell migration and invasion, ECM is degraded preparing the path for cells to migrate, invade and spread to distant areas. Matrix endopeptidases play a crucial role in this process.

Metzincins are a heterogeneous superfamily of Zinc proteolytic proteins of the extracellular matrix. They are characterized by a consensus motif containing three histidines that bind zinc at the catalytic site, and a conserved HEXXHXXGXXH motif within the active site (Bergers and Coussens 2000, Gardel et al. 2010). The C-terminus domain variants are related to their location and their function within the cells and tissues (Bergers and Coussens 2000). Metzincins can be classified into different families on the basis of structural similarity. Among them, MMPs that were the first known zinc-dependent endopeptidases family, ADAMs (α-disintegrin and metalloproteinase) and ADAMTS (α-disintergin and metalloproteinase with thrombospondin motifs) families, have been shown to contribute to tumor progression

(Bergers and Coussens 2000, Gardel et al. 2010). MMPs are zinc-dependent endopeptidases which primary function is tissue remodeling by selective proteolytic degradation (Bourboulia and Stetler-Stevenson 2010). There are 23 human MMPs (24 in mouse), which differ from each other in their structural domain architecture, substrate specificity and their temporal and tissue specific expression patterns (Radisky and Radisky 2010). It is becoming increasingly clear that MMPs do not simply degrade the matrix, but also generate new ligands (Franz et al. 2002).

MMPs have distinct and overlapping substrate specificities and together they possess enzymatic activity against, virtually, all ECM components. Their common characteristics are: dependence on zinc and calcium ions and synthesis as inactive zymogens (or proMMPs) that are activated by limited proteolysis. They are either secreted or expressed as transmembrane proteins, inhibited by tissue inhibitors of metalloproteases (TIMPs) and transcriptionally responsive to cytokines, growth factors, hormones and ECM-derived signals (Gardel et al. 2010).

MMPs can be classified by their cellular localization (17 soluble, secreted enzymes and 6 membrane-associated enzymes), or their substrate specificity: collagenases (MMP1, 8 and 13), gelatinases (MMP2 and 9), and MMPs that degrade a broad spectrum of ECM proteins called stromelysins (MMP3, 10 and 11) or matrilysins (MMP7). Nowadays, as the MMP family grew, a numbering system was adopted and MMPs are now grouped according to their domain structure (Radisky and Radisky 2010, Tallant et al. 2010).

MMPs are constituted by a modular combination of inserts and domains, as indicated in figure 7. This includes, from N- to C-terminus: a signal peptide for secretion, a zymogenic pro-peptide, a zinc- and calcium-dependent catalytic domain, a linker region and a hemopexin-like domain for collagen binding, pro-MMP activation and dimerization (Tallant et al. 2010, Kessenbrock et al. 2011). Most MMPs also possess additional accessory domains that modulate catalytic activity, substrate recognition and cellular localization, which are unique for a number of MMP members (Radisky and Radisky 2010).

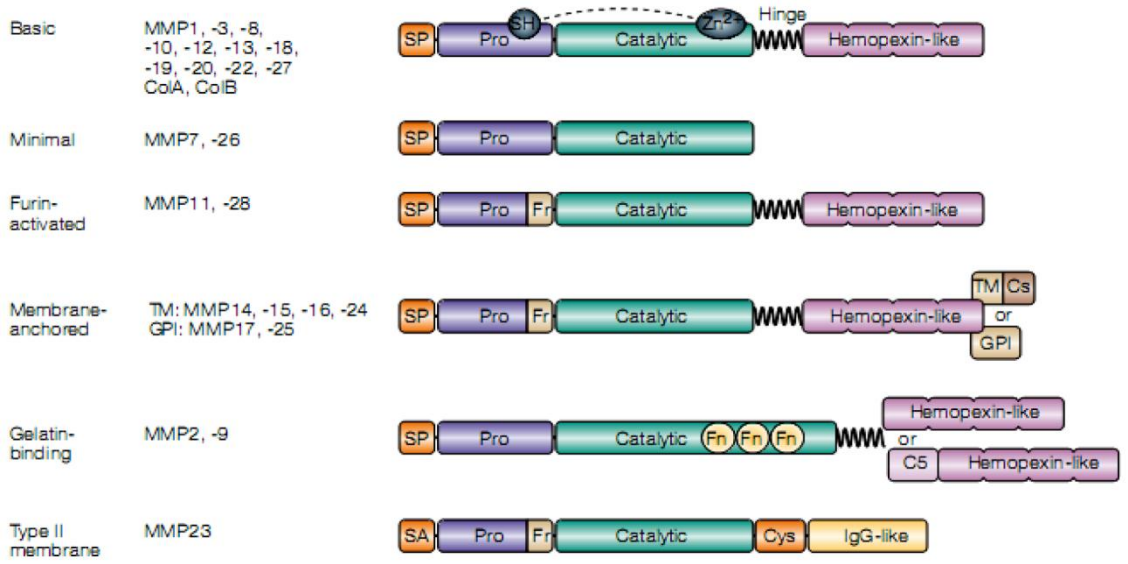

Figure 7-Domain structure of the mammalian MMP family. MMPs can be divided in secreted or anchored in *cis* to the cell surface and those containing a furin-recognition motif (9 MMPs, including all the membrane anchored enzymes) or not (Parks et al. 2004).

Several MMPs are not constitutively transcribed, but are expressed after external induction by cytokines and growth factors (Tallant et al. 2010). They are expressed as inactive pro-enzyme, due to the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site, and disruption of this interaction by a mechanism called cysteine switch, make enzymes proteolytically active (Sternlicht and Werb 2001).

The regulation of MMPs is mainly due to modulation of gene expression, compartmentalization and inhibition of its activity by the endogenous inhibitors, TIMPs. There are four TIMP family members (TIMP-1, -2,-3 and -4) that can inhibit all active MMPs, but not with the same efficiency (Bourboulia and Stetler-Stevenson 2010). MMPs are implicated in a variety of physiological processes, including wound healing, uterine involution and organogenesis, as well as in pathological conditions, such as inflammatory, vascular and auto-immune disorders and carcinogenesis (Gialeli et al. 2011).

During tumor progression, the proteolytic activity of MMPs is required for cancer cells to degrade physical barriers during local expansion and for intravasation at nearby blood vessels, extravasation and invasion at a distant location. Expression of MMPs in the tumor microenvironment is produced by cancer cells, but also by the neighboring stromal cells induced by the cancer cells in a paracrine manner. For example, fibroblasts and macrophages produce matrix-metalloproteases (MMPs) in many tumors, which allows ECM degradation, promoting cancer cell motility and invasion (Wagner and Nebreda 2009, Gialeli et al. 2011).

Increased MMP2 and MMP9 expression and activity are associated with tumor progression of breast, urogenital, brain, lung, skin and colorectal cancer, as well as in other malignancies (Klein and Bischoff 2011). Particularly, in colorectal cancer, several studies have shown that MMP2 and 9 play a role and could be used as prognosis markers and therapeutical targets.

5.2 ROLE OF p38 MAPKs IN ADHESION, MIGRATION AND INVASION

p38 MAPKs play an important role in cell adhesion, migration and invasion, all of them processes involved in tumor progression and metastasis (Wagner and Nebreda 2009, del Barco Barrantes and Nebreda 2012).

p38α negatively regulates cell adhesion in embryonic stem cells (Guo and Yang 2006) and embryonic cardiomyocyte-derived cell lines (Zuluaga et al. 2007) through different mechanisms.

p38α MAPK mediates cell migration in HeLa cells and MEFs (Rousseau et al. 2006) through the regulation of the actin cytoskeleton by MK2. The activation of p38α can also induce cell migration and cytoskeletal remodeling in tumor cells by increasing the phosphorylation of Hsp27 (heat-shock protein 27) (Laferriere et al. 2001). In HGF/Met-triggered cortical neurons, Rac1/p38 cascade is also important for migration (Segarra et al. 2006). In colorectal cells, E-selectin binds and activates death receptor-3, triggering p38 and ERKs activation, which enhances transendotelial migration, survival and the generation of metastasis (Gout et al. 2006). Accordingly, p38 MAPKs play a key role in invasion. For example, in melanoma cells (Estrada et al. 2009) or ovarian cancer cells, p38 MAPK together with EGF and HGF, promote cell migration and invasion through regulation of MMP9 (Zhou et al. 2007). p38α pathway also increases MMP9 activity in breast cancer cells and promotes migration (Suarez-Cuervo et al. 2004). Moreover, p38α induces the expression of MMP1, MMP2, MMP9 and MMP13 in bladder, breast, liver, skin, keratinocytes and prostate cancer (del Barco Barrantes and Nebreda 2012). In head and neck carcinoma, p38α and p38δ promote head and neck squamous cell carcinoma invasion, through the induction of MMP1 and MMP13 expression (Junttila et al. 2007). In hepatocellular carcinoma, head and neck squamous carcinoma, pancreatic or glioma cell lines, p38α also promotes invasion of tumor cells (Wagner and Nebreda 2009, del Barco Barrantes and Nebreda 2012).

p38α plays a central role in the regulation of disseminated tumor cells (DTCs) fate at secondary organs (Bragado et al. 2013), where p38α would be activated, leading to tumor cell dormancy. This allows DCTs to keep in a quiescent state until the microenvironment is favorable for proliferation (Sosa et al. 2014).

p38γ also regulates invasion and metastasis acting as an activator and cofactor for c-Jun-induced MMP9 expression (Loesch et al. 2010).

Related to the invasion process, inhibition of p38 MAPK pathway blocks TGFβ-induced EMT and migration of non-tumor and tumor mammary epithelial cells (Bakin et al. 2002). Moreover, activation of the TGFβ–TRAF6–p38 MAPK pathway promotes c-Jun-

mediated Snail1 expression, which enhances the migratory and invasive capacity of human prostate cancer cells (Thakur et al. 2014). The role of p38 MAPK in the EMT process will be further explained later.

To sum up, p38 MAPKs contribute to the different steps of the metastatic process: epithelial-mesenchymal transition (EMT), migration/invasion, anoikis resistance, extravasation, organ colonization and metastatic dormancy (del Barco Barrantes and Nebreda 2012).

5.3 ROLE OF C3G IN ADHESION, MIGRATION AND INVASION

C3G plays an important role as a mediator of cell adhesion and spreading activated by integrins. As a consequence, C3G deficiency in mouse embryonic fibroblasts (MEFs) impairs cell adhesion and delays cell spreading, while it enhances cell migration (Ohba et al. 2001, Voss et al. 2003). This function of C3G is partially mediated by its main target, Rap1 (Ohba et al. 2001, Kooistra et al. 2007, Huang et al. 2008, Pannekoek et al. 2009). However, C3G, when overexpressed can also promote c-Abl-induced filopodia formation during cell spreading through mechanisms independent of its catalytic activity (Radha et al. 2007). In mammal cells, C3G and/or Rap1, as well as other proteins such as Crk or p130Cas, form multiprotein complexes during integrin-mediated focal adhesion (Bos 2005). So that, Rap1 and its GEFs and GAPs might be responsible for the spatial and temporal regulation of actin dynamics at sites of cell adhesion through their localization and interactions with other molecules inside the cell, like cadherins (Bos 2005). Moreover, Dr. Guerrero´s group in collaboration with our group has recently found that C3G and p38α act through a common pathway promoting cell adhesion in K562 chronic myeloid leukemia cell line, where both proteins interact forming a complex. However, C3G and p38α also display antagonistic roles in the regulation of the expression of some focal adhesion (FA) proteins in these cells. Thus, whereas C3G silencing inhibits their expression, p38α knock-down leads to an increase of some of these proteins (Maia et al. 2013).

As previously mentioned, C3G regulates cell migration, but its particular function can vary depending on the cellular context. C3G deficient MEFs present an enhanced migration (Ohba et al. 2001), while in C3G mutant mice sympathetic preganglionic neurons migration is altered (Yip et al. 2012) and mouse embryos lacking C3G also exhibit a migration defect in cortical neurons (Voss et al. 2008). On the other hand, C3G overexpression results in opposite outcomes in different cell types, so it increases cell migration in glomerular epithelial cells in glomerulonephritis (Rufanova et al. 2009), but decreases cell migration in highly invasive breast carcinoma cells (Dayma and Radha 2011).

C3G also regulates cell-cell interactions, where E-cadherin is important (Kooistra et al. 2007, Pannekoek et al. 2009). C3G binds intracellular E-cadherin to activate Rap1 and induces E-cadherin translocation (Pannekoek et al. 2009). Moreover, because C3G competes with β-catenin in the binding to E-cadherin (Kooistra et al. 2007), β-catenin and E-cadherin functions can be additionally modulated by C3G, which might be also important to regulate cell migration.

Recent data also suggest that C3G, acting through Rap1, would promote invasion of epithelial ovarian cancer cells through increasing MMP2 and 9 secretion (Che et al. 2015).

6. EPITHELIAL-MESENCHYMALTRANSITION

Epithelial-mesenchymal transition (EMT) together with its reverse process, mesenchymal-epithelial transition (MET), allows the dissemination of cells from solid tumors and the colonization of distant sites (Pereira et al. 2015). In the metastatic process, cells from the primary tumor that suffer an EMT process, access to the circulatory system and go to distant sites. To form a secondary solid tumor, cells extravasation requires recognition and adhesion to endothelial cells and matrix degradation to invade the secondary tissue (Tse and Kalluri 2007). Once finished colonization, cells are thought to redifferentiate via MET and regain the organization of cells present in the primary tumor (Pereira et al. 2015).

EMT is a process that allows epithelial cells to acquire a fibroblastoid, motile and invasive phenotype. This involves a loss of polarity, downregulation of epithelial-specific proteins (for example, adherens and tight-junction proteins like E-cadherin and zona occludens 1 (ZO-1), respectively) and expression of mesenchymal proteins (for example, N-cadherin or vimentin) and MMPs (figure 8). Moreover, EMT may invoke the expression of stemness markers (Zeisberg and Neilson 2009). EMT is important during embryogenesis, organogenesis and for tissue repair during adult life. EMT is also induced in carcinoma cells undergoing a phenotypic conversion to make them invasive (Grunert et al. 2003).

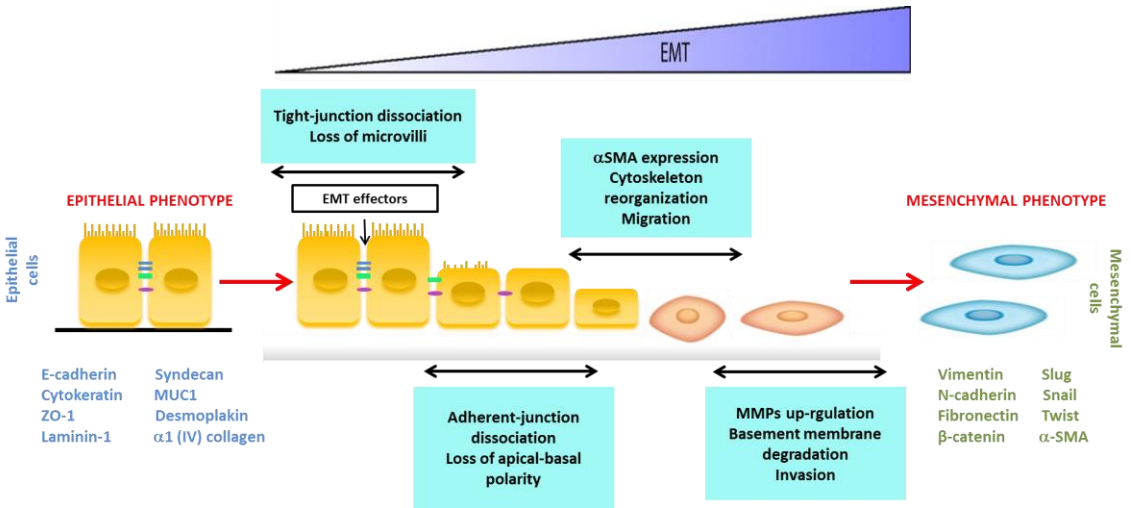

Figure 8-Schematic representation of EMT process steps. EMT involves a functional transition of polarized epithelial cells into mobile mesenchymal cells. Commonly used epithelial and mesenchymal cell markers are listed. Co-localization of these two sets of distinct markers defines an intermediate phenotype.

Several markers change their expression and/or localization during the EMT process. Some of them are listed in table 1. The switch from E-cadherin to N-cadherin expression is used as a marker of EMT. E-cadherin is the main responsible for adherent junctions between cells, forming a “molecular zipper”. It binds to either β-catenin or γ-catenin and subsequently to α-catenin in the cytoplasm, forming a complex with cellular cytoskeleton microfilament network (Tsanou et al. 2008). In the cytoplasm, β-catenin stabilizes cell junctions, meanwhile in the nucleus it activates the transcriptional activity of LEF-1/TCFs, which are required to induce EMT, mainly by increasing Snail1 expression (Zeisberg and Neilson 2009).

Loss of E-cadherin-mediated adhesion may act as a promoter of tumor cell detachment from the primary site, concurrent invasion of adjacent normal tissue and dissemination to distant places (Tse and Kalluri 2007).

The zona occludens proteins (ZO-1, ZO-2 and ZO-3) are tight junction associated proteins that belong to the family of membrane-associated guanlyate kinase (MAGUK) homologs. They have three PDZ (postsynaptic density/disc-large/ZO-1) domains, an SH3 domain, a GK (guanylate kinase) domain, an acidic domain and an actin binding region (Gonzalez-Mariscal et al. 2000). ZO-1 is a junctional adaptor protein that interacts with other tight junction components, such as proteins from JAM (junctional adhesion molecule) family and F-actin (Bazzoni et al. 2000, Fanning and Anderson 2009, Fanning et al. 2012). It regulates cadherin-mediated intercellular junctions and F-actin cytoskeletal distribution, as well as endothelial cell migration and angiogenic potential, so that, cells lacking ZO-1 show a reduced migration (Tornavaca et al. 2015). ZO-1 knock-out mice are embryonic lethal, due to its requirement for normal blood vessel formation in the yolk sac (Katsuno et al. 2008). Accordingly, ZO-1 depletion reduces endothelial sprouting (Tornavaca et al. 2015).

Snail1/2 transcription factors are common downstream targets of signaling pathways regulating EMT. Snail not only suppresses E-cadherin expression, but it also increases that of mesenchymal markers such as fibronectin and vitronectin and decreases the expression of other epithelial markers like claudins, occludins and cytokeratins, apart from other effects (de Herreros et al. 2010). Twist is another transcription factor involved in EMT induction. It is a basic helix-loop-helix protein that is upregulated during early embryonic morphogenesis, tissue fibrosis and cancer metastasis. In metastasis, Twist can act independently of Snail to downregulate E-cadherin and upregulate Fibronectin and N-cadherin (Zeisberg and Neilson 2009). The ZEB family of transcription factors have an essential role regulating signaling mechanisms of the epithelial-mesenchymal transition during cancer progression. They are involved in the maintenance of cancer cells stemness and their expression is linked with increased aggressiveness, metastasis and angiogenesis in mice models and primary human tumors (Sanchez-Tillo et al. 2011). For instance, ectopic expression of ZEB proteins in mammary epithelial cells is sufficient to induce the dissociation of adherens junctions, presumably through suppressing the expression of genes encoding Plakophilin-2 and ZO-3 (Eger et al. 2005). Moreover, ZEB transcriptional factors also increase the expression of genes encoding matrix metalloproteases (Miyoshi et al. 2004).

Different micro-RNAs are up- or down-regulated in tumor processes, existing a double-negative feedback loop between mi-RNAs and the transcription factors that drive EMT. Different micro-RNAs control E-cadherin or Vimentin expression, as well as that of transcription factors regulating EMT. These include Snail, which is regulated by the miR-30 and miR-34 family and ZEB 1 and 2 by the miR-200 family. Other genes targeted by mi-RNAs are those from cytoskeletal components, Wnt pathway and stemness (Findlayet al. 2014).

Acquired or maintained markers	Attenuated markers
Cell-surface proteins	
N-cadherin α5β1 integrin αVβ6 integrin Syndecan-1	E-cadherin ZO-1
Cytoskeletal markers	
FSP1 α-SMA Vimentin β-Catenin	Cytokeratins 8/18/19
ECM proteins	
α1(I) collagen α1(III) collagen Fibronectin Laminin 5 MMP2 MMP9	α1(IV) collagen Laminin 1 Desmoplakin
Growth Factors	
TGFβ FGF-1,-2,-8	
Transcription factors	
Snail 1 (Snail) Snail 2 (Slug) ZEB 1/2 Twist FOXC2	
MicroRNAs	
miR10b miR-21	miR-200 family

Table 1-Main markers of cells undergoing EMT.

Although TGFβ is an important suppressor of tumorigenesis due to its role inducing apoptosis and cell cycle arrest in the initial stages, it is an important EMT inducer, enhancing tumor progression and metastasis (Tse and Kalluri 2007). Smad-independent signaling pathways activated by TGFβ such as RhoA, p38 MAPK and PI3K/Akt pathways are involved in EMT induction (Boyer et al. 1999, Bhowmick et al. 2001, Strutz et al. 2002, Yu et al. 2002). p38 MAPK pathway also collaborates with Smad 2/3 in the regulation of TGFβ1-dependent expression of fibronectin and collagen I in pulmonary epithelial cells and its inhibition blocks twist expression (Kolosova et al. 2011). Moreover, p38 MAPK, together with Smad, NF-κB and ZEB 2 mediate N-cadherin up-regulation induced by TGFβ1 in combination with TWEAK (Itoigawa et al. 2015). In contrast, other data indicate that p38α maintains E-cadherin expression in

human primary mesothelial cells by suppressing TAK1–NFκB signaling, which impairs EMT (Strippoli et al. 2010). Other studies support a role for p38 MAPK in the regulation of EMT in breast cancer cells through the control of the expression of miRNAs, Twist, Snail, Slug and ZEB 2 (Antoon et al. 2013).

In colorectal cancer, Wnt/*β*-catenin signaling pathway and the switch from E- to N-cadherin neo-expression are considered the major inducers of EMT and metastatic progression (Cavallaro et al. 2002, Thiery 2002, Rosivatz et al. 2004, Pancione et al. 2012). Wnt pathway is activated in stem-like tumors and cell lines, which co-expressed markers of intestinal and colorectal stem cells and EMT genes (Merlos-Suarez et al. 2011). Moreover, Snail is often amplified in colon cancer (Hidaka et al. 2000, Nakao et al. 2001). Although TGF*β*1 can induce EMT in colon cancer cell lines bearing a wild type TGFβR2 (Pancione et al. 2012), most colorectal cancer cells appear to be resistant to the induction of EMT by TGFβ (Calon et al. 2015). However, the presence of the EMT signature in these tumors associates with a worse prognosis and less sensitivity to conventional chemotherapy (Pereira et al. 2015).

7. STROMAL REGULATION IN CANCER

Cancer cells interact with the host stromal microenvironment, which contributes to support tumor growth and to induce metastasis. The stroma is formed by the extracellular matrix and cellular components including fibroblasts (myofibroblasts), glial, epithelial, fat, immune, vascular, smooth muscle and immune cells (Zeisberg et al. 2007, Gout and Huot 2008).

Cells from the stroma influence the initiation and promotion of cancer by secreting cytokines and growth factors and/or by expressing their receptors, so that, contributing to stimulate tumor cell growth and recruitment of precursor cells. At the same time, cancer cells modulate the stroma signaling (Li et al. 2007, Zeisberg et al. 2007).

Tumor cells recruit and activate fibroblasts through various mechanisms, becoming myofibroblasts or cancer associated fibroblasts (CAFs) (Condeelis and Pollard 2006, Gout and Huot 2008). Several cytokines and growth factors, including TGFβ, PDGF, IL-4 and IGF-2, have been reported to induce this myofibroblastic process. Other required signals for this process are reactive oxygen species (ROS), matrix proteins and mechanical stress arising from integrin-dependent cell interactions (Conti and Thomas 2011). CAFs are perpetually activated and express α-SMA. CAFs can themselves initiate cancer in response to HGF or TGFβ and they can contribute to proliferation and progression of cancer cells through the production of growth factors, chemotactic factors, angiogenic factors and MMPs. Additionally, these signals also lead to the transformation of endothelial cells into CAFs (Condeelis and Pollard 2006).

Other types of cells recruited to the primary tumor are tumor-associated macrophages that came from monocytes recruited into tumors in response to cancer-associated inflammation. They produce VEGF, HGF, MMP2 or IL-8 (Barbera-Guillem et al. 2002).

Many tumors gain the ability to produce TGFβ, to stimulate tumor growth and invasion in an autocrine way, promoting angiogenesis and avoiding the immune system (Siegel and Massague 2003, Li et al. 2007). In response to TGFβ, CAFs proliferate and secrete cytokines, which will have further effects in tumors. In colorectal cancer, mesenchymal stem cells (MSC) that integrate into the tumor stroma have been reported to induce an EMT process of these cancer cells, which is mediated by TGFβ (Mele et al. 2014). TGFβ in colorectal cancer cell lines enhances cell growth (Sun et al. 2013). In addition, TGFβ produced by stroma increases the frequency of tumor-initiating cells and increases the efficiency of organ colonization during the initial phase of metastasis (Calon et al. 2012, Calon et al. 2015).

The chemokine stroma-derived factor (SDF-1/CXCL12) is constitutively produced by mesenchymal and marrow-derived stromal cells. In cancer, SDF-1 acting through CXCR4 receptor promotes tumor growth, angiogenesis, tumor metastasis and contributes to the immunosuppressive response (Li et al. 2007, Kim 2012, Hu et al. 2014). The SDF-1/CXCR4 pathway is also involved in colorectal cancer progression, inducing invasion and EMT through activation of the Wnt/β-catenin signaling pathway (Hu et al. 2014). Stromal cells are positive for SDF-1 and cancer cells also have a strong expression of SDF-1 and its receptor CXCR4 (Kim 2012).

Stromal fibroblasts, macrophages, and epithelial cells secrete MMPs that hydrolyze the extracellular matrix proteins of the surrounding tissue. For example, fibroblasts can produce MMP2 and macrophages MMP9. MMPs inhibitors, such as TIMP-1 can be also produced by stromal cells (Li et al. 2007).

8. EPIGENETIC REGULATION AND DNA-METHYLASES

8.1 EPIGENETIC: DNA METHYLATION

The term epigenetic has been defined as the inheritance of changes in gene function without any change in the DNA nucleotide sequence (Hammoud et al. 2013). Epigenetic regulation controls gene expression in response to environmental changes and developmental status (Golbabapour et al. 2011).

Epigenetic modifications comprise DNA methylation, post-translational histone modifications and nucleosome positioning, chromatin remodeling and post-transcriptional modification of certain non-coding RNAs, among other mechanisms (Portela and Esteller 2010, Tost 2010). Epigenetic regulation is considered a key mechanism to maintain normal development and tissue homeostasis, so abnormalities in this regulation have been detected in different pathologies, being remarkable those found in cancer (Tost 2010).

The most widely studied epigenetic modification in human is cytosine methylation. DNA methylation occurs in the 5 position of the pyrimidine ring of cytosines in the context of the dinucleotide sequence CpG, clustered in CpG islands. However, DNA methylation does not occur exclusively at CpG islands (Portela and Esteller 2010). In general, CpG-island methylation is associated with gene silencing. DNA methylation can inhibit gene

expression by different mechanisms. First, DNA methylation inhibits the association of the transcriptional machinery to promoter regions (figure 9). In addition, methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins, leading to the formation of histone modifying and chromatin-remodeling complexes (Bird and Wolffe 1999, Portela and Esteller 2010). In fact, chromatin assembled on methylated DNA associates with hypoacetylated histones and becomes transcriptionally silent (Bird and Wolffe 1999, Esteller 2002).

In several types of cancer, hypermethylation of CpG islands in promoter regions of tumor suppressor genes has been described and it is considered an important mechanism for inactivation of these genes.

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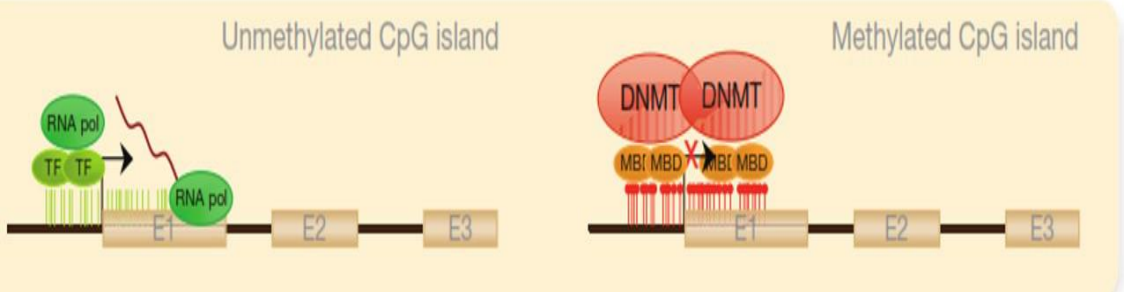

Figure 9-DNA methylation of CpG islands at promoters impairs transcriptional activation. DNA methylation can occur in different regions of the genome. Methylation of regulatory regions of the genome impairs transcription (Portela and Esteller 2010).

8.2 DNA METHYLTRANSFERASES (DNMTs)

DNA methylation is mediated by the DNMT family of enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine to CpG (Robertson 2001). In mammals, five members of the DNMT family have been reported so far: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. DNMT can be classified into de novo DNMTs (DNMT3A and DNMT3B) and maintenance DNMTs (DNMT1) (Golbabapour et al. 2011).

DNMT1 methylates both imprints and non-imprints genes (Golbabapour et al. 2011). DNMT1 has a preference toward hemimethylated DNA and is the most abundant methyltransferase in somatic cells. It is responsible for maintaining the methylation patterns following DNA replication (Robertson 2001). DNMT3 functions as a novo methyltransferase, with the same preference for unmethylated and hemimethylated DNA, and consists of two related proteins encoded by distinct genes, dnmt3a and dnmt3b. DNMT3A and DNMT3B are thought to be responsible for establishing the pattern of methylation in germ cells during embryonic development (Portela and Esteller 2010). DNMT2 and DNMT3L do not show methyltransferase activity. DNMT2 seems to play a regulatory role in DNA methylation and it might methylate RNA instead

of DNA, but this remains unclear (Newell-Price et al. 2000, Golbabapour et al. 2011, Subramaniam et al. 2014).

As outlined in figure 10, DNMTs are comprised of two regions: the conserved C-terminal catalytic domain with 500 amino acids and the N-terminal region with a large multi-domain of variable size, which has regulatory functions. The catalytic domain shares a common structure called “Ado-Met dependent methyltransferase”, involved in cofactor binding and catalysis (Newell-Price et al. 2000).

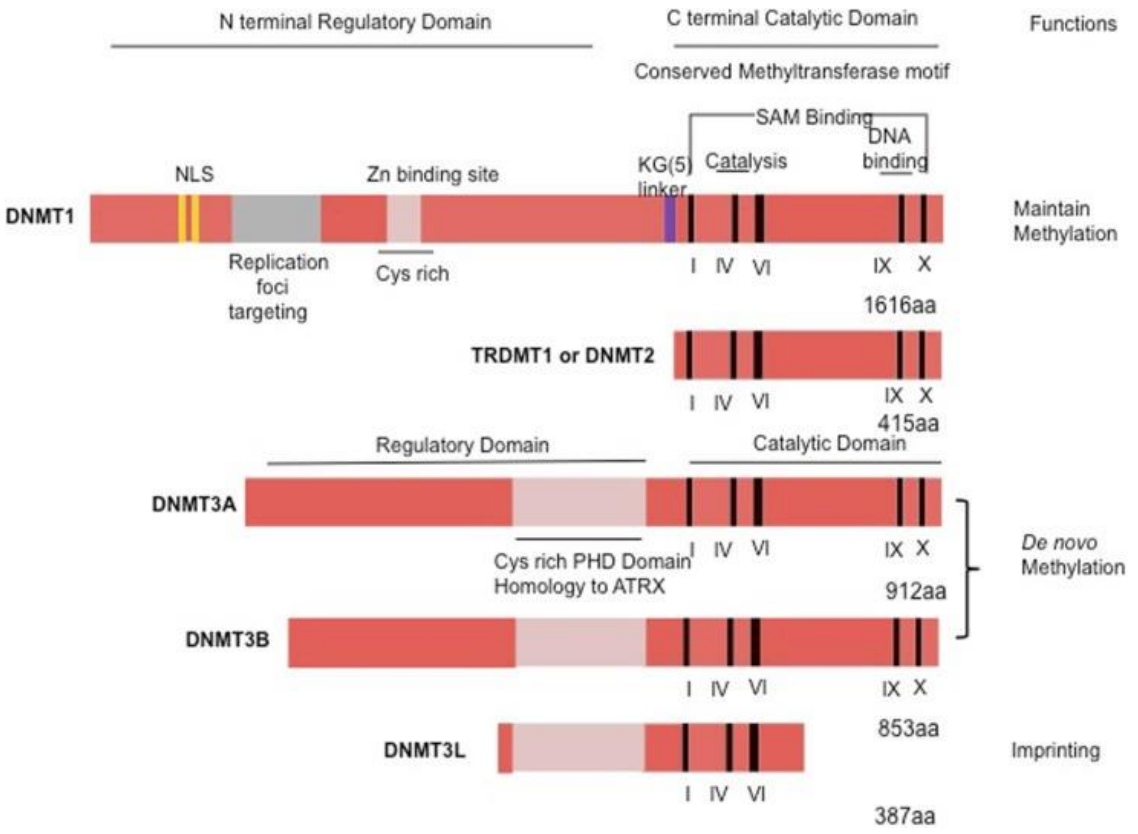

Figure 10-Schematic representation of the structure of human DNMTs. DNMT1, DNMT2, DNMT3A, 3B and 3L structure is shown. The N-terminal region contains motifs of interaction with proteins or DNA and the C-terminal contains the conserved methyltransferases domains. PHD means plant homology domain (Subramaniam et al. 2014).

DNMT3 (A and B) levels can be regulated, being of relevance its post-transcriptional regulation (Denis et al. 2011). It has been described that HuR protein, an RNA-binding protein that stabilizes and/or modulates the translation of target mRNAs, binds to the 3´UTR of dnmt3b mRNA at an HuR-recognition motif. This interaction stabilizes dnmt3b mRNA, leading to an increase in DNMT3B protein levels in colorectal RKO cells (Lopez de Silanes et al. 2009).

Human DNMTs levels, specially, those of DNMT3A and 3B, are often increased in several cancers such as colon, prostate, breast, liver and leukemia. In colon cancer, DNMT1 and DNMT3 modulate different histone modification complexes (Newell-Price et al. 2000) and there has been found several hypermethylated gene promoters such as *MLH1*, retinoblastoma, p16, rarrb, and sfrp promoters (Nosho et al. 2009). dnmt3b

expression in colorectal cancer is significantly associated with the CpG island methylator phenotype pathway (Nosho et al. 2009). Moreover, DNMT3B activation has been implicated in aberrant de novo methylation of CpG islands in this type of cancer (Okano et al. 1999, Kanai and Hirohashi 2007, Linhart et al. 2007), being also capable of inducing colon tumors in mice (Linhart et al. 2007).

GROUP BACKGROUND

1. FUNCTION OF p38α PATHWAY IN CELL DEATH. CROSSTALK WITH C3G

p38 MAPKs can act mediating cell death or survival depending on different factors, such as the cell type, the stimulus and the p38 isoform. Some years ago, there were many studies indicating that p38 MAPKs promoted cell death, although a few suggested that p38 MAPKs could also play a pro-survival role (Nebreda and Porras 2000). However, the precise function of p38 MAPKs regulating cell death was unclear.

During the last years, our group focused their studies on the characterization of the function of p38α MAPK pathway in the regulation of cell death and its functional interaction with other pathways using embryonic cardiomyocytes and MEFs-derived cell lines from p38α deficient mice. Data from those studies have demonstrated that p38α plays a relevant role as a mediator of apoptosis in response to several stimuli through both, the up-regulation of some pro-apoptotic proteins (Porras et al. 2004) and the down-regulation of the activity of the survival pathways, ERKs (Porras et al. 2004) and Akt (Zuluaga et al. 2007).

In collaboration with Dr. Carmen Guerrero's group (Centro de Investigación del Cáncer de Salamanca), we also studied the potential crosstalk between p38α and C3G pathways. We have found a new functional relationship between C3G and p38α MAPK regulating cell death in MEFs (Gutierrez-Uzquiza et al. 2010) and in the K562 chronic myeloid leukemia (CML) cell line (Maia et al. 2009). In both cell models C3G regulates apoptosis through down-regulation of p38α activity. In K562 CML cells, C3G silencing enhances STI-571-induced apoptosis through the up-regulation of p38α activity by a mechanism dependent on Rap1 (Maia et al. 2009). In MEFs, C3G knock-down also through the up-regulation of p38α activity either promotes cell survival in response to oxidative stress or cell death upon serum-deprivation, but through a Rap1 independent mechanism as shown in figure 11 (Gutierrez-Uzquiza et al. 2010).

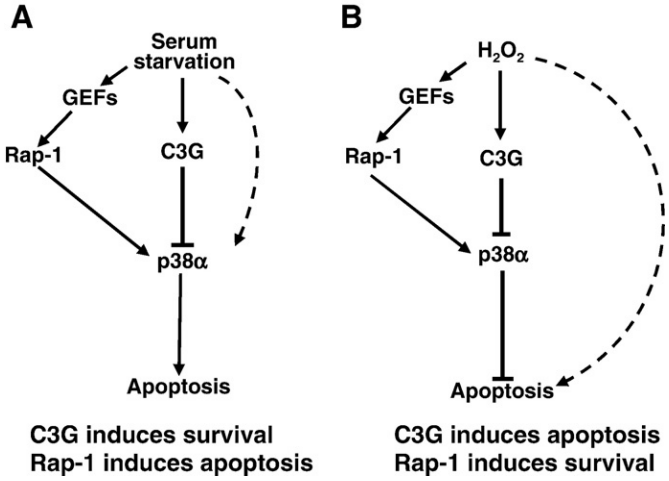

Figure 11-Regulation of apoptosis by C3G and Rap1 through p38α MAPK. Model showing the regulation of p38α by C3G and Rap1 in MEFs and their effects on the balance between apoptosis and cell survival. (A) Upon serum deprivation, C3G through the negative regulation of p38α mediates cell survival, while activation of Rap1 by other GEFs, leads to p38α activation inducing apoptosis. (B) Upon treatment with H_2O_2, C3G mediates apoptosis through inhibition of p38α activity and Rap1 (activated by other GEFs) induces cell survival through p38α (Gutierrez-Uzquiza et al. 2010).

Moreover, the group has revealed a novel pro-survival role for p38α in response to low or moderate doses of H_2O_2, which involves the induction of antioxidant enzymes such as superoxide-dismutase-2 (SOD-2) and catalase preventing from a high accumulation of ROS. As shown in figure 12, p38α allows cells to survive under mild levels of oxidative stress in MEFs. It was also found that mTOR/p70S6K activation is dependent on p38α activation in this oxidative microenvironment (Gutierrez-Uzquiza et al. 2012). This pro-survival role of p38α could be a key event in the control of tumor growth in different types of tumors, including the colorectal cancer.

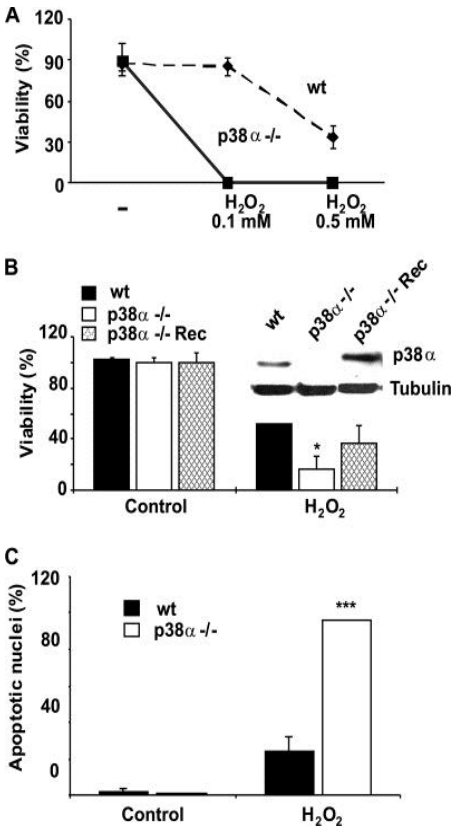

Figure 12-p38α protects from H_2O_2-induced cell death. MEFs (*wt* and p38α −/−) maintained in the presence of serum were treated with H_2O_2 when indicated. (A) p38α expression increases cell viability in cells treated with H_2O_2 (0.1 or 0.5 mM) for 24 h. (B) p38α reconstitution in p38α−/− cells (Rec) rescues cells from cell death upon treatment with H_2O_2 (1 mM) for 6 h. The data correspond to cell viability expressed as the percentage of control value in *wt* cells. p38α expression was determined by Western blot and normalized with tubulin. (C) Loss of p38α increases the number of apoptotic nuclei in cells treated with H_2O_2 (0.5 mM) for 3 h. *p<0.05 and ***p<0.001, p38α −/− versus *wt* MEFs upon treatment with H_2O_2 (Gutierrez-Uzquiza et al. 2012).

2. ROLE OF p38α MAPK IN THE REGULATION OF APOPTOSIS IN THE HUMAN COLON CARCINOMA HCT116 CELL LINE

To assess the role of p38α as a regulator of cell death in response to some chemotherapeutical compounds in the human colon carcinoma HCT116 cell line, permanent p38α knock-down cells were used. Data revealed that p38α is a key player in cisplatin-induced apoptosis. Hence, as shown in figure 13, in response to cisplatin, p53 is activated, increasing their levels, which leads to a p53-mediated ROS production that activates p38α MAPK. Through this cascade, p38α induces apoptosis and enhances the initial p53 activation, leading to a subsequent p38α/p53 positive feedback loop (Bragado et al. 2007).

Therefore, p38α is mediating cell death in HCT116 cells and we wondered if p38α pathway could be a major regulator of other cellular processes in these cells identifying mechanisms for its actions.

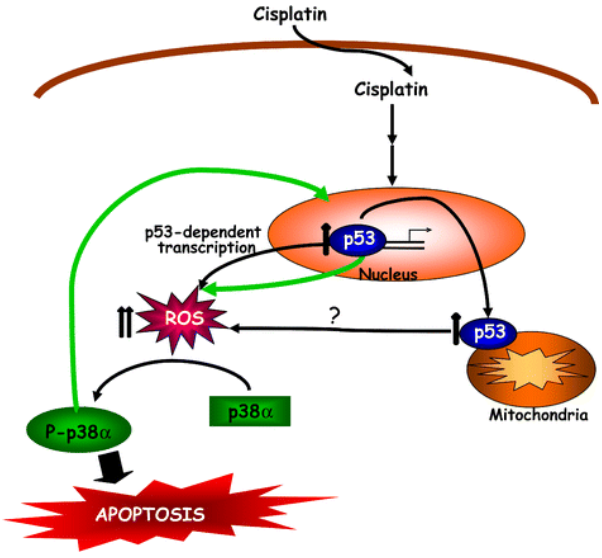

Figure 13-Cisplatin-induced apoptosis in HCT116 cells through the p53/ROS/p38α MAPK cascade. p53 increases in response to cisplatin, which leads to ROS accumulation leading to p38α MAPK-mediated apoptosis. p38α MAPK enhances p53 activation, which results in a p38α MAPK/p53 loop of amplification (Bragado et al. 2007).

3. FUNCTION OF p38α MAPK IN CELL ADHESION AND MIGRATION. NEW TARGETS OF p38α

p38 MAPKs also play a key role in the regulation of cell adhesion and migration. In agreement with this, our group showed that p38α through down-regulation of Rac-1 activity negatively regulates cell adhesion in embryonic cardiomyocytes derived cell lines (Zuluaga et al. 2007). Moreover, in collaboration with Dr. Carmen Guerrero's group, we found that C3G and p38α acting through a common regulatory cascade promote cell adhesion in the K562 CML cell line regulating focal adhesion proteins (Maia et al. 2013). However, C3G and p38α display antagonistic roles in the regulation of focal adhesion complex formation (Maia et al. 2013). These data, together with the function of C3G/p38 in the control of cell death, prompted us to investigate if C3G acting through p38α could be also regulating migration and invasion processes in MEFs (M.Arechederra´s Thesis) and in the human colon carcinoma HCT116 cell line. Results derived from this analysis in MEFs revealed that C3G inhibits migration and invasion through down-regulation of p38α activity.

In addition, to uncover novel mechanisms of action of p38 MAPKs in cell adhesion and migration/invasion, we have previously performed DNA microarray analyses using *wt* and p38α knock-out MEFs (A. Gutiérrez´s Thesis). Data derived from these studies showed up changes in the expression of a number of genes and some of them encoded proteins involved in the regulation of these processes such as Fibulin 3. In fact, Fibulin 3 was highly up-regulated in p38α knock-out MEFs. These results were validated by RT-PCR and RT-qPCR in MEFs. Therefore, based on the strong negative regulation of Fibulin 3 by p38α, it was studied how p38α MAPK regulated Fibulin 3 expression and the implication of this p38α/Fibulin 3 pathway in the regulation of cell migration and invasion in MEFs (M. Arechederra´s Thesis). In particular, p38α was shown to silence Fibulin 3 expression through methylation of regulatory sequences of the gene, which enhanced migration/invasion. In parallel, this was also studied in a tumor model, the human colon carcinoma HCT116 cell line.

AIMS

General Aim:

The main aim of this research project is to study the role played by C3G/p38α MAPK cascade in the control of migration, invasion and tumor growth in a human colon carcinoma model, as well as to determine if Fibulin 3 could be a target of p38α to control these processes.

Specific Aims:

1. To determine if p38α MAPK regulates fibulin 3 expression, how it does it and the role of Fibulin 3 as a potential mediator of p38α actions on cell migration, invasion and tumor growth.

2. To analyze whether C3G could act through p38α MAPK to regulate migration, invasion and tumor growth, identifying the mechanisms involved.

MATERIALS AND METHODS

1. CELL LINES

In our studies, we have used the human colon carcinoma cell line HCT116 obtained from ATCC (CCL-247), which were authenticated by microsatellite markers analysis. HCT116 cells with permanent p38α knock-down, previously generated in our laboratory by transfection of a p38α shRNA inserted in pSuper.retro.puro vector (OligoEngineVEC-PRT-0001) (Bragado et al. 2007), were also used. Additionally, these cells were, either permanently or transiently, genetically modified.

Organism	Homo sapiens, human
Tissue	Colon ascendens
Morphology	Epithelial
Culture Properties	Adherent
Disease	Colorectal carcinoma. Derived from primary tumor
Age	Adult (48-year-old)
Gender	Male
Stage	Dukes´D
Characteristics	This cell line has a mutation in codon 13 (G13D) of the Ras proto-oncogene, in exon 20 (H1047R) of the p110α catalytic subunit of PI3K (PIK3CA) and premature truncations in codon 216 of the E-cadherin gene (CDH1). Other relevant mutations: Cyclin-dependent kinase inhibitor 2A (CDKN2A) and β-catenin (CTNNB1). *wt* for APC, B-RAF, PTEN and TP53. Positive for CpG island methylator phenotype and negative for chromosomal instability. Microsatellite instability.

Table 2- Characteristics of HCT116 cell line. From Ahmed et al. 2013 and ATCC.

1.1 FIBULIN 3 KNOCK-DOWN:

Permanent fibulin 3 knock-down (in parental and p38α knock-down HCT116 cells) was performed by transfection with a human fibulin 3 shRNA (OriGeneTechnologies, TR30018) inserted in the pGFP-B-RS vector. To do it, cells at a confluence around 50-60% were used. The medium was replaced by a fresh one (with serum) and the transfection was done using Metafectene-Pro reagent (Biontex, T040-0.2) following the commercial protocol. 48 h after transfection, cells were trypsinized and splitted to select clones expressing the shRNA using a blasticidin (2 µg/ml) containing medium. Different clones were picked, expanded and analyzed for fibulin 3 expression. As a control, cells transfected with the empty vector were also generated.

Transient fibulin 3 knock-down was also performed using a second human fibulin 3 shRNA (Sigma, NM-004105). Cell assays were initiated 48 h after transfection.

1.2 C3G PERMANENT KNOCK-DOWN:

Permanent C3G knock-down (in parental and p38α knock-down HCT116 cells) was done by infection with lentiviral particles containing a mixture of different human C3G shRNAs (Santa Cruz Biotechnology, sc-29863-V) or a control shRNA (Santa Cruz Biotechnology, sc-134220). Cells were seeded 12 h prior to the viral infection at a density that guaranteed 50-60% confluence the next day. Then, the medium was replaced and 75.000 infectious units of lentiviral particles were added in the presence of 10 µg/ml polybrene. Cells were incubated overnight and the medium was removed and a fresh one was added. After 48 h cells were selected with 2µg/ml puromycine. Different clones were picked, expanded and analyzed for C3G protein levels.

C3G knock-down was also performed with an alternative infection with lentiviral particles containing a shRNA (5´GATCCCCCCACTATGATCCCGACTATTTC AAGAGAATAGTCGGGATCATAGTGGTTTTTGGAAA3´) kindly supplied by Dr. Guerrero´s group inserted in the pLVTHM vector containing GFP. GFP expressing cells were selected.

1.3 RAP1 PERMANENT KNOCK-DOWN:

Permanent Rap1 knock-down (in parental and p38α knock-down HCT116 cells) was performed using two different shRNAs against human Rap1 (Sigma, TRCN0000029784 and TRCN0000029788) inserted in the pLKO.1-puro plasmid. Transfection was done using Metafectene-Pro reagent, as described above. Cells were selected and maintained with 2µg/ml puromycine.

1.4 RE-EXPRESSION OF DNMT3A:

To re-express DNMT3A in HCT116 shp38α cells, transient transfections were performed using dnmt3a inserted in the pcDNA3 vector using Metafectene-Pro reagent, as described above. Experiments were performed 48-72 h after transfection.

2. CELL CULTURE CONDITIONS AND PRESERVATION

2.1 CELL CULTURE CONDITIONS

HCT116 cells were grown in McCoy's medium (Invitrogen, 16600-082) supplemented with 10% fetal bovine serum (FBS) (Gibco, 26140-079), 20 mM Hepes (pH 7.4) and antibiotics: penicillin (12 µg/ml), streptomycin (10 µg/ml), amphotericin B (0.25 µg/ml) and MycoZap (Lonza, VZA-2012). HCT116 cells with permanent p38α, C3G and Rap1 knock-down were grown in the same medium, in the presence of puromycin (2 µg/ml) (Sigma-Aldrich, P8833). HCT116 cells with permanent Fibulin3 knock-down were grown in the presence of blasticidin (2 µg/ml) (Invitrogen, R210-01). Antibiotics were removed from the medium 48h before performing the experiments.

Cells were splitted when they reached 90% confluence by trypsinization. Cells were washed with phosphate-buffered saline (PBS) (Gibco, 70011) and Trypsin 0,25%-EDTA 0,02% (Gibco, 25200-056) was added. Trypsin action was stoped with10% FBS supplemented medium. The cellular suspension was splitted out following the required conditions for each experiment. Cells were maintained at 37ºC with 5% CO_2 in a humidified atmosphere.

2.2 FREEZING, CRYOPRESERVATION AND THAWING OF CELLS

Cells were stored in 10% DMSO-FBS in liquid nitrogen (-170 ºC). Cells were progressively frozen: -20 ºC (for 20-30 min), -80 ºC (for 12-16 h) and liquid nitrogen. Thawing was performed quickly at 37ºC and cells were placed in the appropriated culture medium. When cells were attached, medium was replaced by fresh medium to remove DMSO.

2.3 CELL TREATMENTS

Cells were treated with the following compounds:

Inhibitors:

- p38α/β inhibitor, SB203580 (Calbiochem, 559389), at 5 µM (mainly for p38α) or 10 µM (for p38α and β).

- 5-aza-2´-deoxicytidine (Sigma, 3656), at 0.5-1 µM for 48 h (24 h of pretreatment and 24 h of treatment in the absent of FBS) to inhibit DNA methylation.

- Broad spectrum MMPs inhibitor, marimastat, at 0,01 µM.

Stimuli:

- Serum: 10% FBS for 10 min after serum-deprivation (24h) for signalling experiments.

- Hepatocyte growth factor (HGF): 40 ng/ml for different time periods (10, 20 and 60 min) in serum deprived cells for signalling experiments or for 24-72h to promote migration and invasion.

- Hydrogen peroxide (H_2O_2): 2 mM treatment for 4h to assess cell viability.

3. PROTEIN ANALYSIS

3.1 PREPARATION OF CELL EXTRACTS

Depending on the assay, cell culture medium was collected into tubes and stored at -80ºC until it was processed. Then, cells were washed twice with cold PBS and lysed with the following buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin,10 µg/ml leupeptin, 1 mM $NaVO_3$ and 20 mM NaF.

Cells were detached from the plate by scraping, collected in an Eppendorf tube and maintained on ice for 10 min. Every 5 min, tubes were shaken using a vortex. Then, cell lysates were centrifuged at 13000 rpm for 10 min at 4°C. The supernatant (total protein extracts) was transferred to a new tube and stored at -80°C.

3.2 PROTEIN QUANTIFICATION

Protein quantification was performed using the method described by Bradford in 1976. Five min after addition of Bradford reagent (diluted 1:5 in ultrapure water) to the samples, the absorbance at 595 nm was measured. For each experiment, a standard curve with known concentrations of bovine serum albumin (0 to 5 µg/µl) was used to extrapolate sample values.

3.3 WESTERN-BLOT ANALYSIS

3.3.1 Protein electrophoresis

Protein electrophoresis was performed using SDS-polyacrylamide gels (SDS-PAGE) and non-SDS-polyacrylamide gels (Anderson gels) (Table 1). The last ones allow a better separation of phosphorylated proteins with similar electrophoretic mobility. Acrylamide concentrations were chosen according to the proteins size to be studied:

for proteins of a relatively small molecular weight (30-80 KDa), we used higher percentages of the separating gel (12-15%) and for proteins higher than 80-100 KDa, we used lower percentages of the separating gel (7.5%-10%). Protein samples were prepared by adding Laemmli buffer 4X (Tris-HCl pH 7.6, 10% (w/v) glycerol, 1% (w/v) SDS, 0.002% (w/v) bromophenol blue, 2 mM β-mercaptoethanol) to the protein extracts. Then, they were heated at 95ºC and loaded into the gel, as well as the molecular weight markers.

Electrophoresis was developed at a constant amperage of 20 mA using the following running buffers:

- For SDS-PAGE gels: 25 mM Tris-HCl (pH 8.3), 200 mM Glycine and 0.1% SDS.
- For Anderson gels: 50 mM Tris-HCl (pH 8.3), 400 mM Glycine and 0.1% SDS.

SDS-PAGE gels composition (Vf=10ml)

Composition	Separating Gel 10%	Separating Gel 12%	Separating Gel 15%	Stacking gel (Vf=5ml)
30% Acrylamide/ Bisacrylamide	3.3 ml	4 ml	5 ml	0.83 ml
H_2O	4 ml	3.3 ml	2.3 ml	3.4 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	-
1 M Tris pH 6.8	-	-	-	0.63 ml
10% SDS (w/v)	100 µl	100 µl	100 µl	50 µl
10% Ammoniumpersulfate	100 µl	100 µl	100 µl	50 µl
TEMED	4 µl	4 µl	4 µl	5 µl

Anderson gels composition (Vf=10 ml)

Composition	Separating Gel 7.5%	Separating Gel 10%	Separating Gel 15%	Stacking gel (Vf=5 ml)
30% Acrylamide	2.52 ml	3.36 ml	5.03 ml	0.833 ml
1% Bis-acrylamide	1.95 ml	1.31 ml	0.87 ml	0.667 ml
H_2O	3.02 ml	2.82 ml	1.59 ml	2.875 ml
1.5 M Tris pH 8.8	2.52 ml	2.52 ml	2.52 ml	-
1 M Tris pH 6.8	-	-	-	0.625 ml
10% Ammonium persulfate	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl

Table 3- SDS-PAGE and Anderson gels composition.

3.3.2 Proteins transfer

Proteins from the gels were transferred to a nitrocellulose membrane using a semi-dry equipment. The nitrocellulose membrane was activated by immersion into distilled water and it was assembled as follows from bottom to top: 3 Whatman papers,

nitrocellulose membrane, acrylamide gel and 3 Whatman papers, which were all previously soaked in transfer buffer (20% methanol, 50 mM Tris, 400 mM glycine and 0.1% SDS). Then, an electrical current of 15 V for 30-45 min (depending on the number/size of gels) was applied. Transfer was confirmed by staining the membrane with a Ponceau S solution (0.5% in 1% acetic acid).

3.3.3 Proteins immunodetection

Membranes were washed several times with distilled water, and twice with Tween-Tris-buffered saline (TTBS) (10 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS) with 0.05% Tween-20). Then, they were incubated in the blocking solution (either 5% non-fat dry milk or 5% BSA in TTBS) for one hour at room temperature. After this time, they were rinsed with TTBS and incubated with the primary antibody in 5% non-fat milk-TTBS or BSA-TTBS (for phospho-proteins) 1 h at room temperature (RT) for non-phosphorylated proteins or overnight at 4 ºC for phospho-proteins at the indicated dilution (Table 2). Then, membranes were washed 3 times with TTBS (5 min/each) and incubated for 1 h at RT with the secondary antibody at a dilution of 1:5000 in the same buffer as the primary antibody. Finally, they were washed 3 times with TTBS and incubated with a chemiluminescent solution (Pierce ECL from Thermo Scientific, 32106) to visualize proteins in an X-ray film.

Primary antibody	Laboratory	Dilution	Secondary antibody	Dilution
p38α	Santa Cruz Biotechnology sc-535	1:1000	Rabbit	1:3000
C3G (H-300)	Santa Cruz Biotechnology sc-15359	1:1000	Rabbit	1:3000
Fibulin 3	Santa Cruz Biotechnology sc-99177	1:1000	Rabbit	1:3000
Phospho-p38 MAPK	Cell Signaling Technology 9211	1:1000	Rabbit	1:2500
Phospho-ERK1/2	Cell Signaling Technology 9101	1:1000	Rabbit	1:3000
Phospho-Ser473-Akt	Cell Signaling Technology 9271	1:1000	Rabbit	1:3000
E-cadherin	BD Transduction Laboratories 610181	1:1000	Mouse	1:3000
N-cadherin	BD Transduction Laboratories 610920	1:1000	Mouse	1:3000
ZO-1	Invitrogen 33-9100	1:1000	Rabbit	1:3000
DNMT3A	Cell Signaling Technology 2160	1:500	Rabbit	1:3000
HuR	Santa Cruz Biotechnology sc-5161	1:1000	Rabbit	1:3000
β-Actin	Sigma 4-5441	1:1000	Mouse	1:5000

Table 4- Primary and secondary antibodies used for Western-Blot.

4. ANALYSIS OF mRNA EXPRESSION

4.1 TOTAL RNA ISOLATION

Cells were washed twice with cold PBS and total RNA was isolated using RNeasy Mini Kit (Quiagen, 74104) following manufacturer instructions. DNase (Quiagen, 79254) treatment was included to avoid possible genomic DNA contaminations. Finally, RNA was eluted with ultrapure water and stored at -80ºC until its use. RNA concentration was determined by spectrophotometry absorbance measurement at 260 nm (one unit of absorbance at 260 nm corresponds to 40 µg of RNA per ml). To estimate RNA purity, the ratio between the absorbance at 260 and 280 nm was calculated. A ratio A260/A280 of 1.8-2 is accepted for pure RNA.

4.2 c-DNA SYNTHESIS

Total RNA (1-3 µg) was reverse-transcribed to generate the cDNA using the Super Script III RT kit (Invitrogen, 18080-040) following instructions from the manufacturer. Essentially, RNA mixed with oligo (dT) (0.5 mM) and random primers (2.5 µM), was incubated at 65 ºC for 5 min to ensure RNA denaturation and then, placed on ice for at least one min. cDNA synthesis mix (5X RT buffer, 20 Units of RNase inhibitor, 5 mM DTT and 200 Units of SuperScript III) was added to each sample and incubated at 50ºC for 1h. The reaction was stopped by heating at 70 ºC for 15 min to inactivate the enzyme. c-DNA was stored at -20 ºC for its use for qPCR analysis.

4.3 QUANTITATIVE PCR

Real time PCR or quantitative PCR (qPCR) was performed using specific primers and Fast Start Universal SYBR Green Master (Rox) (Roche, 04913850001) to detect DNA in the 7900 Fast Real Time System (Life Technologies, 4329001). PCR reactions were done in triplicate using specific primers (Table 3). GAPDH was used as the normalizing gene, as its expression was constant under the experimental conditions, and different negative controls were used prepared with RNA or water instead of the cDNA.

A qPCR curve has typically an exponential phase followed by a plateau phase. To interpret the fluorescence emission, a fluorescence threshold bigger than background signal is determined during the exponential phase of real-time PCR. To process the data, the fractional number of PCR cycles required to reach this threshold is defined as the cycle threshold, or Ct. The Ct measure needs to be in the exponential phase of amplification, where the curve is linear.

To perform the analysis of the data, ΔCt value for each sample and primer is obtained as follows: Ct for a primer minus Ct for the normalizing control (GAPDH in this case) under a particular experimental condition. Then, the values are referred to the control

condition, obtaining the ΔΔCt value (sample ΔCt-control ΔCt=ΔΔCt). Finally, quantification of RNA levels was performed using the relative quantification (RQ), calculated as follows: $RQ= 2^{-\Delta\Delta Ct}$.

We consider a RQ significant when there is a minimum of two-fold change: RQ of more than 2 or less than 0,5.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Human GAPDH	CATCGAAGGTGGAAGAGTGG	CATCAAGAAGGTGGTGAAGC
Human Fibulin 3	TGGCGGCTTCCGTTGTTATCCA	TGGGGCAGTTCTCGGCACAT
Human TGFβ 1	GAGCCTGAGGCCGACTACTA	CGGAGCTCTGATGTGTTGAA
Human TGFβ RECEPTOR 1	CTTGTAAGCCAAGTTTTCACCC	CTCCACATGCTTAGGGGTGT
Human TNFα	GGCGCTCCCCAAGAAGACA	AAGTGCAGCAGGCAGAAGAG
Human CXCL12 (SDFα-1)	TCTGAGAGCTCGCTTGAGTG	GTGGATCGCATCTATGCATG
Human CXCR4	GCCTTATCCTGCCTGGTATTGTC	GCGAAGAAAGCCAGGATGAGGAT
Human MMP2	TCGCCCATCATCAAGTTCCC	TTCTTTAGTGTGTCCTTAGCA
Human MMP9	CGGTGATTGACGACGCCT	ACCAAACTGGATGACGATGT
Human MMP10	CATTGCTAGGCGAGATAGGGG	CAGTCACAGAACATGCAGGA
Human MMP13	TATGACTATGCGTGGCTGAA	TGTGTCCCATTTGTGGTGTGG
Human E-cadherin	CAACGACCCAACCCAAGAA	CACACACGVTGACCTCTAA
Human N-cadherin	GCGTGAAGGTTTGCCAGTGT	AGCACAAGGATAAGCAGGATGA
Human Snail 1	CTATGCCGCGCTCTTTCCTC	CTGCTGGAAGGTAAACTCTGGA
Human Twist 1	CAAAGAAACAGGGCGTGGGG	CAGAGGTGTGAGGATGGTGCC
Human Zeb 1	GGCATACACCTACTCAACTACGG	TGGGCGGTGTAGAATCAGAGTC
Human Zeb 2	AATGCACAGAGTGTGGCAAGGC	ATCTGGCGTTCCAGGGACTCAT

Table 5- Sequences of the primers used in quantitative PCRs.

5. ADHESION, MIGRATION AND INVASION ASSAYS

5.1 ADHESION ASSAY

50,000 cells were seeded in 12 multiwell plates and left at 37 ºC for 30 min, 1 h or 2 h. Then, the culture medium was removed, cells were washed twice with PBS and incubated for 30 min at RT with a solution of 0.2% crystal violet (w/v in 2% ethanol, Sigma-Aldrich, C-0775). To remove crystal violet excess, plates were washed with distilled water and dried. Then, cells adhered to the plate were quantified.

5.2 WOUND HEALING ASSAY

Cell migration was evaluated using wound healing assays. Confluent cells were pre-treated with mitomycin C (25 µg/ml) (Sigma-Aldrich, M0503) for 30 min to inhibit cell growth. Then, they were washed with PBS and a straight scratch was done. Cells were washed again and a fresh medium supplemented with 2% FBS was added (control conditions) and cells were allowed to migrate for 12–72 h at 37 °C and 5% CO_2 under these conditions or in the presence of HGF (40 ng/ml) to promote cell migration. Migration was followed over time by a phase-contrast microscope coupled to a digital camera. Photographs were taken at different time points (0 h, 12 h, 24 h, 48 h and 72 h) and the percentage of wound healing closure was quantified using TScratch program and referred to 0 h value.

5.3 INVASION ASSAY

To study the invasive capacity of the cells through matrigel, Boyden chambers with transwells inserts (8 µm filter, BD, 353097) coated with matrigel (444 µg/cm^2) (BD Biosciences, 356234) were used. 50.000 cells were seeded in the upper chamber in a serum-free medium. Depending on the assay, cells were treated with SB203580 or marimastat inhibitors. In the lower chamber, HGF (40 ng/ml) was added to the medium to act as chemoattractant and cells were left in the incubator for 24 h at 37 °C and 5% CO_2. Then, the medium and matrigel from the upper chamber were removed and cells present in the lower chamber were fixed with 4% paraformaldehyde, washed several times with PBS and stained with 0.2% crystal violet w/v. Inserts were washed with distilled water and dried. To count the cells, a phase-contrast microscope was used.

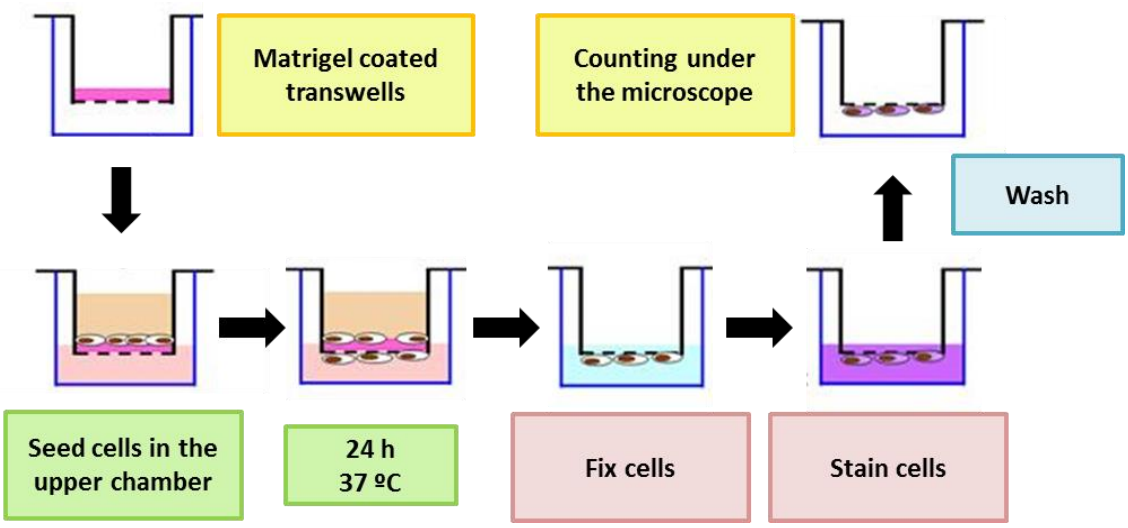

Figure 14- Schematic representation of invasion assay through matrigel.

6. QUANTIFICATION OF MMP2 AND MMP9 ACTIVITIES BY ZYMOGRAPHY

MMP2 (gelatinase A) and MMP9 (gelatinase B) activities were determined by zymography. 80% confluent cells were serum-deprived for 24-48h and the culture medium was collected. To establish the volume of medium to be used, total protein or RNA levels were measured to normalize. Samples were prepared using a non-reducing loading buffer 4X (250 Mm Tris-HCl pH 6.8, 25% glycerol, 2.5% SDS and 1 mg/ml Bromophenol blue) and under non-reducing conditions. Then, they were loaded in a 8% SDS polyacrylamide gel, polymerized in the presence of 0.1% gelatin.

The electrophoresis was carried out at constant voltage of 80 V for 3-4 h. Then, gels were washed with 2.5% Triton X-100 for 30 min to remove SDS, rinsed with substrate buffer (0.2 M NaCl, 5mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3, 50 mM Tris pH 7.5) and incubated in this buffer at 37°C overnight to allow protein renaturation and MMP activation. To visualize gelatin degradation areas as clear bands, the gel was stained with Coomassie Brilliant Blue (BioRad, 161-0400).

7. ANALYSIS OF CELL VIABILITY AND APOPTOSIS

7.1 ANALYSIS OF CELL VIABILITY

HCT116 cells (40.000) were seeded in 24 multiwell plates in triplicate. At a confluence of 70%, cells were deprived and treated with 2 mM H_2O_2 for 4 h. The medium was removed, cells were washed twice with PBS and incubated with crystal violet (0.2% w/v in 2% ethanol solution) for 20 min. Then, cells were washed several times with distilled water until the excess staining was eliminated. The plate was air-dried, the stained cells were lysed in 1% SDS and absorbance at 560 nm was measured. Results are expressed as the percentage of viable cells as compared to control cells (100%).

7.2 ANALYSIS OF ANOIKIS

To quantify cell viability and apoptosis, HCT116 cells were maintained detached for 6 h (under soft shaking to prevent adhesion) and then were replated for 4h, the number of cells was counted and the nuclei morphology was analyzed. Nuclei were stained by incubation for 30 min (in the dark) with 0,25µg/ml propidium iodure in PBS containing 50 µg/ml RNasa. Previously, cells were washed twice with cold PBS, fixed at RT for 30 min using a solution of cold methanol:acetic acid (dilution 3:1) and washed again with PBS. Finally, cells were embedded in Vecta shield mounting medium (Vector laboratories, 101098-042) to visualize the preparations. Total number of nuclei and the number of apoptotic nuclei (condensed and/or fragmented) were counted.

8. TUMORIGENESIS STUDIES

8.1 ANCHORAGE DEPENDENT GROWTH

To measure anchorage-dependent growth, HCT116 cells (300) were seeded in a 6 cm dish in duplicate. After 8-10 days, the medium was removed and cells foci were fixed and stained with a 0.2% crystal violet w/v in 2% ethanol solution. The total number of foci was quantified using Image J program and their size using Open CFU program.

8.2 ANCHORAGE INDEPENDENT GROWTH

To measure anchorage independent growth in soft-agar, cells were cultured in 24 multiwell dishes in triplicate, containing two agar layers. 3000 cells were resuspended in 0.7% agar (BD, 214530) diluted in complete medium and poured onto a 0.5 % layer of agar (diluted in medium). To prevent agar drying, fresh medium was added to the top layer every 3 days. After 10-15 days colonies were stained with 0.005% crystal violet (w/v) and counted.

8.3 XENOGRAFTS ASSAYS

HCT116 cells resuspended in McCoy's medium (10^6/100 µl) were injected subcutaneously into the flank of eight-week-old male nude mice (Harlan Laboratories). Tumor growth was monitored twice a week for 6 weeks. Tumor size was calculated by the formula ((L/2)x(W/2))xπ, where L and W are the longest and the shortest diameter in cm, respectively. All animal experiments were carried out in compliance with the institutions guidelines.

8.4 SPHERE FORMATION ASSAYS

80.000 cells with or without p38α, C3G or double knock-down, were seeded in triplicate in 6 multiwell plates in DMEM advanced medium (Life Technologies, 12491-023) supplemented with antibiotics and the following complements: hFGF2 at 20 ng/ml (Almone labs, F-170) and insulin (1 mg/L), transferrin (0.55 mg/L) and sodium selenite (0.67 µg/L) (Life Technologies, 41400-045). Photographs were taken every 24 h and the number of spheres was counted under a phase-contrast microscope.

9. CONFOCAL MICROSCOPY ANALYSIS

9.1 F-ACTIN STAINING

F-actin staining was performed using rhodamine-conjugated phalloidin. Cells were cultured in sterile glass coverslips, previously coated with 2% gelatin in PBS, which were placed inside a 24 multiwell plate. After 24-48 h, the medium was removed and cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, cells were stained for 1 h with rhodamine phalloidin-TRITC (Invitrogen, R415. 300U) diluted 1:1000 in PBS 0.1% BSA. Then, glasses were washed twice with PBS and mounted in Vecta shield mounting medium to visualize the preparations in a Leica TCS-SL confocal microscope with a 63X objective.

9.2 IMMUNOFLUORESCCENCE ANALYSIS OF E-CADHERIN AND ZO-1 SUBCELLULAR LOCALIZATION

The subcellular localization of E-cadherin and ZO-1 proteins was analyzed by confocal microscopy using the same antibodies used for western-blots. Cells were seeded on 2% gelatin-coated glass coverslips. After 24-48 h, they were fixed with 4% paraformaldehyde at RT for 30 min. Additionally, to detect ZO-1, cells were permeabilized with 0.1% triton X-100 in 0.1% BSA-PBS for 20 min. Then, fixed cells were washed with PBS and incubated in blocking solution (2% BSA in PBS, 1h at RT), followed by an incubation with mouse anti-E-Cadherin or rabbit anti-ZO-1 (dilution 1:50) in 0.1% BSA PBS for 1 h at RT. After washing with PBS, cells were incubated for 1h at RT with FITC-labelled anti-mouse or anti-rabbit Alexa 594 (dilution 1:200) and DAPI 1:1000 in 0.1% BSA PBS. After washing with PBS, cells were mounted in Vecta shield mounting medium and visualized in a Leica TCS-SL confocal microscope with a 63X objective.

10. ANALYSIS OF TUMORS FROM XENOGRAFTS

Tumors from xenografts assays were fixed with 4% PFA overnight at 4ºC, washed with PBS three times and left 1h in ethanol 50% at 4ºC. Then, they were kept in ethanol 70% at 4ºC to preserve the samples until they were embedded in paraffin and cut in sections. Next, paraffin was removed from the sections for immunohistochemistry and immunofluorescence analyses as well as for tunel assays.

10.1 IMMUNOHISTOCHEMISTRY ANALYSIS

First, samples were hydrated by incubation of the sections in the following way: xylene for 5 min (twice), ethanol 100% for 5 min (twice), ethanol 90% for 3 min, ethanol 80% for 3 min, ethanol 70% for 3 min and finally water for 3 min. Then, samples were transferred to a citrate buffer (10 mM, pH=6) and heated for 12 min. After cooling down to RT, the sections were washed with water for 5 min and with PBS for 5 min. Next, they were treated in the following way: ethanol 90% for 3 min, ethanol 100% for 3 min twice, 3% H_2O_2 in methanol 100% for 20 min, ethanol 100% for 3 min, ethanol 90% for 3 min in order to block endogenous peroxidase and afterwards, samples were washed with water for 5 min and twice with PBS for 5 min. Tissue samples were incubated in a blocking buffer (30 g/L bovine serum albumin in PBS and additionally, goat serum 0.015% (Invitrogen, PCN5000)) for 30 min at RT. Then, samples were incubated with the primary antibody anti-αSMA (DAKO, M0851) at 1:100 and ZO-1 (Life technologies, 339100) at 1:50 in blocking buffer overnight at 4ºC. After washing 5 min with PBS, samples were incubated with the secondary antibody (anti-mouse IgG 1:2000 in blocking buffer) for 1 h at RT and then, washed with PBS for 5 min. Afterwards, they were incubated with the avidin/biotin reagent mix (Vectastain Elite ABC Kit, PK-6100) for 30 min at RT in the dark and washed again with PBS for 5 min. Next, one drop of hydrogen peroxide (in a buffer of pH=7.5) and two drops of the dAB reagent (Peroxidase Substrate Kit, SK-4100) diluted in distillated water were added to the samples and left for 1 min and removed by washing with water for 5 min.

To carry out the hematoxylin staining, slides were placed in Harris (cytosolic) for 10 sec or Mayers (nuclear) hematoxylin for 5 min. The excess of hematoxylin was rinsed with water several times for 5 min. Finally, slides were dehydrated in the following way: incubation with 70% ethanol for 3 min, 80% ethanol for 3 min, 90% ethanol for 3 min, 100% ethanol for 3 min (twice) and xylene 2 min (twice). The samples were then mounted using Vectamount H-5000 mounting medium and dried.

10.2 IMMUNOFLUORESCENCE ANALYSIS

Slides were hydrated in the following way: incubation with xylene for 10 min (twice), ethanol 100% for 3 min (twice), ethanol 90% for 3 min, ethanol 80% for 3 min, ethanol 70% for 3 min and finally water for 10 min twice. They were transferred to a citrate buffer (10 mM, pH=6) and heated for 12 min. After cooling down to RT, the sections were washed with water for 5 min and with PBS for 5 min. Next, slides were incubated with 0.1% Triton X-100 in PBS for 5 min and washed with PBS for 5 min. Then, samples were incubated in blocking buffer (30 g/L bovine serum albumin in PBS and additionally, goat serum 0.015% (Invitrogen, PCN5000)) plus 200 µL of serum for 30 min. Sections were then washed with PBS and incubated with the primary antibody E-Cadherin (cell signaling, 3195S), CD14 (BD Pharmirgen, 553739) and MECA 32 (BD Pharmigen, 550563) at 1:50 in blocking buffer overnight at 4ºC. Afterwards, samples were washed first with 0.01% Tween-PBS for 5 min and then, with PBS for 5 min and incubated with the secondary antibody: Alexa fluor 488 Gota anti-rabbit (Life technologies, A11034) for E-Cadherin and Alexa Fluor 488 Goat anti-rat (Life technologies, A11006) for CD14 and MECA 32, for 1 h at RT. Next, samples were

washed again with 0.01% Tween-PBS for 5 min. Samples were mounted using Vectamount H-5000 mounting solution.

10.3 TUNEL ASSAY

First, sections were hydrated by treatment in the following way: xylene for 5 min (twice), ethanol 100% for 3 min (twice), ethanol 70% for 3 min, ethanol 50% for 3 min and finally, they were washed with water for 3 min (twice). Then, slides were washed with PBS and incubated with Proteinase K (Invitrogen, AM2546) in PBS (60 µg/µl) for 15 min at RT. Proteinase K activity, was stopped by incubation with TBS for 5 min and samples were first washed with PBS for 5 min at RT and afterwards, with water for 3 min (twice). Then, samples were treated with H_2O_2 3% (diluted in methanol) for 10 min in order to block endogenous peroxidase. Next, slides were washed with water for 3 min (twice) and PBS for 3 min. TdT reaction was performed at 37ºC for 1 h, using a reaction mixture containing: TdT buffer and TdT enzyme (80 U/ml) (Fermentas Life Sciences, EP0162), 150 mM NaCl, 2 µM biotin 16-dUTP (Roche, 11093070910) and RNase free H_2O. Slides were rinsed in PBS and incubated with the avidin/biotin enzyme mix (Vectastain Elite ABC Kit, PK-6100) 30 min at RT. Then, they were washed with PBS for 3 min, incubated with DAB peroxidase substrate (Sigma, D-4293) for 5-10 min at RT or until color is developed and washed with water to stop the reaction. Finally, slides were incubated with a methyl green solution (1% in water) for 5 min and then, transferred directly to 100% ethanol to wash them twice. Samples were dehydrated in xylene for 1 min (twice) and dried. The samples were mounted using Cytoseal 60 Mounting Medium (Richard-Allan Scientific, 8310-4).

11. STATISTICAL ANALYSIS

Data have been represented as the mean value of 3-10 independent experiments ±S.E.M. When comparisons were made between two experimental groups, unpaired Student's t-test was used. To compare more than two groups, analysis of variance (ANOVA) was performed.

RESULTS

1. p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH METHYLATION OF DNA REGULATORY SEQUENCES. ROLE IN MIGRATION, INVASION AND TUMOR GROWTH

As indicated in the group background section, Affymetrix microarrays analyses revealed that fibulin 3 mRNA levels were up-regulated in p38α-deficient MEFs. This was also validated by RT-qPCR (M. Arechederra´s Thesis).

p38α (Wagner and Nebreda 2009, del Barco Barrantes and Nebreda 2012) and Fibulin 3 (Hu et al. 2009, Kim et al. 2012, Chen et al. 2014), are both involved in the regulation of migration and invasion of non-tumor and tumor cells. Moreover, fibulin 3 expression is deregulated in several tumors, including colorectal cancer (Tong et al. 2011, Obaya et al. 2012). Hence, we decided to characterize the regulation of fibulin 3 expression by p38α MAPK and to study if p38α could act through Fibulin 3 to regulate migration and invasion, as well as tumor growth in HCT116 cells.

1.1 p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION IN HCT116 CELLS

First, we quantified fibulin 3 mRNA expression by RT-qPCR in non-silenced and p38α knock-down HCT116 cells in order to know if p38α was regulating fibulin 3 in these cells. As shown in figure 15A, there was an increase in fibulin 3 mRNA levels in HCT116 cells with permanent p38α knock-down as compared to non-silenced cells. Similarly, western-blot analysis using total cell extracts or mediums (to analyze both intracellular and secreted Fibulin 3) showed that the levels of the secreted and intracellular Fibulin 3 protein were higher in p38α knock-down HCT116 cells (Fig. 15B and 15C).

Moreover, to further demonstrate that these changes in fibulin 3 expression were dependent on p38α MAPK, the effect of the selective p38α/β inhibitor SB203580 was determined. As shown in figure 15D, treatment with SB203580 also up-regulated Fibulin 3 secretion in non-silenced HCT116 cells, while it had no effect on p38α knock-down cells. These results confirmed that p38α is a negative regulator of fibulin 3 expression. As SB203580 had no effect on p38α knock-down HCT116 cells, it is very likely that p38β is not a relevant regulator of fibulin3 expression as compared to p38α.

Therefore, all these data indicate that p38α MAPK is a negative regulator of fibulin 3 expression in this human colon carcinoma cell line.

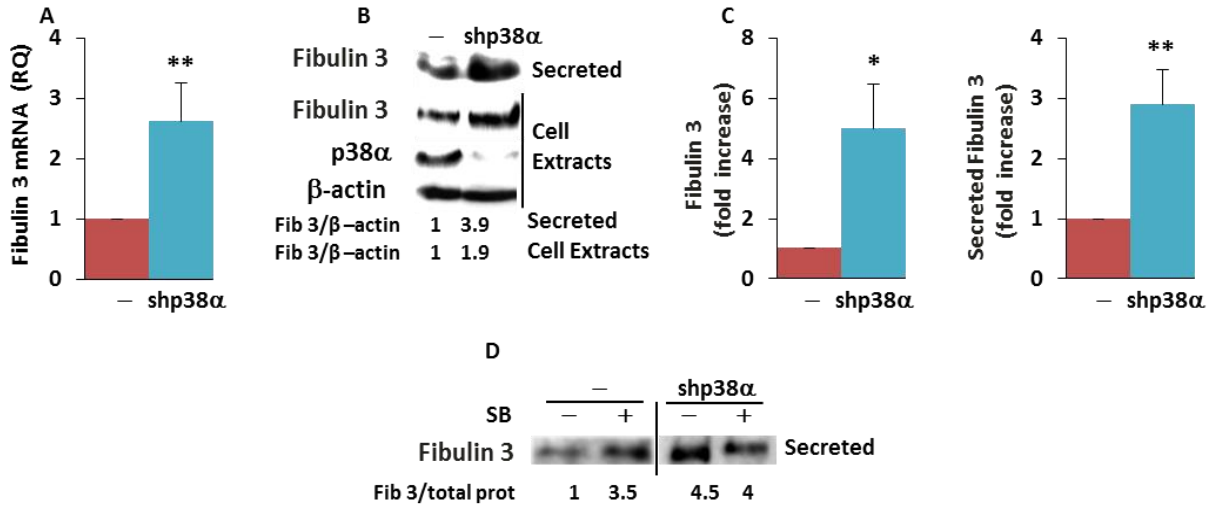

Figure 15-p38α MAPK is a negative regulator of fibulin 3 expression in HCT116 cells. HCT116 cells (non-silenced and p38α knock-down (shp38α)) were maintained in the absence of serum for 24h and then, total RNA or proteins were isolated. (A) Analysis by RT-qPCR of fibulin 3 mRNA levels in HCT116 cells. Results represent the mean ± S.E.M. of RQ values (n=3-8). ***p<0.001 as compared with non-silenced cells. (B) Western-blot analysis of Fibulin 3 protein levels in the culture medium (secreted) or in cell extracts as indicated. β-actin was used to normalize cell extracts and p38α as a control of its expression. (C) Histograms show the mean ± S.E.M. of the densitometric analyses of the blots normalized with β-actin (n=3-8). *p<0.05, **p<0.01, ***p<0.001 as compared with non-silenced cells. (D) Effect of SB203580 (5 μM) on Fibulin 3 secretion to the culture medium referred to total protein levels. Fib3/β-actin ratios were calculated by densitometric analysis of the blots and expressed as a fold increase.

1.2 p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH METHYLATION OF DNA REGULATORY SEQUENCES

Data from the literature indicated that Fibulin 3 expression could be regulated through DNA methylation. For example, Blackburn et al. in 2003, described important upstream regulatory motifs in fibulin 3 gene regulating its transcription in adult human and mouse retina. Furthermore, fibulin 3 expression is down-regulated in some tumors as a consequence of promoter methylation (Tong et al. 2011, Kim et al. 2012), which leads to changes in the invasive capacity (Kim et al. 2012). So, we wondered whether p38α might down-regulate fibulin 3 expression through methylation of DNA regulatory sequences.

To analyze it, we first studied the effect of the DNA methylation inhibitor, 5-aza-2´-deoxicytidine (5A2dC). 5A2dC treatment induced an increase in the levels of the secreted Fibulin 3 protein in non-silenced HCT116 cells, while it had no effect in knock-down HCT116 cells (Fig. 16A). Moreover, fibulin 3 mRNA levels were up-regulated in non-silenced HCT116 cells treated with 5A2dC (at 0.5 and 1 μM) up to the levels of p38α knock-down cells, where they remained unchanged (Fig. 16B). These results suggest that p38α might down-regulate fibulin 3 expression through a mechanism dependent on DNA hypermethylation. This p38α-mediated DNA methylation was demonstrated in MEFs by other members of the group (M. Arecehederra´s thesis).

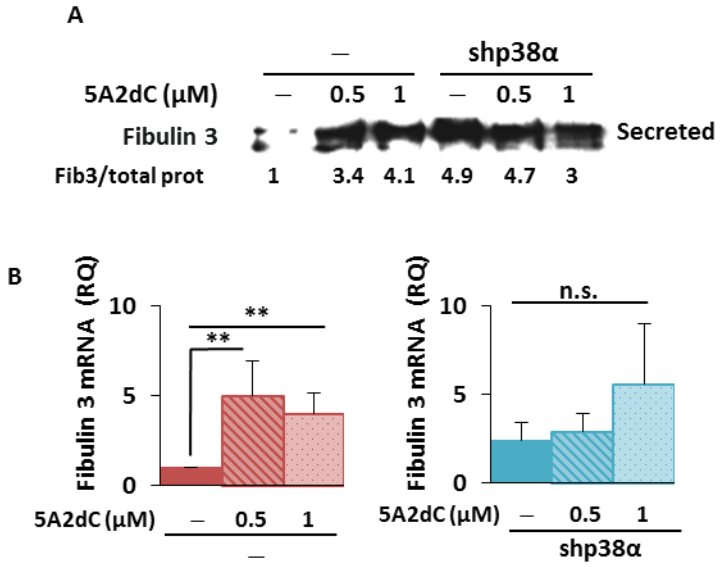

Figure 16-p38α down-regulates fibulin 3 expression through DNA methylation. HCT116 cells (non-silenced and p38α knock-down) were maintained in the absence of serum for 24h, and then, total RNA or proteins were isolated. When indicated, cells were treated with 5-aza-2´-deoxicytidine (5A2dC) at 0,5μM or 1μM for 48h (24h pretreatment plus 24h treatment in the absence of serum). (A) Western-blot analysis of Fibulin 3 protein levels in the culture medium. Fib3/total prot ratio was calculated by densitometric analysis of the blots referred to total proteins and expressed as a fold increase. (B) Analysis by RT-qPCR of fibulin 3 mRNA levels. **p<0.01 compared as indicated.

DNA methylation results from the activity of DNA methyltransferases (DNMTs) (Robertson 2001). Among DNMTs, DNMT3A/3B are de novo DNA methyltransferases, whose levels can be regulated by different mechanisms, being of relevance their post-transcriptional regulation (Denis et al. 2011). In particular, binding of HuR protein to the 3´UTR of dnmt3B mRNA, enhances its stability (Dai et al. 2012) and increases its protein levels in colorectal RKO cells (Lopez de Silanes et al. 2009). p38α MAPK can phosphorylate HuR, which enhances its binding to certain mRNAs such as p21 mRNA, increasing its protein levels (Lafarga et al. 2009). Thus, we hypothesized that p38α MAPK could be regulating DNMT3A/3B protein levels through a HuR-dependent mechanism. So, we analyzed DNMT3A protein levels in non-silenced and p38α knock-down HCT116 cells. We found a significant decrease in DNMT3A protein levels in p38α knock-down HCT116 cells as compared to their corresponding non-silenced cells (Fig. 17A).

These results indicate that p38α MAPK positively regulates DNMT3A protein levels, which inversely correlates with Fibulin 3 levels, suggesting that DNMT3A would be responsible for the hypermethylation of fibulin 3 gene regulatory sequences and the subsequent down-regulation of Fibulin 3. To prove it, DNMT3A was re-introduced in p38α knock-down HCT116 cells by transfection of a dnmt3a construct, which led to a strong decrease in Fibulin 3 levels (Fig. 17B).

As HuR could be involved in dnmt3a mRNA stabilization, we first measured total HuR protein levels and we found a decrease in HuR protein levels in p38α knock-down HCT116 cells (Fig. 17C). This would be in agreement with the low DNMT3A protein levels. Hence, these results suggest that p38α MAPK might positively regulate DNMT3A protein levels through HuR-mediated stabilization, which could result in the hypermethylation of fibulin 3 gene regulatory sequences.

In fact, this is the case in MEFs, where HuR knock-down prevents p38α-dependent DNMT3A expression (M. Arechederra´s Thesis).

Therefore, although we cannot exclude the possibility of the participation of other DNA methyltransferases, it is very likely that p38α-HuR mediated DNMT3A expression is responsible for hypermethylation of fibulin 3 DNA regulatory sequences.

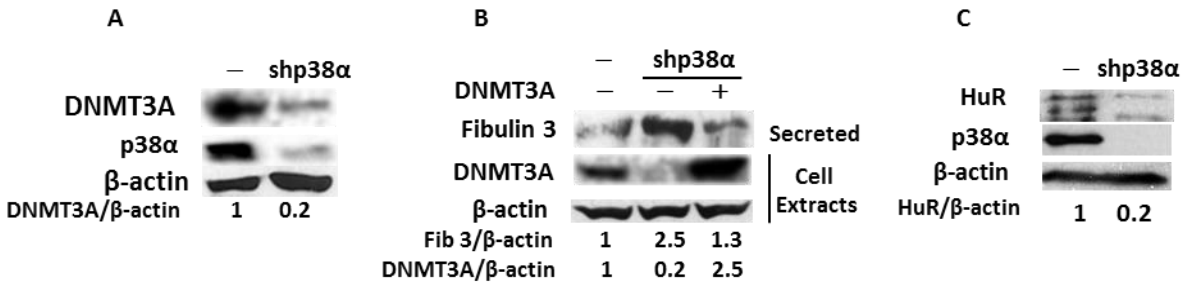

Figure 17-DNMT3A expression correlates with Fibulin3 down-regulation. Regulation by p38α. HCT116 cells were maintained in the absence of serum for 24h, and then, proteins were isolated. Western-blot analysis of DNMT3A (A) and HuR (C) protein levels in cell extracts. β-actin was used to normalize and p38α as a control of its expression. (B) Changes in Fibulin 3 protein levels upon DNMT3A expression in p38α knock-down HCT116 cells. Western-blot analysis of Fibulin 3 (secreted) and DNMT3A (in cell extracts) normalized with β-actin levels in cell extracts. DNMT3A/β-actin, Fib3/β-actin and HuR/β-actin ratios were calculated by densitometric analysis of the blots and expressed as a fold increase.

1.3 FIBULIN-3 KNOCK-DOWN INHIBITS MIGRATION AND INVASION OF HCT116 CELLS

Fibulin 3 is involved in the regulation of migration and invasion in different tumor cells, either promoting or inhibiting cell invasiveness depending on the tumor type (Hu et al. 2009, Kim et al. 2012). Moreover, changes in fibulin 3 gene expression by promoter methylation were shown to occur in advanced stages of colorectal cancer, which correlates with the induction of metastasis (Tong et al. 2011). p38α also regulates cell migration and invasion (Nebreda and Porras 2000, Wagner and Nebreda 2009). So, we evaluated the function of Fibulin 3 in the processes of migration and invasion in HCT116 cells and whether it mediates p38α effects. To analyze it, permanent knock-down of fibulin 3 gene was performed using a human fibulin 3 shRNA, selecting different clones. Secreted Fibulin 3 protein levels were decreased in both, non-silenced and p38α knock-down HCT116 cells upon fibulin 3 silencing (Fig. 18A). As shown in figure 18B and 18C, non-silenced HCT116 cells migrate faster than p38α knock-down cells and fibulin 3 knock-down impaired migration of parental cells and slightly reduced that of p38α knock-down cells, either in the absence or presence of HGF. Similarly, a transient fibulin 3 knock-down (Fig. 19A) using a second human fibulin 3 shRNA also prevented migration (Fig 19B).

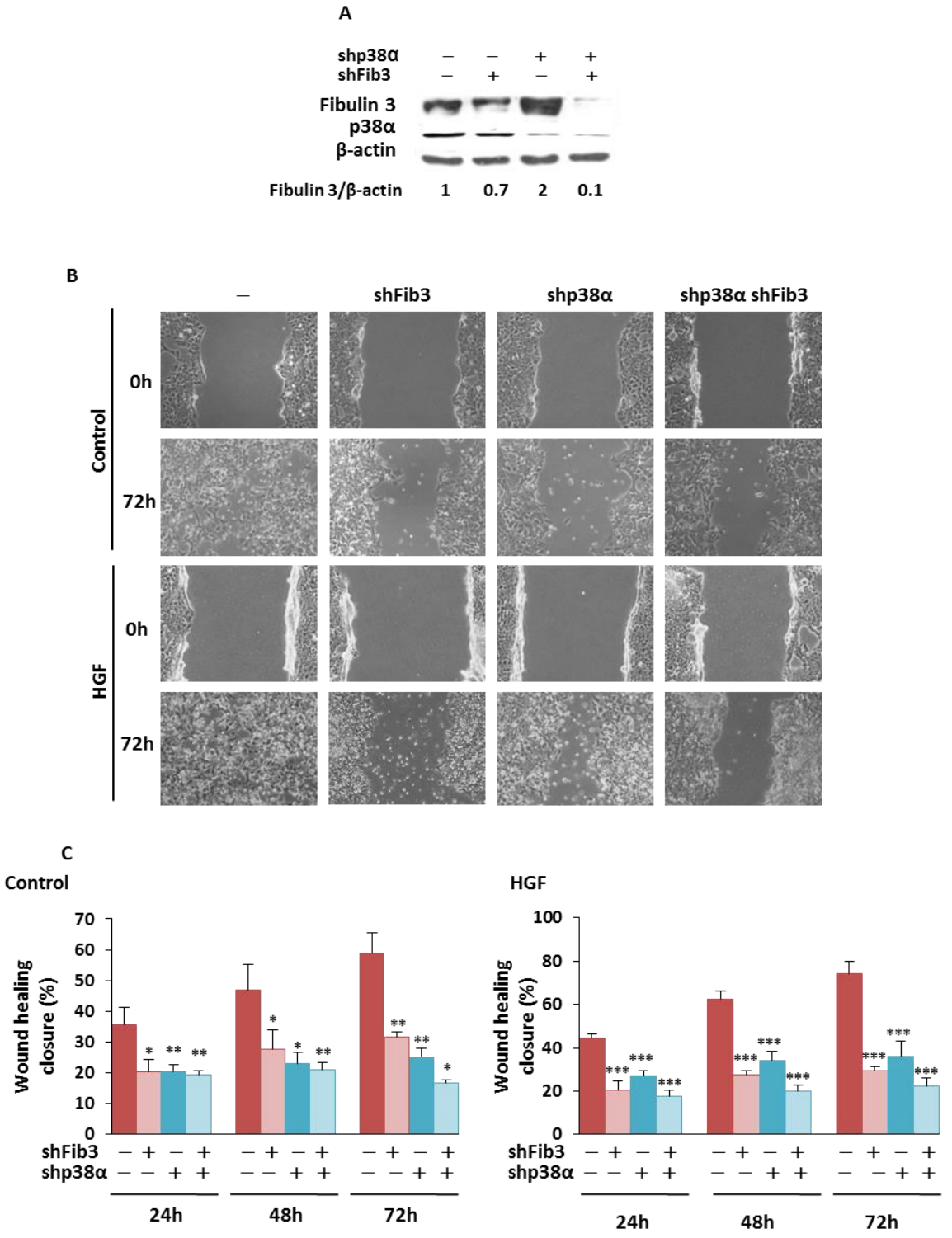

Figure 18-Fibulin 3 knock-down inhibits migration of HCT116 cells expressing p38α. HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down) were maintained in 2% serum-supplemented medium, untreated or treated with HGF (40 ng/ml) when indicated. (A) Western-blot analysis of Fibulin 3 protein levels in the culture medium normalized with β-actin from cell extracts. p38α was used as a control of its expression. Fibulin 3/β-actin ratio was calculated by densitometric analysis of the blots and expressed as a fold increase. (B and C) Wound healing assay. A wound was performed in confluent cells. Then, cells were allowed to migrate in the presence (HGF) or absence of HGF (control). (B) Representative images from phase contrast microscope after 0 and 72 h of migration. (C) Histograms show the mean ± S.E.M. of the percentage of wound closure at 24, 48 and 72 h (n=5-8). *p<0.05, **p<0.01, ***p<0.001 as compared with non-silenced cells.

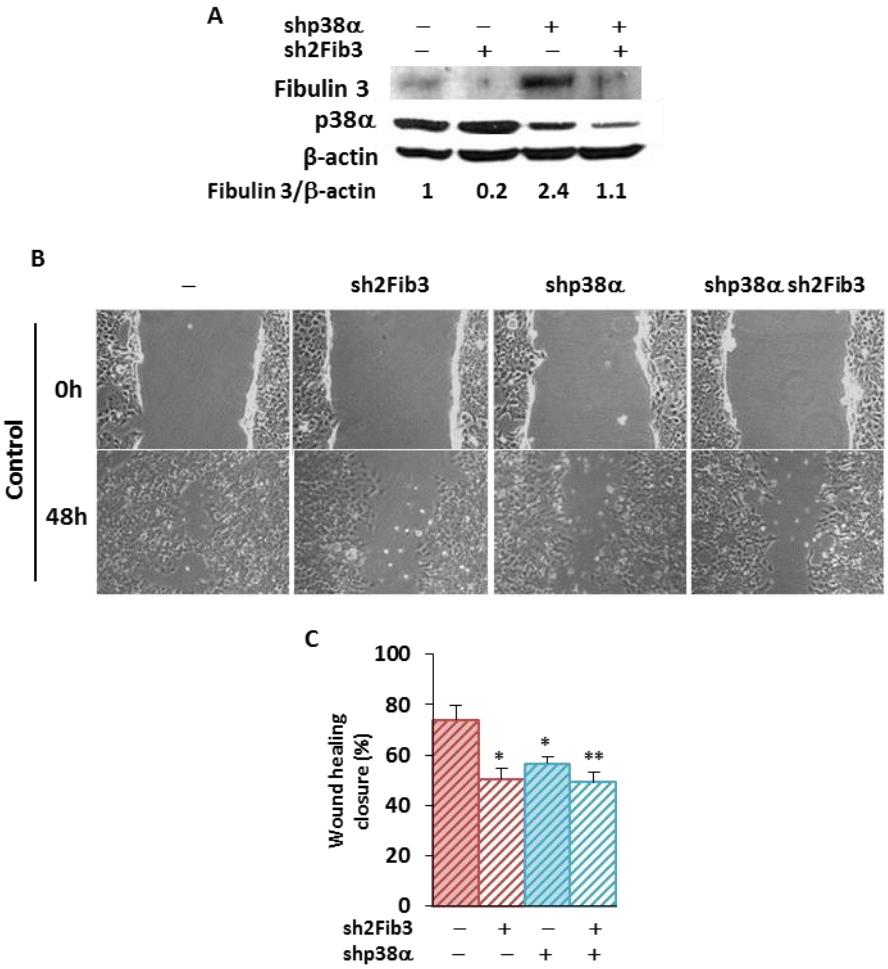

Figure 19-Fibulin 3 knock-down inhibits migration of HCT116 cells expressing p38α. HCT116 cells (non-silenced and p38α knock-down (shp38α); with (sh2Fib3) or without fibulin 3 knock-down) were maintained in 2% serum-supplemented medium. (A) Western-blot analysis of Fibulin 3 protein levels in the culture medium normalized with β-actin from cell extracts. p38α was used as a control of its expression. Fibulin 3/β-actin ratio was calculated by densitometric analysis of the blots and expressed as a fold increase. (B and C) Wound healing assay. A wound was performed in confluent cells. Then, cells were allowed to migrate. (B) Representative images from phase contrast microscope after 0 and 48 h of migration. (C) Histogram shows the mean ± S.E.M. of the percentage of wound closure at 48 h (n=3). *p<0.05, **p<0.01 as compared with non-silenced cells.

The effect of Fibulin 3 knock-down on the invasive capacity of HCT116 cells was also evaluated. Similar to migration assays, p38α know-down decreased basal and HGF-induced invasion through matrigel and fibulin 3 silencing blocked basal and HGF-induced invasion only in cells expressing p38α (Fig. 20A and 20B).

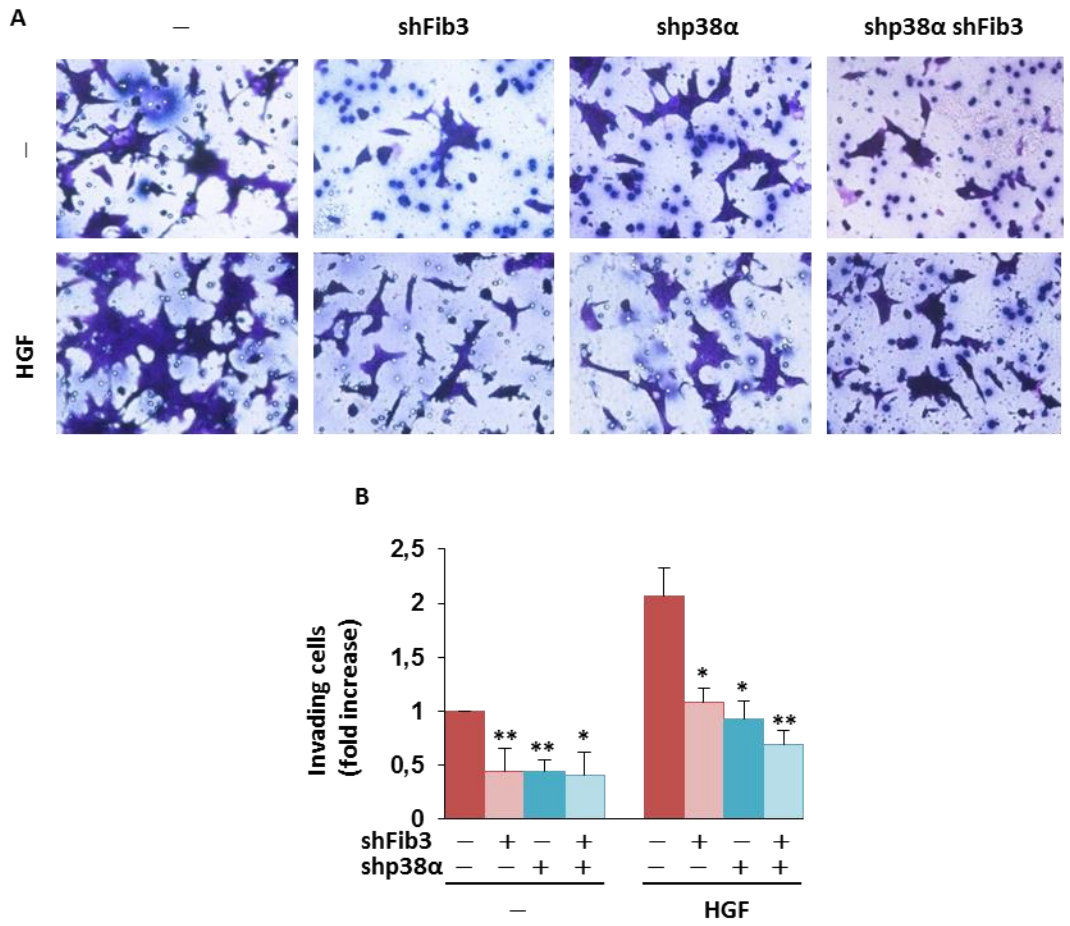

Figure 20-Effect of Fibulin 3 knock-down reduces invasion of HCT116 cells expressing p38α. Invasion through matrigel of HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down) using HGF as a chemoattractant. (A) Representative phase contrast microscope images of invading cells after staining with crystal violet. (B) Histograms show the mean ± S.E.M. of the number of invading cells expressed as the fold increase of control values (n=3-8). *p<0.05, **p<0.01 as compared with non-silenced cells.

1.4 MECHANISMS INVOLVED IN THE REGULATION OF CELL MIGRATION AND INVASION BY FIBULIN 3 IN HCT116 CELLS. ROLE OF p38α/β

In order to analyze potential mediators of p38α and/or Fibulin 3 actions on invasion, the participation of MMPs was evaluated as they play a relevant role in extracellular matrix degradation during cell migration/invasion (Bourboulia and Stetler-Stevenson 2010, Kessenbrock et al. 2011). Hence, we initially evaluated MMP2 and MMP9 activities. MMP9 activity was highly decreased upon fibulin 3 knock-down in both non-silenced and p38α knock-down cells (Fig. 21A and 21B). p38α silencing also reduced MMP9 activity, but to a lower extend (Fig. 21A and 21B), which correlated, at least partially, with invasion results. MMP2 activity was slightly reduced in fibulin 3 knock-down cells (Fig. 21A and 21B), suggesting its contribution to invasion as well.

To evaluate the relevance of MMPs in the invasion of HCT116 cells, the effect of the broad spectrum MMP inhibitor, marimastat, was assessed. As shown in figures 22A and 22B, HGF-induced invasion was impaired by marimastat treatment. Therefore,

changes in the activity of others MMPs, together with MMP2 and 9 activities, would mediate the pro-invasive effect of Fibulin 3 in HCT116 cells.

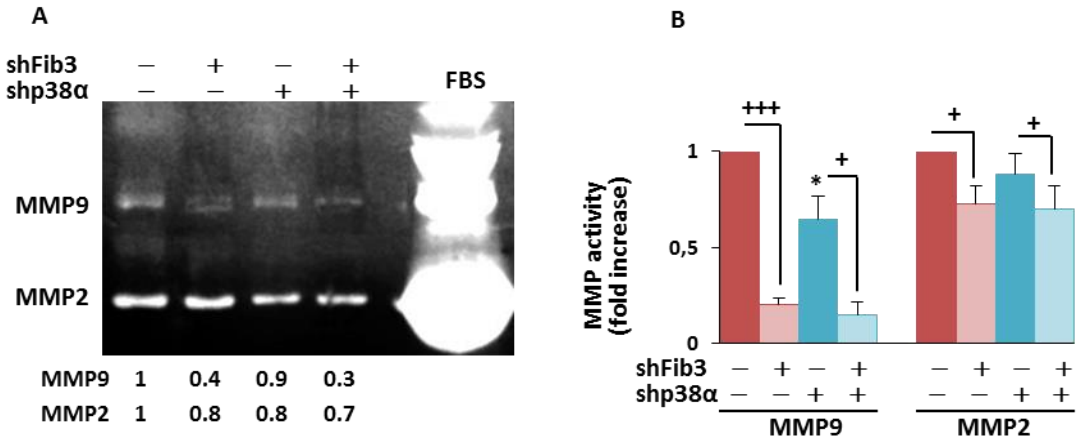

Figure 21-Effect of Fibulin 3 knock-down on MMP2 and 9 activities. Zymographic analysis of MMP2/9 activities using gelatin as substrate and FBS as a control. (A) Representative zymogram. (B) Histograms show the mean value ± S.E.M. of the densitometric analysis of gelatinase areas (n=3). *p<0.05 as compared with non-silenced cells; $^{+}$p<0.05, $^{+++}$p<0.001 compared as indicated.

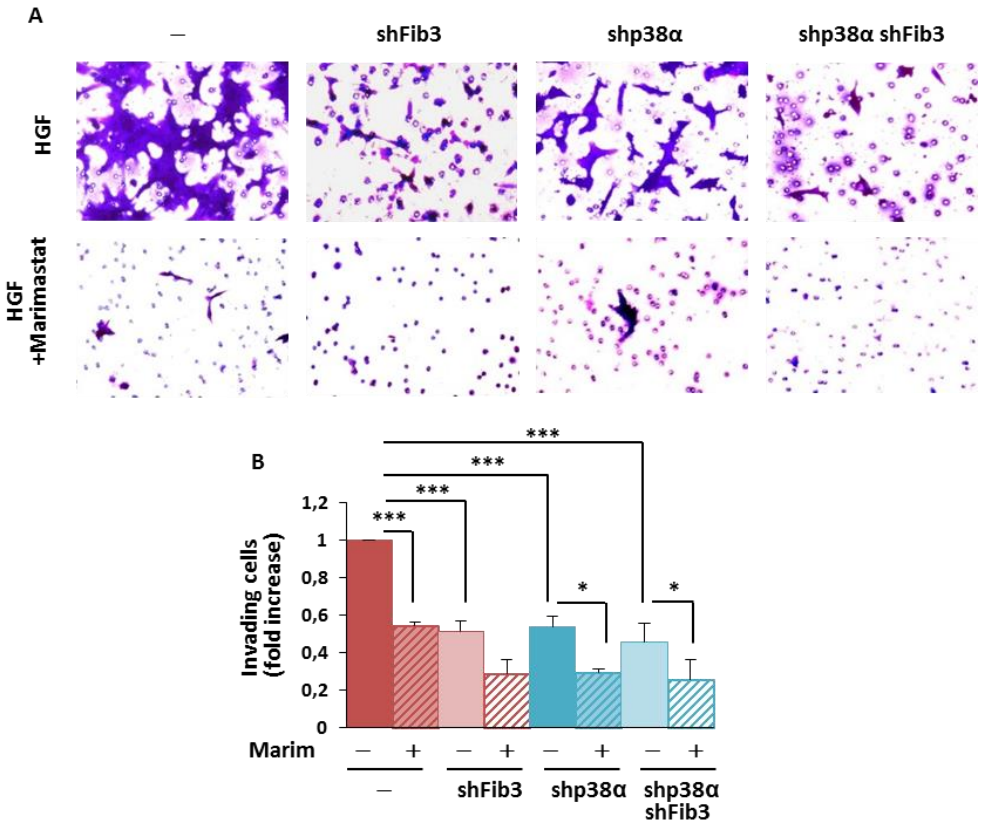

Figure 22-Inhibition of MMPs prevents invasion of HCT116 cells. Invasion through matrigel of HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down) using HGF as a chemoattractant and the MMP inhibitor, marimastat (marim), when indicated. (A) Representative phase contrast microscope images of invading cells after staining with crystal violet. (B) Histogram shows the mean ± S.E.M. of the number of invading cells expressed as the fold increase of control values (n=3). *p<0.05, ***p<0.001 compared as indicated.

Even though Fibulin 3 levels were lower in non-silenced cells, as compared to p38α knock-down HCT116 cells, its silencing inhibited migration and invasion in these cells. In fact, Fibulin 3 appears to be a positive regulator of cell migration and invasion, mainly in cells expressing p38α. So, we analyzed the effect of fibulin 3 knock-down on p38 MAPKs activation. As shown in figure 23, we found that in non-silenced HCT116 cells, HGF induced the activation of p38α MAPK and another isoform with a lower mobility (probably p38β), which was the only one activated in p38α knock-down cells. Interestingly, Fibulin 3 knock-down highly reduced the activation of both p38 MAPKs isoforms, which correlates with the decrease in migration and invasion.

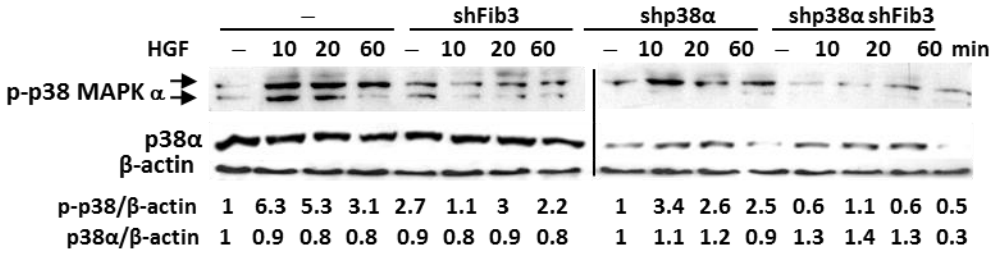

Figure 23-Effect of Fibulin 3 knock-down on p38 signaling pathway. Western-blot analysis of phospho-p38 MAPK levels from HCT116 cells (non-silenced, p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down) normalized with extracts β-actin. p-p38/β-actin and p38α/β-actin ratios were calculated by densitometric analysis of the blots and expressed as a fold increase. p38α was used as a control.

Together these results indicate that Fibulin 3 promotes migration and invasion of HCT116 cells through a mechanism that appears to require p38α and/or p38β activation. At the same time, p38α limits Fibulin 3 expression, which could represent a negative feed-back loop. As expected, p38α mediates migration and invasion in these cells. MMPs would be mediators of Fibulin 3/p38α effects on these processes.

1.5 ROLE OF FIBULIN 3 IN THE TUMORIGENIC CAPACITY OF HCT116 CELLS

Once we knew the function of Fibulin 3 regulating migration and invasion of HCT116 cells, we next wanted to determine the role of Fibulin 3 in the regulation of the tumorigenic capacity of these cells. First, we carried out anchorage dependent growth assays. As shown in figure 24A (left panel), fibulin 3 knock-down significantly reduced the number of foci in both parental and p38α knock-down cells, although the effect was more prominent in parental HCT116 cells. This effect was confirmed using a second human fibulin 3 shRNA to transiently knock-down fibulin 3 gene (figure 24B). Moreover, foci size was smaller in cells with Fibulin3 down-regulation (Fig. 24A, right panel). The

number of foci was also slightly reduced upon p38α knock-down, but differences were not statistically significant.

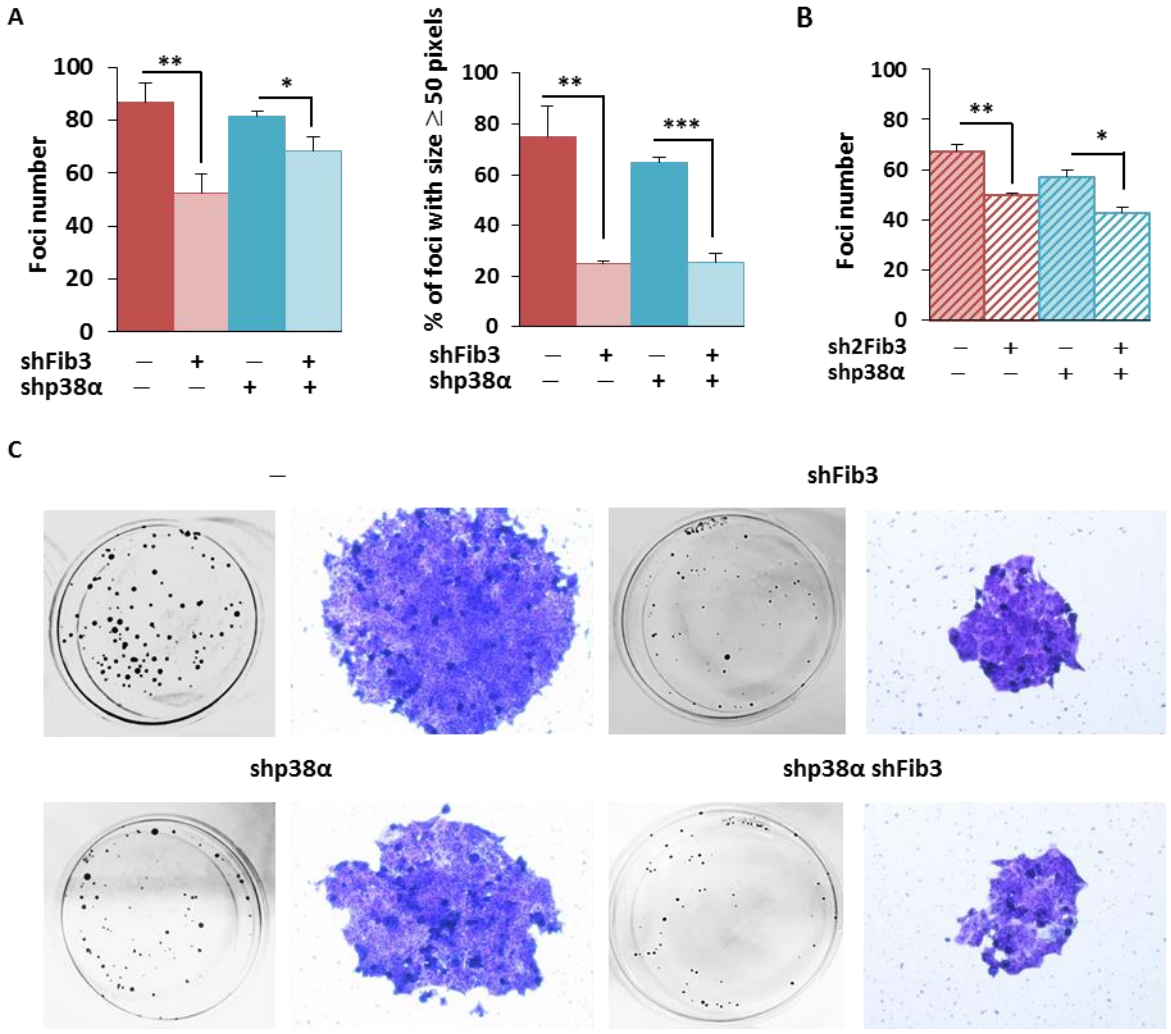

Figure 24-Fibulin 3 knock-down inhibits focus formation. Anchorage dependent growth of HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) (in A and C) or (sh2Fib3) (in B) or without fibulin 3 knock-down). Histograms show the mean ± S.E.M. of foci number (A and B) and the percentage of those with a size ≥50 pixels (B) (n=5). *p<0.05, **p<0.01, ***p<0.001 compared as indicated. (C) Representative images of foci from a plate (left) and individual foci (right) at 13 days.

To further characterize the potential role of fibulin 3 in tumor growth, we also determined the ability of these cells to grow in soft-agar. The number of foci was significantly reduced upon either fibulin 3 or p38α knock-down (Fig. 25A), as well as foci size (Fig. 25B). To further confirm the function of p38α promoting cell growth in soft-agar, HCT116 cells were treated with SB203580 to inhibit p38α/β. As shown in figure 25A, SB203580 treatment highly reduced anchorage independent cell growth only in cells expressing p38α. Thus, SB203580 had no further effect when fibulin 3 or p38α were knocked-down. To demonstrate *in vivo* this pro-tumorigenic function of Fibulin 3 and p38α in HCT116 cells, we also carried out xenografts assays in nude mice. As shown in figures 26A and 26B, non-silenced HCT116 cells led to tumor

formation 14 days after injection and tumor size progressively increased over time. Tumor sizes were significantly decreased by fibulin 3 knock-down in non-silenced cells, but not in p38α knock-down cells, where the size of the tumors was highly reduced, independently of fibulin 3 silencing. These data are in agreement with data derived from soft-agar assays, although the effect of p38α knock-down is higher, probably due to differences in the microenviroment and/or the influence of the angiogenic processes.

Therefore, these results indicate that Fibulin 3 promotes tumor growth in HCT116 cells through a mechanism dependent on p38α. Moreover, in these cells p38α is a potent positive regulator of tumor growth.

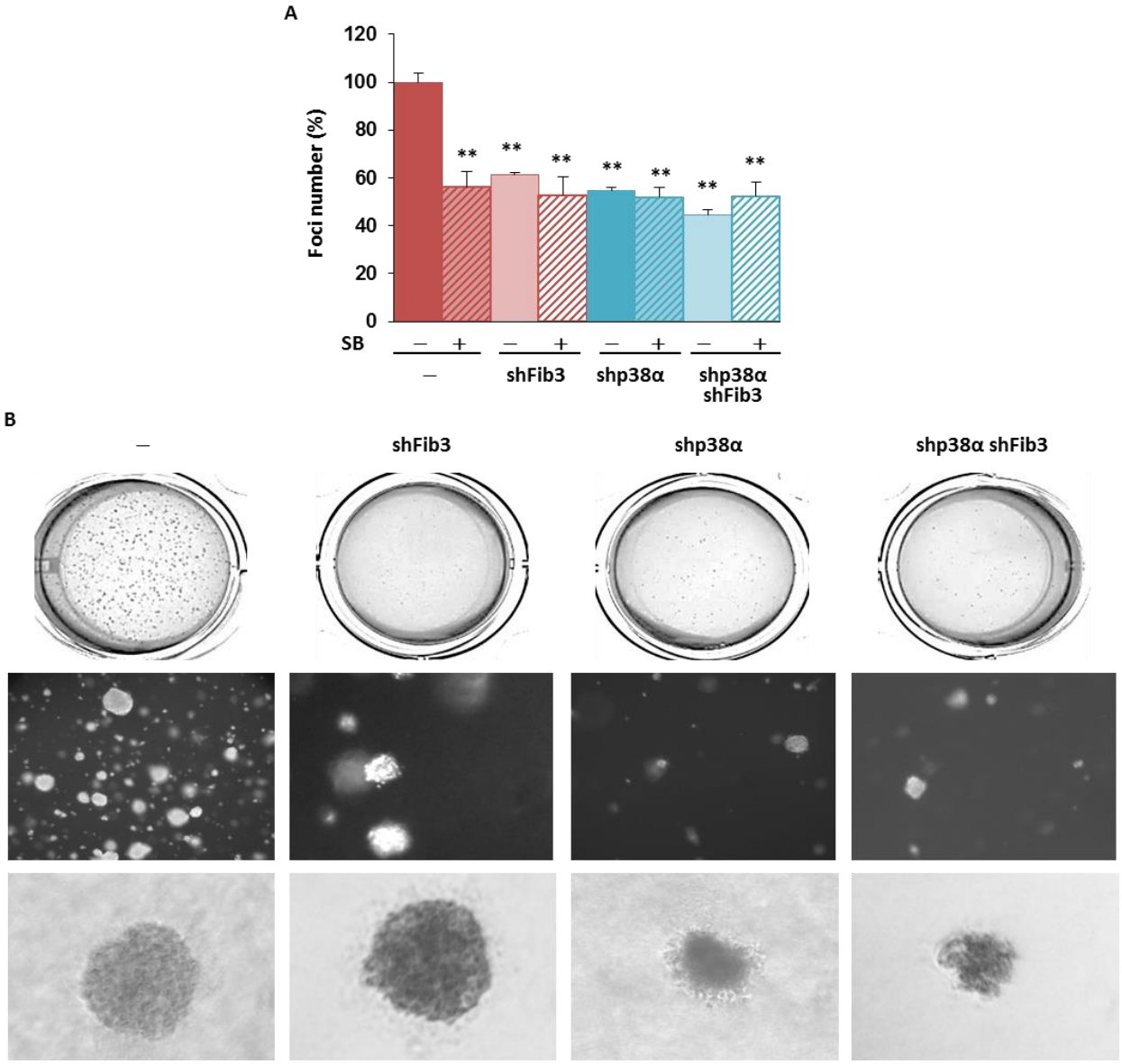

Figure 25- Fibulin 3 and p38α knock-down inhibit anchorage independent growth of HCT116 cells. Anchorage independent growth of HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down) in soft agar was analyzed at 14 days. When indicated, cells were treated with SB203580 (5μM). (A) Histogram shows the mean ± S.E.M. of the foci number expressed as the percentage of non-silenced cells (n=3). **p<0.01 as compared with non-silenced cells. (B) Representative images of several (upper panel) or individual foci (lower panel).

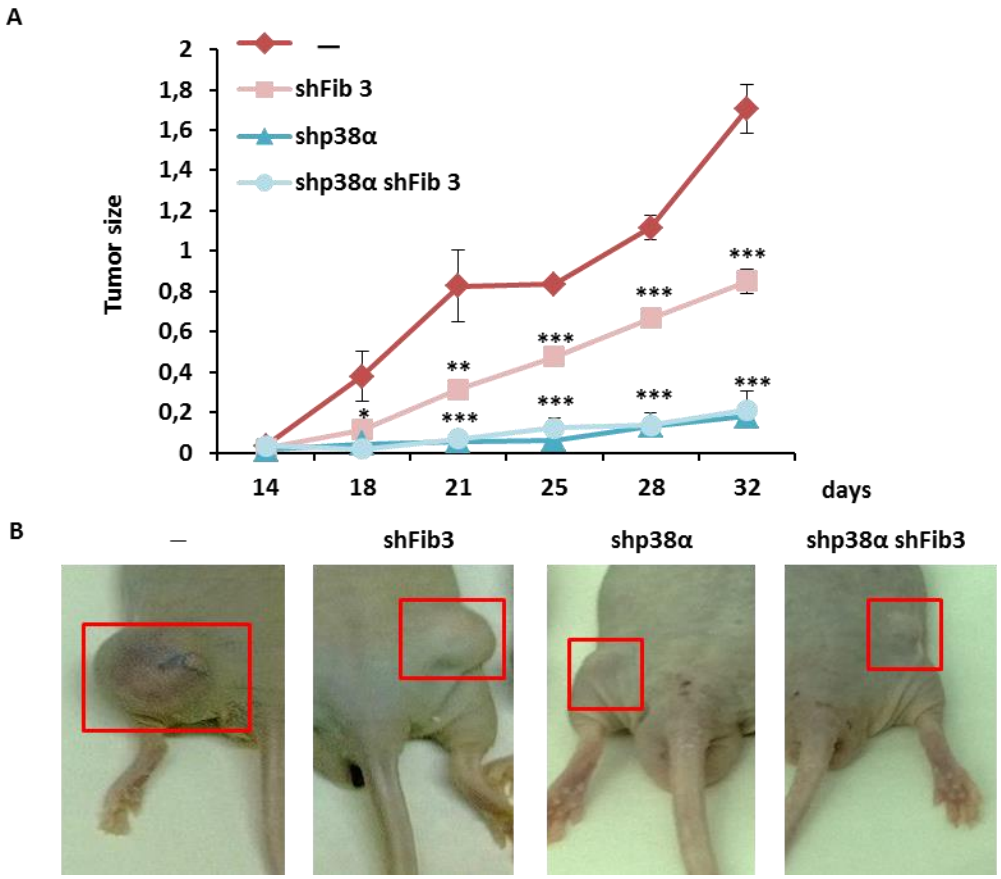

Figure 26- Fibulin 3 and p38α knock-down inhibit *in vivo* HCT116 cells tumor growth. Xenograft assay. Immunodeficient mice were injected subcutaneously with HCT116 cells (non-silenced and p38α knock-down (shp38α); with (shFib3) or without fibulin 3 knock-down). Tumor size was calculated by the formula ((L/2)x(W/2))xπ, where L and W are the longest and the shortest diameter in cm, respectively. (A) Graph shows the mean ± S.E.M. of tumor size at the indicated time points (n=6). *p<0.05, **p<0.01, ***p<0.001 as compared with non-silenced cells. (B) Representative images of tumors.

2. C3G REGULATES CELL ADHESION, MIGRATION AND INVASION THROUGH DOWN-REGULATION OF p38α MAPK ACTIVITY BY A MECHANISM NOT MEDIATED BY RAP1. INVOLVEMENT IN TUMOR GROWTH

As it was mentioned in the introduction and the group background section, we have previously described a crosstalk between C3G and p38α MAPK to regulate apoptosis in CML (Maia et al. 2009) and MEFs (Gutierrez-Uzquiza et al. 2010). Both p38α MAPK (Rousseau et al. 2006, Cuenda and Rousseau 2007, Wagner and Nebreda 2009) and C3G (Ohba et al. 2001, Rufanova et al. 2009, Dayma and Radha 2011) regulate migration. p38α MAPK is also relevant for cell adhesion (Guo and Yang 2006, Zuluaga

et al. 2007), as well as C3G (Ohba et al. 2001, Voss et al. 2003, Guo and Yang 2006, Pannekoek et al. 2009).

Moreover, our group in collaboration with Dr. Guerrero´s group found that C3G and p38α act through a common pathway to regulate cell adhesion in CML (Maia et al. 2013). As previously indicated, C3G also regulates the tumorigenic and/or the migratory and invasive capacity of different cancer cells (Hirata et al. 2004, Gutierrez-Berzal et al. 2006, Okino et al. 2006, Che et al. 2015). Additionally, in colon carcinoma, C3G gene was reported to be demethylated (Samuelsson et al. 2011), suggesting that it could play a role in this type of cancer. Based on all this, we wondered if C3G could act though p38α to control cell migration, invasion and tumor growth in colorectal cancer and whether Rap1, the main C3G target, could be mediating these effects.

2.1 C3G INHIBITS CELL MIGRATION AND INVASION OF HCT116 CELLS THROUGH DOWN-REGULATION OF p38α ACTIVITY BY A MECHANISM NOT MEDIATED BY RAP1. FUNCTION IN CELL ADHESION

Based on Oncomine database, C3G expression can be deregulated in colon carcinoma. Therefore, we analyzed C3G protein expression in different colorectal cancer cell lines with distinct invasive capacities: HCT116, SW480 and SW620 cells. As shown in figure 27, HCT116 cells, which has a lower invasive capacity, show higher levels of C3G, while SW480 and SW620 cells that are more invasive cells, express a very low level of C3G. Therefore, there was an inverse correlation between C3G protein levels and the invasive capacity of colon carcinoma cell lines.

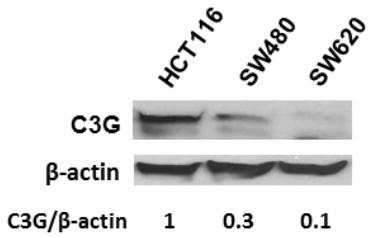

Figure 27-C3G levels in different colorectal cancer cell lines. Western-blot analysis of C3G levels HCT116, SW480 and SW620 cells normalized with β-actin. C3G/β-actin ratio was calculated by densitometric analysis of the blots and expressed as a fold increase.

As C3G down-regulation correlates with an invasive phenotype in colon carcinoma, we have characterized the function of C3G in the regulation of the migratory and the invasive capacities of human HCT116 colon carcinoma cells and whether it acts through p38α. To do it, we performed a permanent C3G knock-down in parental and p38α-silenced cells, using a mixture of different shRNAs, selecting different clones. C3G protein levels significant decreased in both parental and p38α knock-down cells

after silencing (Fig. 28A). Moreover, in response to serum, a significant up-regulation of the levels of phospho-p38α MAPK, phospho-Akt and phospho-ERKs was observed upon C3G silencing (Fig. 28B). This indicates that C3G negatively regulates p38α activity in HCT116 cells as it was previously found in MEFs (Gutierrez-Uzquiza et al. 2010).

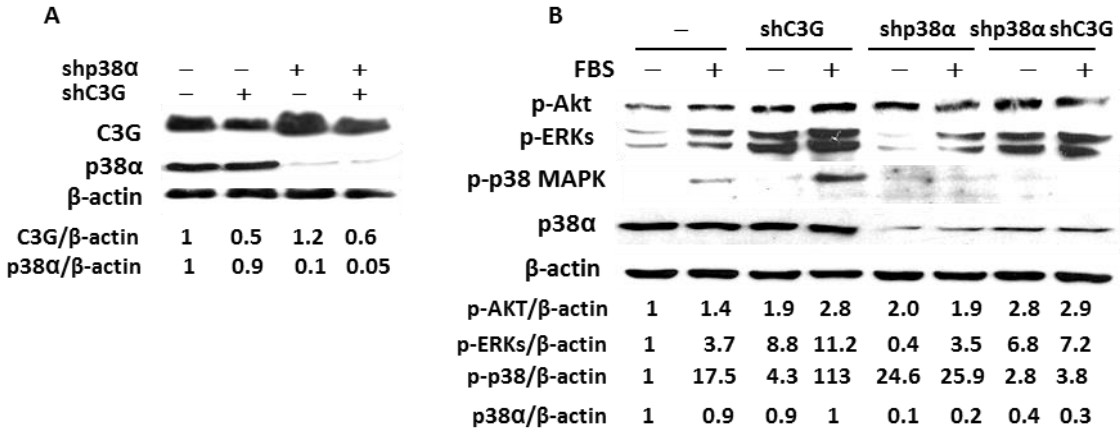

Figure 28- Effect of C3G knock-down on different signaling pathways. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were maintained in the absence of serum for the last 16 h. When indicated, cells were stimulated with serum (FBS) for 10 min. (A) Western-blot analysis of C3G levels. (B) Phospho-Akt, phospho-ERKs and phospho-p38 MAPK levels normalized with β-actin. p38α was used as a control of its expression. C3G/ β-actin, p38α/ β-actin and p-protein/β-actin ratios were calculated by densitometric analysis of the blots and expressed as a fold increase.

Hence, we next determined the effect of C3G knock-down on migration and whether this effect was dependent on p38α activity. As shown in figures 29A and 29B, C3G silencing enhanced migration in HCT116 cells expressing p38α, but not in those subjected to p38α depletion. Similarly, using another shRNA against C3G we also observed an increase in migration in cells expressing p38α (Fig. 29C). Moreover, inhibition of p38α/β with SB203580 prevented the enhancement of migration induced by C3G knock-down in cells expressing p38α and decreased migration of non-silenced cells.

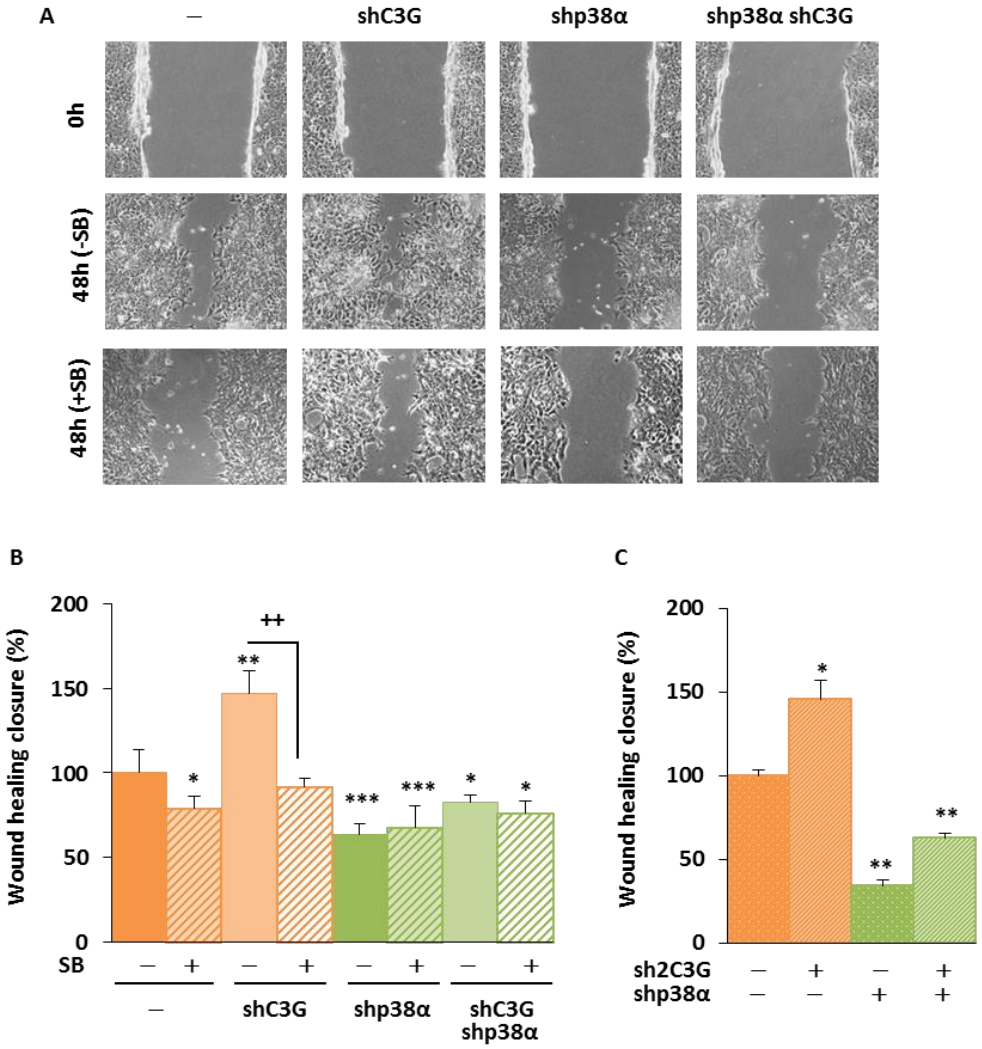

Figure 29- C3G knock-down enhances migration of HCT116 cells through a mechanism dependent on p38α. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) (in A and B) or (sh2C3G) (in C) or without C3G knock-down) were used. Wound healing assays. After doing the wound, cells were maintained in 2% of serum with or without the p38α/β inhibitor, SB203580 (10μM)) and were allowed to migrate. (A) Representative images from phase contrast microscope after 0 and 48 h of migration. (B and C) Histograms shows the mean ± S.E.M. of the percentage of wound closure (n=5-8). *p<0.05, **p<0.01, ***p<0.001 versus non-silenced cells; $^{++}$p<0.01 compared as indicated.

To assess if C3G was acting through its main target Rap1 in this context, Rap1 was knocked-down using two different shRNAs. Figure 30A shows that Rap1 levels were significantly reduced by both shRNAs, which led to a significant reduction in migration (Fig. 30B and 30C). This indicates that Rap1 does not mediate C3G effects on migration.

We also studied C3G function in the regulation of adhesion in HCT116 cells. As shown in figures 31A and 31B, C3G knock-down decreased adhesion of HCT116 cells, while p38α knock-down or p38α chemical inhibition (Fig. 31C) and Rap1 knock-down increased it. Thus, C3G knock-down cells expressing p38α showed the lowest adhesion, which is in agreement with its increased migration. All this indicates that C3G promotes adhesion through down-regulation of p38α activity, while Rap1 exerts an opposite effect.

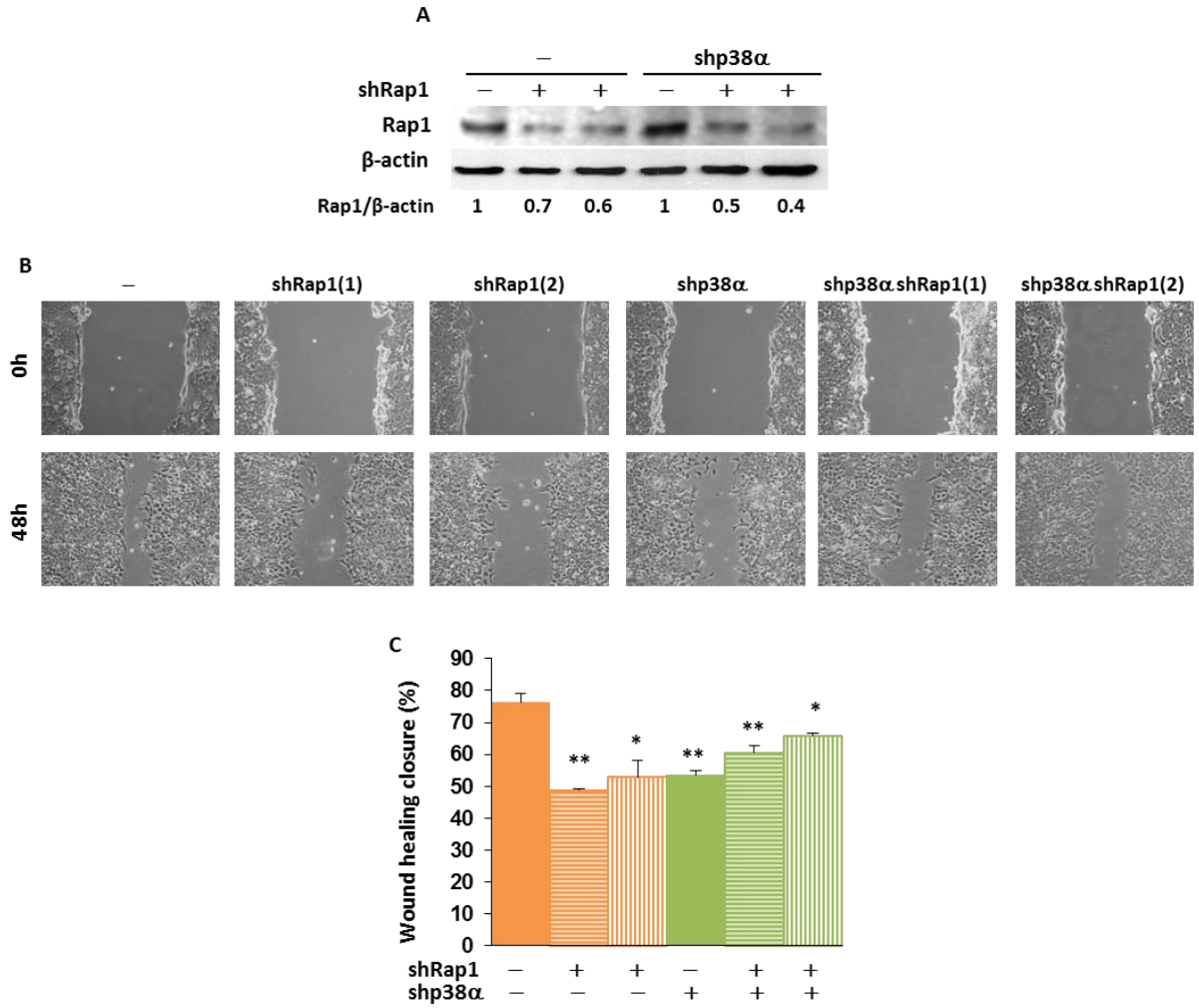

Figure 30- C3G knock-down enhances migration of HCT116 cells through a mechanism not mediated by Rap1. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shRap1(1) and shRap1(2)) or without Rap1 knock-down) were used. (A) Western-blot analysis of Rap1 levels normalized with β-actin. Rap1/β-actin ratio was calculated by densitometric analysis of the blots and expressed as a fold increase. (B and C) Wound healing assay. After doing the wound, cells were maintained in 2% of serum and were allowed to migrate. (B) Representative images from phase contrast microscope after 0 and 48 h of migration. (C) Histogram shows the mean ± S.E.M. of the percentage of wound closure (n=3). *p<0.05, **p<0.01, ***p<0.001 versus non-silenced cells.

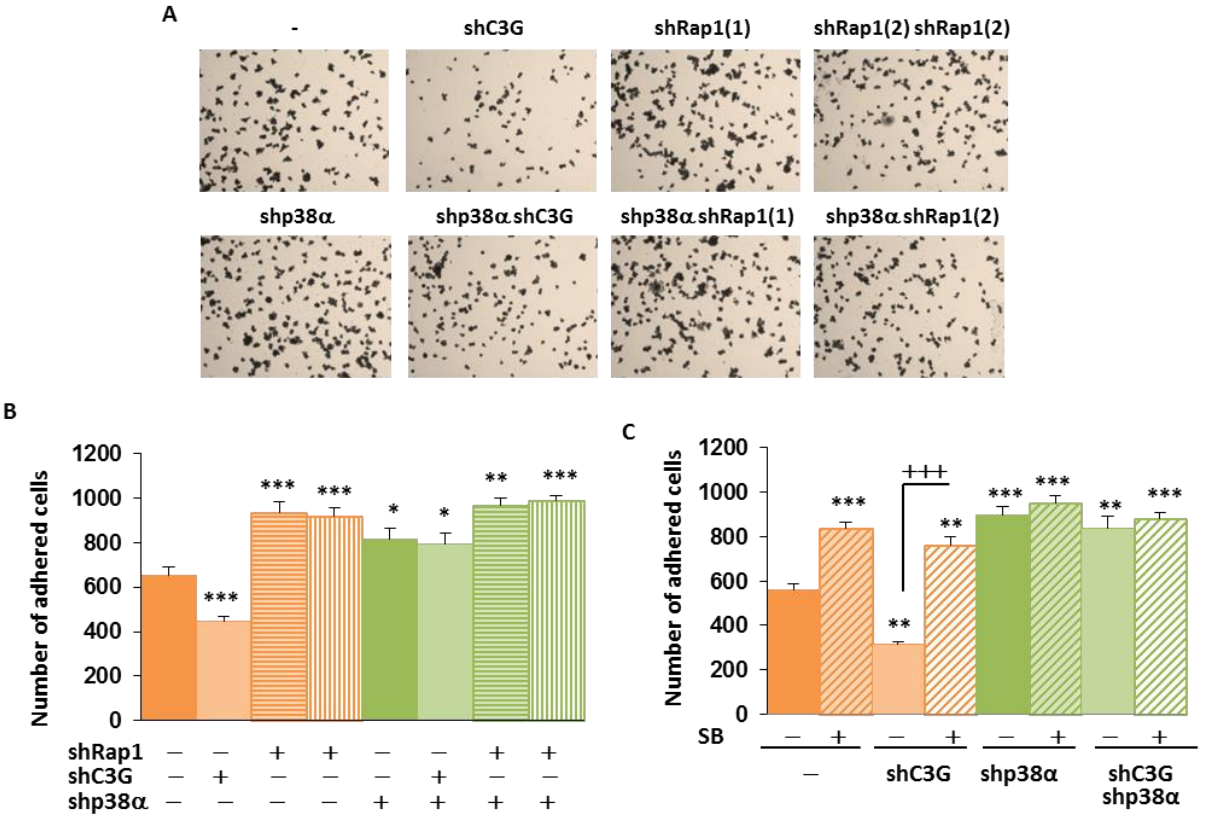

Figure 31- Effect of C3G, p38α and Rap1 knock-down on adhesion. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down; with (shRap1) or without Rap1 knock-down) were used. Adhesion assay. Cells with (C) or without (B) the p38α/β inhibitor, SB203580 (10μM)) were seeded in a medium supplemented with 10% FBS and 1 h after, adhered cells were counted. (A) Representative images from phase contrast microscope. (B and C) Histograms show the mean ± S.E.M. of the number of adhered cells (n=3). *p<0.05, **p<0.01, ***p<0.001 versus non-silenced cells; +++p<0.001 compared as indicated.

Next, we studied invasion of HCT116 cells through matrigel using HGF as chemoattractant. As shown in figures 32A and 32B, in C3G knock-down cells expressing p38α, invasion was increased, but not in those where p38α was also silenced. Accordingly, inhibition of p38α/β with SB203580 impaired it. Single p38α knock-down also reduced invasion.

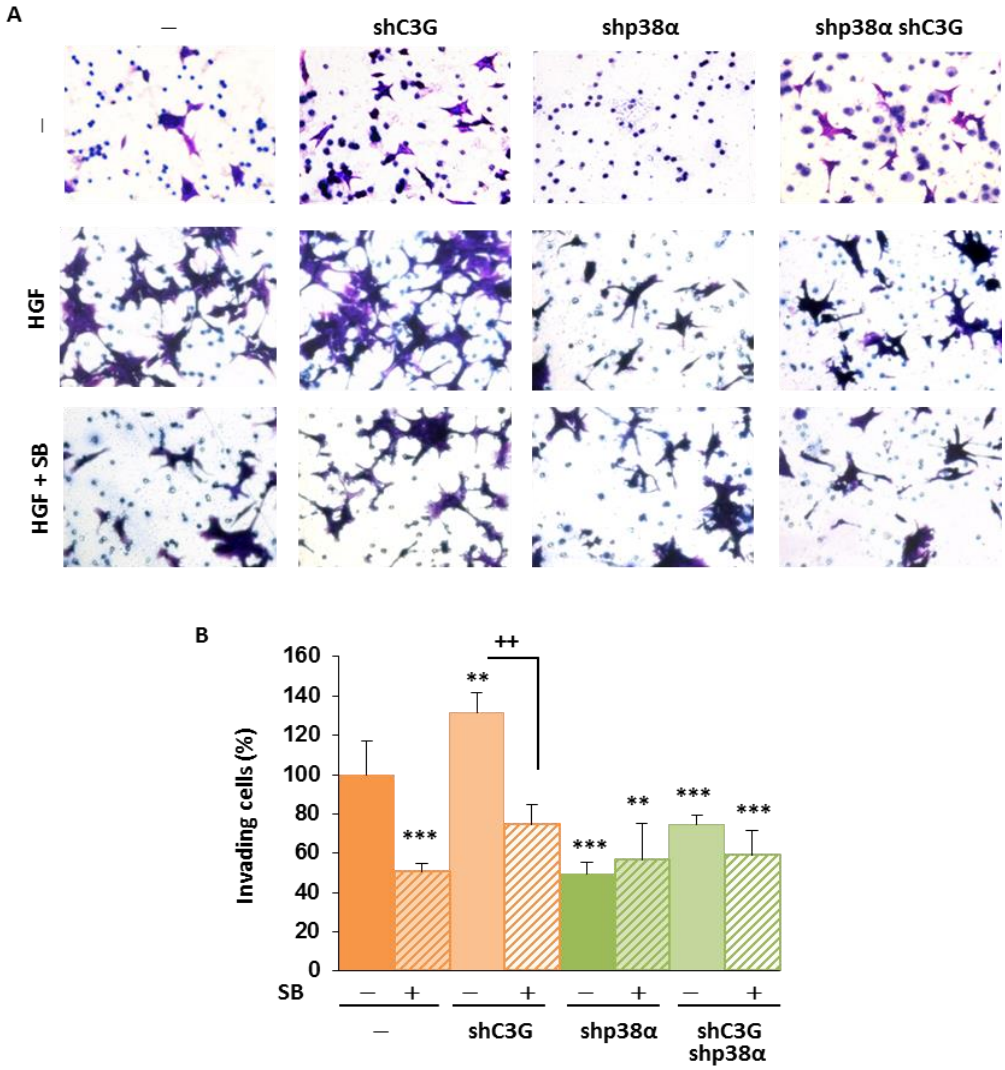

Figure 32- C3G silencing promotes invasion of HCT116 cells by a mechanism mediated by p38α. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were maintained in the absence of serum and in the presence or absence of the p38α/β inhibitor, SB203580 (5μM). Invasion through matrigel using HGF as chemoattractant. (A) Representative images of invading cells after staining with crystal violet (phase contrast microscope). (B) Histogram showing the mean value ± S.E.M. of the percentage of invading cells (n=5). **p<0.01; ***p<0.001 versus non-silenced cells; $^{++}$p<0.01 compared as indicated.

We also studied if C3G could be acting through Rap1 to regulate invasion. However, Rap1 knock-down abrogates invasion (Fig. 33A and 33B). This indicates that C3G is not acting through a Rap1 dependent mechanism.

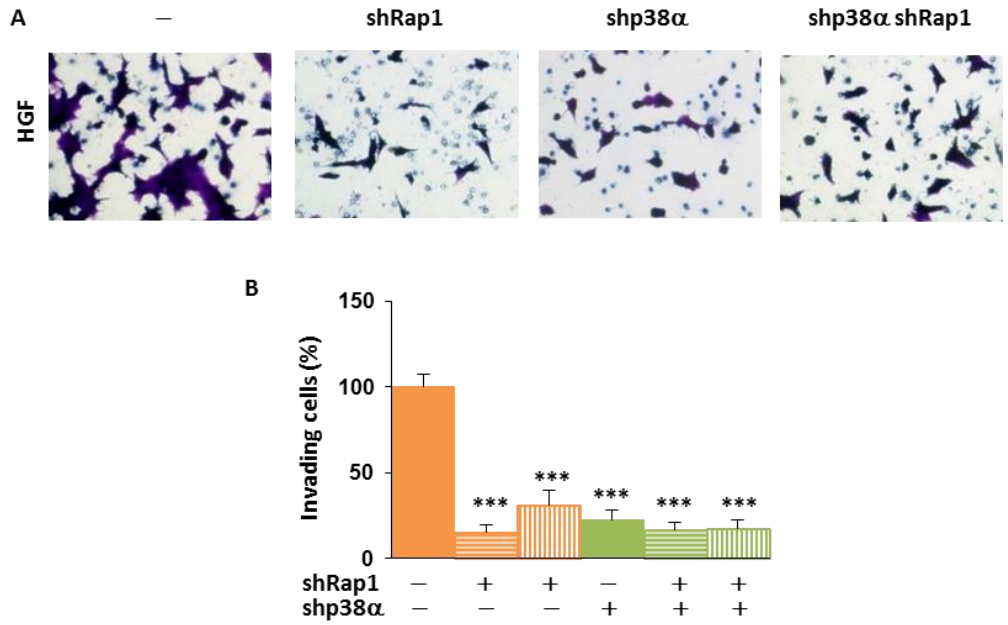

Figure 33-C3G silencing promotes invasion of HCT116 cells by a mechanism not mediated by Rap1. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shRap1) or without Rap1 knock-down) were maintained in the absence of serum. Invasion through matrigel using HGF as chemoattractant. (A) Representative images of invading cells after staining with crystal violet (phase contrast microscope). (B) Histogram showing the mean value ± S.E.M. of the percentage of invading cells (n=3). ***p<0.001 versus non-silenced cells.

All these results indicate that C3G inhibits migration and invasion and promotes adhesion of HCT116 cells through down-regulation of p38α activity. In contrast, Rap1 would play an opposite role, therefore C3G effects on these processes are not mediated by Rap1.

2.2 ROLE OF MMPS AS MEDIATORS OF C3G EFFECTS ON CELL INVASION

MMPs are important for extracellular matrix degradation during cell migration/invasion and play a key role in colorectal cancer progression (Conti and Thomas 2011). Therefore, we determined if they could be responsible of the pro-invasive effect of C3G knock-down in HCT116 cells mediated by p38α.

First, we analyzed the mRNA expression of various MMPs reported to be important for invasion of colon carcinoma cells such as MMP2, 9, 10 and 13 (Fig. 34). C3G silencing significantly reduced MMP10 mRNA levels, while it had no significant effect on the rest.

p38α knock-down decreased MMP 9 and 10 mRNA levels, as well as the double C3G and p38α knock-down, which also decreased MMP13 mRNA levels.

Hence, these changes would explain the reduced invasion observed in cells depleted of p38α and in those with C3G/p38α silencing, but they do not support the C3G effect on invasion when p38α is expressed. Therefore, changes in MMPs activity might account for the changes in invasion.

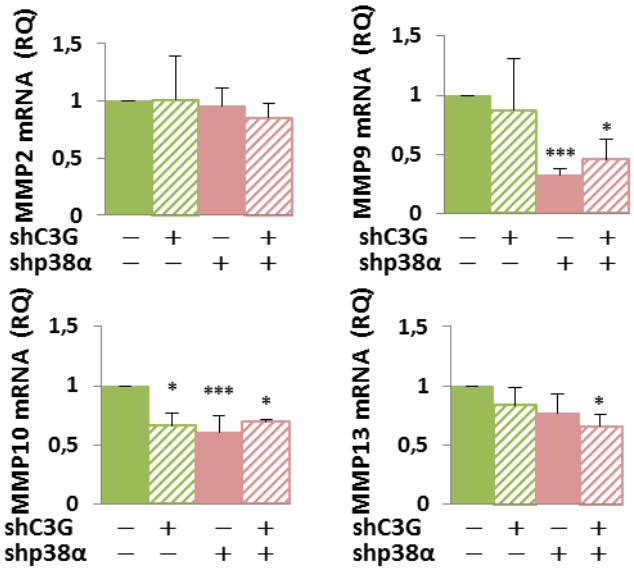

Figure 34-Effect of C3G and p38α knock-down on different MMPs expression. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. Analysis by RT-qPCR of MMP2, 9, 10 and 13 mRNA levels. Histograms show the mean ± S.E.M. of RQ values (n=4). *p<0.05, ***p<0.001 as compared with non-silenced cells.

Hence, we also analyzed MMP2 and MMP9 activities in HCT116 cells by zymography. As shown in figures 35A and 35B, MMP2 and 9 activities were higher in HCT116 cells expressing p38α than in those with p38α silencing. C3G knock-down further increased these activities, but only in cells expressing p38α. These changes in MMP2/9 activities correlate with those in the invasive ability of these cells. Moreover, MMP2 and 9 activities also decreased in cells with Rap1 silencing, regardless of the presence of p38α.Therefore, the regulation of MMP2 and 9 activities by C3G, p38α and/or Rap1 might represent a mechanism to control invasion.

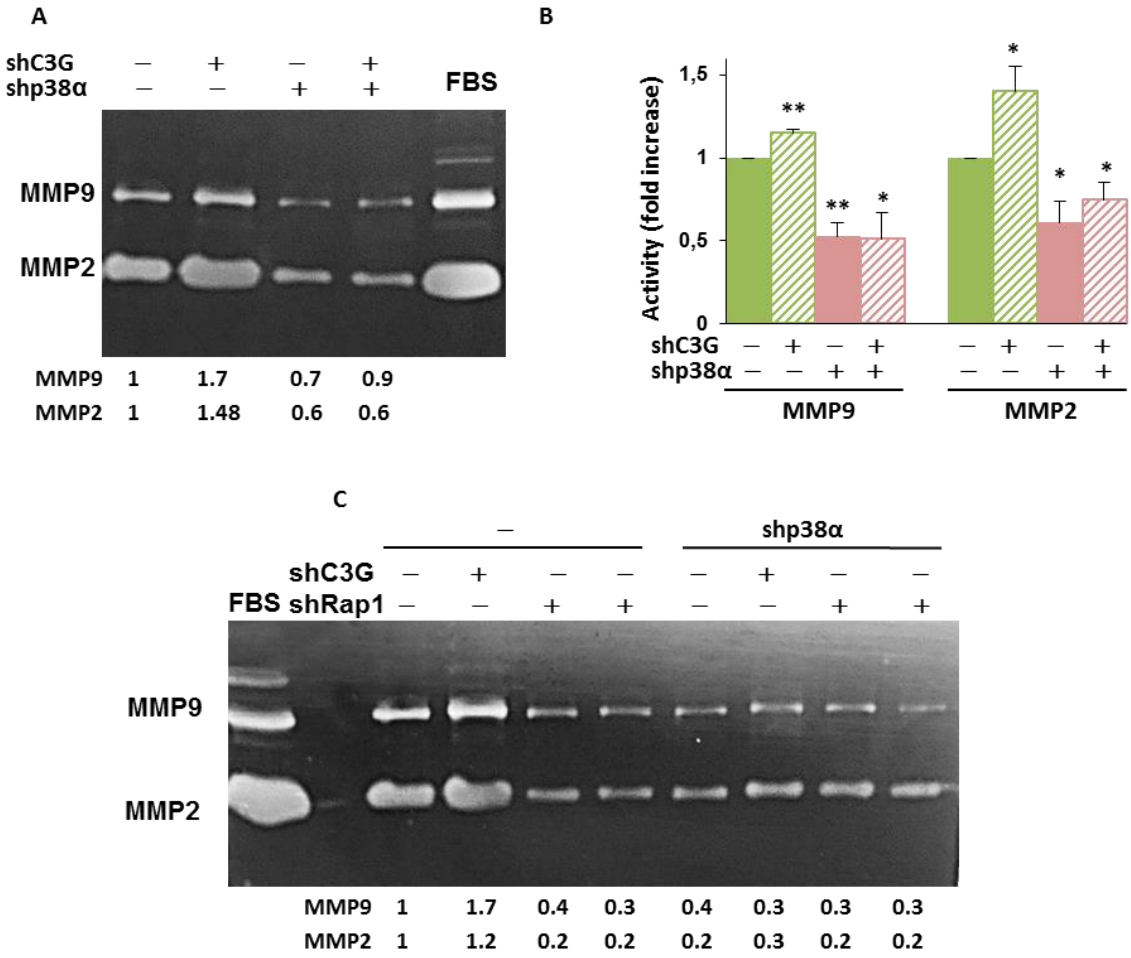

Figure 35- Effect of C3G, p38α and Rap1 knock-down on MMP2/9 activities. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down; with (shRap1) or without Rap1 knock-down) were used. Zymographic analysis of MMP2/9 activities using gelatin as substrate and FBS as a control. (A and C) Representative zymograms. (B) Histogram showing the mean ± S.E.M. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value (n=4). *p<0.05, **p<0.01 versus non-silenced cells.

2.3 MECHANISMS INVOLVED IN THE REGULATION OF MIGRATION AND INVASION BY C3G AND p38α

Cell migration and invasion involve changes in the expression and/or subcellular localization of cell-cell contact proteins (Thiery et al. 2009, Lamouille et al. 2014) and the re-organization of F-actin cytoskeleton (Parsons et al. 2010). Moreover, induction of a migratory and invasive phenotype is often associated with epithelial-mesenchymal transition (EMT).

An important protein present in adherent junctions is E-cadherin, playing a key role in cell-cell interactions. In fact, E-cadherin loss is a key event of many EMT processes (Tsanou et al. 2008, Pancione et al. 2012). C3G interacts with E-cadherin cytoplasmic domain, which participates in the maturation of E-cadherin-based cell-cell contacts

(Hogan et al. 2004, Kooistra et al. 2007). Thus, we studied E-cadherin as a potential mediator of C3G and/or p38α on migration and invasion. As shown in figure 36A, E-cadherin protein levels substantially decreased in HCT116 cells subjected to C3G knock-down, with or without p38α depletion. Silencing of p38α also led to E-cadherin down-regulation, so that, the largest reduction in E-cadherin levels was observed in cells with C3G and p38α knock-down. Similarly, E-cadherin mRNA levels also decreased upon C3G and p38α Knock-down, but double knock-down had no further effect (Fig. 36B). This decrease in E-cadherin did not correlate with the migratory and invasive capacity of the cells. Hence, we then, analyzed changes in E-cadherin subcellular localization by confocal microscopy. In all cases, E-cadherin was mainly located in the membrane, although in the double knock-down it was partially internalized (Fig. 36C).

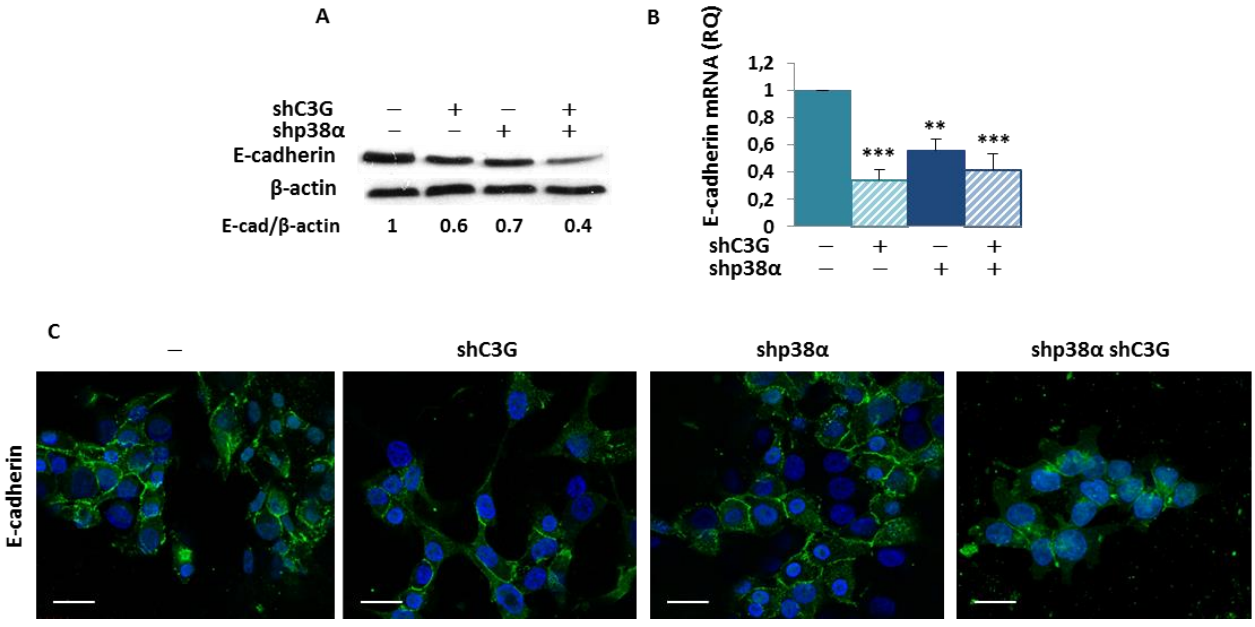

Figure 36-Effect of C3G knock-down on E-cadherin expression and subcellular localization in HCT116 cells. Function of p38α MAPK. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. (A) Representative western-blot analysis of E-cadherin levels normalized with β-actin. E-cadherin/β-actin ratio was calculated by densitometric analysis of the blots and expressed as a fold increase. (B) Analysis by RT-qPCR of E-cadherin mRNA levels. Histogram showing the mean ± S.E.M. of RQ values (n=5). **p<0.01, ***p<0.001 as compared with non-silenced cells. (C) Representative confocal microscopy images of E-cadherin immuno-detection using a specific antibody and a FITC-labeled anti-mouse (green). Nuclei were stained with DAPI. Scale bar=20μm

We also analyzed the levels and localization of ZO-1 protein, a main component of tight junctions, which is crucial to maintain cell-cell contacts and is also regulated in EMT processes (Polette et al. 2007). Although no significant changes in total ZO-1 protein levels were observed upon C3G knock-down in cells expressing p38α (Fig. 37A), ZO-1 was found to be internalized (Fig. 37C). In contrast, in p38α silenced HCT116 cells,

ZO-1 was mainly present in the membrane, as it was also observed in cells with the double C3G/p38α knock-down, where its total level was reduced (Fig. 37A and 37B).

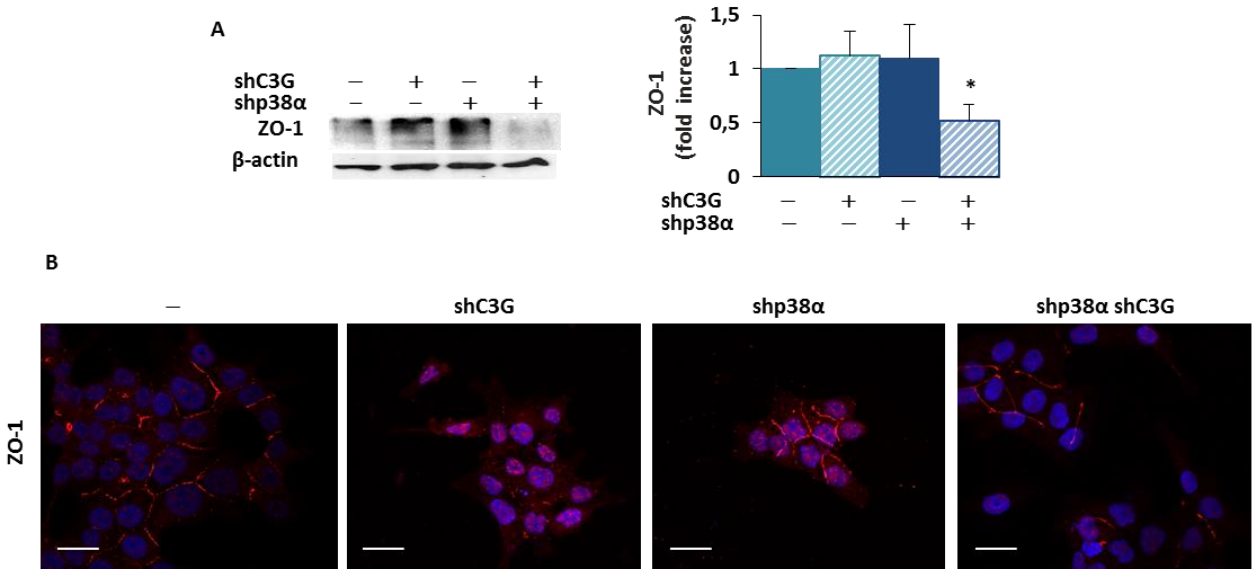

Figure 37-Effect of C3G knock-down on ZO-1 expression and subcellular localization in HCT116 cells. Function of p38α MAPK. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. (A) Representative western-blot analysis of ZO-1 levels normalized with β-actin (left panel). Histogram (right panel) showing the mean ± S.E.M. of the densitometric analysis of blots (n= 4). *p<0.05 as compared with non-silenced cells. (B) Representative confocal microscopy images of ZO-1 immunodetection using a specific antibody and anti-rabbit Alexa 594 (red). Nuclei were stained with DAPI. Scale bar=20μm.

These data suggest that cell-cell contacts, specially tight junctions, are partially disrupted in C3G knock-down HCT116 cells expressing p38α, which would favor migration and invasion processes.

EMT is involved in the generation of metastasis as it allows epithelial cells to acquire a migratory and invasive phenotype defined by the loss of epithelial-specific proteins, including tight- and adherens-junction proteins (Grunert et al. 2003). Therefore, the reduction in E-cadherin levels and ZO-1 internalization observed upon C3G knock-down could be associated with an EMT process. Thus, we next determined the expression of the mesenchymal marker, N-cadherin. Its protein (Fig. 38A) and mRNA levels (Fig. 38B) increased upon C3G, p38α and double C3G/ p38α knock-down in HCT116 cells. These results suggest that C3G, p38α and C3G/p38α knock-down could induce an EMT process, which it is not correlated in all cases with migratory and invasive capacities of the cells.

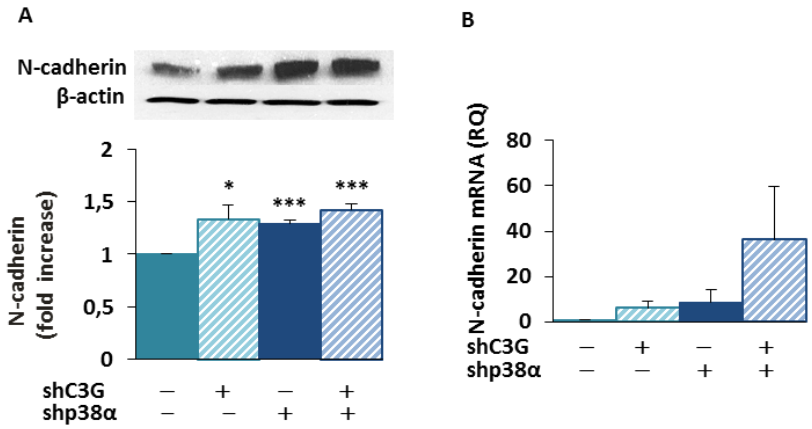

Figure 38-Effect of C3G knock-down on N-cadherin expression in HCT116 cells. Function of p38α MAPK. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. (A) Representative western-blot analysis of N-cadherin levels normalized with β-actin and histogram showing the mean ± S.E.M. of the densitometric analyses of blots (n=4). (B) Analysis by RT-qPCR of N-cadherin mRNA levels. Histogram represents the mean ± S.E.M. of RQ values (n=5). *p<0.05, ***p<0.001 as compared with non-silenced cells.

To gain a further insight into the potential function of C3G regulating EMT, we studied the expression of some transcription factors involved in this process. In particular, Snail 1, Twist 1, Zeb 1 and Zeb 2 mRNA levels were quantified by RT-qPCR. Snail 1 and Zeb 1 mRNAs levels increased in C3G knock-down HCT116 cells expressing p38α (Fig. 39). On the other hand, p38α knock-down induced a significant increase in Zeb 1 and Zeb 2 mRNA levels, while double C3G/p38α silencing did not significantly modify these mRNA levels, although a tendency to increase them was observed (Fig. 39). These data suggest that C3G knock-down might be inducing EMT, which is correlated with the migratory and invasive properties of C3G depleted cells. However, p38α single knock-down might also have an effect inducing EMT, which does not correlate with the migratory and invasive properties of shp38α HCT116 cells.

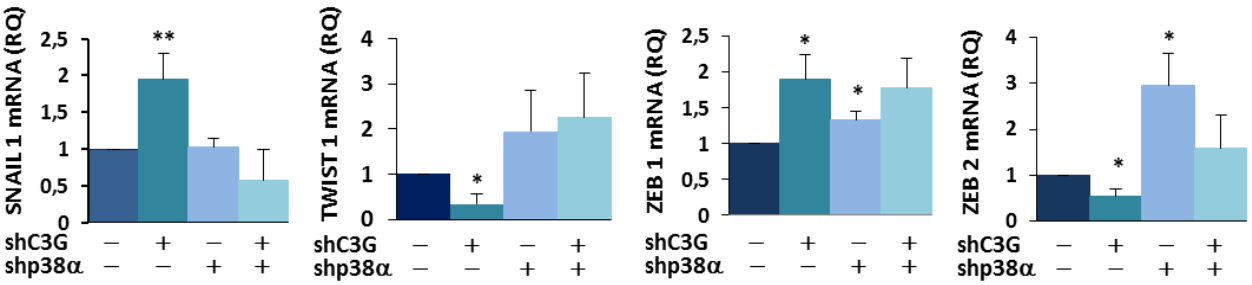

Figure 39-Effect of C3G and p38α knock-down on the expression of transcription factors involved in EMT. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were maintained in the absence of serum for 24 h and then, total RNA were isolated. Analysis by RT-qPCR of Snail 1, Twist 1, Zeb 1 and Zeb 2 mRNA levels. Results represent the mean ± S.E.M. of RQ values (n=4). *p<0.05, **p<0.01 as compared with non-silenced cells.

Hence, we next studied F-actin cytoskeleton organization by confocal microscopy due to its relevance in migration. F-actin enriched structures including ruffles, filopodia and lamelipodia are usually present in migratory cells as a consequence of the actin re-organization (Franz et al. 2002), which can be regulated by p38α (Huot et al. 1998, Okada et al. 2005, del Barco Barrantes and Nebreda 2012).

As shown in figure 40, the presence of filopodia and lamelipodia was more noticeable in C3G knock-down HCT116 cells expressing p38α (see arrows), which would promote migration of these cells.

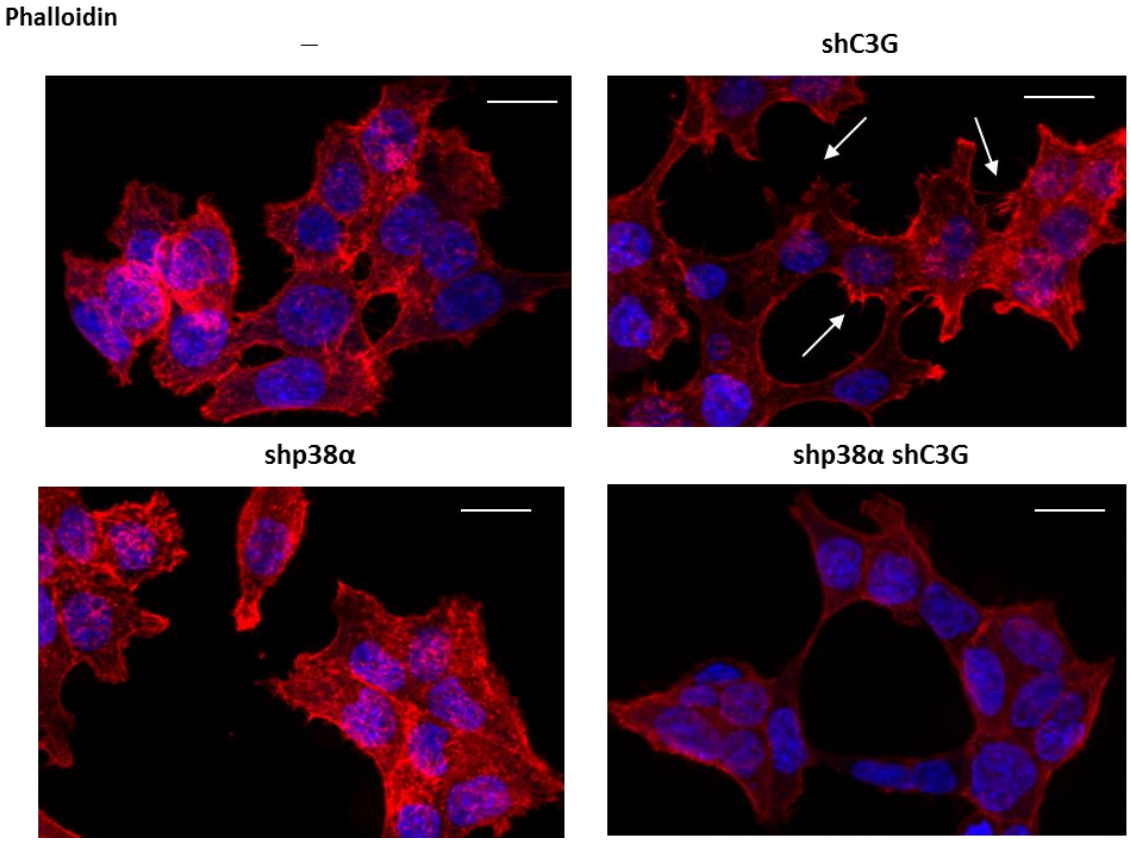

Figure 40-Effect of C3G knock-down on actin-organization in HCT116 cells. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. Representative confocal microscopy images of actin staining, using rhodamine conjugated phalloidin. Scale bar=20μm. Arrows indicates the presence of filopodia.

All these studies evidence a disruption of cell-to-cell contacts in C3G knock-down HCT116 cells expressing p38α due to both ZO-1 internalization and partial E-cadherin loss. All this together with the actin cytoskeleton re-organization produced in these cells could explain the enhancement in migration and invasion.

2.4 ROLE OF C3G IN THE REGULATION OF THE TUMORIGENIC CAPACITY OF HCT116 CELLS

To study the function of C3G and p38α on the tumorigenic ability of HCT116 cells, we first carried out anchorage dependent growth assays. As shown in figure 41A, the assays revealed a reduction in the number of foci in C3G knock-down cells, which was more prominent in cells expressing p38α. The number of foci was also reduced when p38α was silenced. These results suggest that both C3G and p38α promote foci formation probably through different pathways. Moreover, inhibition of p38α with SB203580 decreased the number of foci in non-silenced cells (Fig. 41A). In contrast, in C3G knock-down cells, the foci number was increased by SB203580 treatment, while having no effect on double C3G/p38α knock-down cells. This effect of p38α/β inhibition might be a consequence of the increase in adhesion (Fig. 31C). We also studied the number of cells per focus and its morphology. As shown in figure 41B, both C3G and p38α knock-down decreased the number of cells per focus and SB203580 only reduced them in non-silenced cells. Moreover, the foci formed by C3G knock-down cells, especially those expressing p38α, showed a decrease in cell-cell contacts, so that, cells were more disperse than those expressing C3G (Fig. 41C).

We next asked if Rap1 was mediating C3G actions on foci formation. As shown in figures 42A and 42B, Rap1 knock-down led to a significant increase in the number of foci in p38α knock-down HCT116 cells, but not in cells expressing p38α, even though there was a tendency to increase it. Thus, C3G and Rap1 knock-down have different effects on anchorage dependent growth of HCT116 cells.

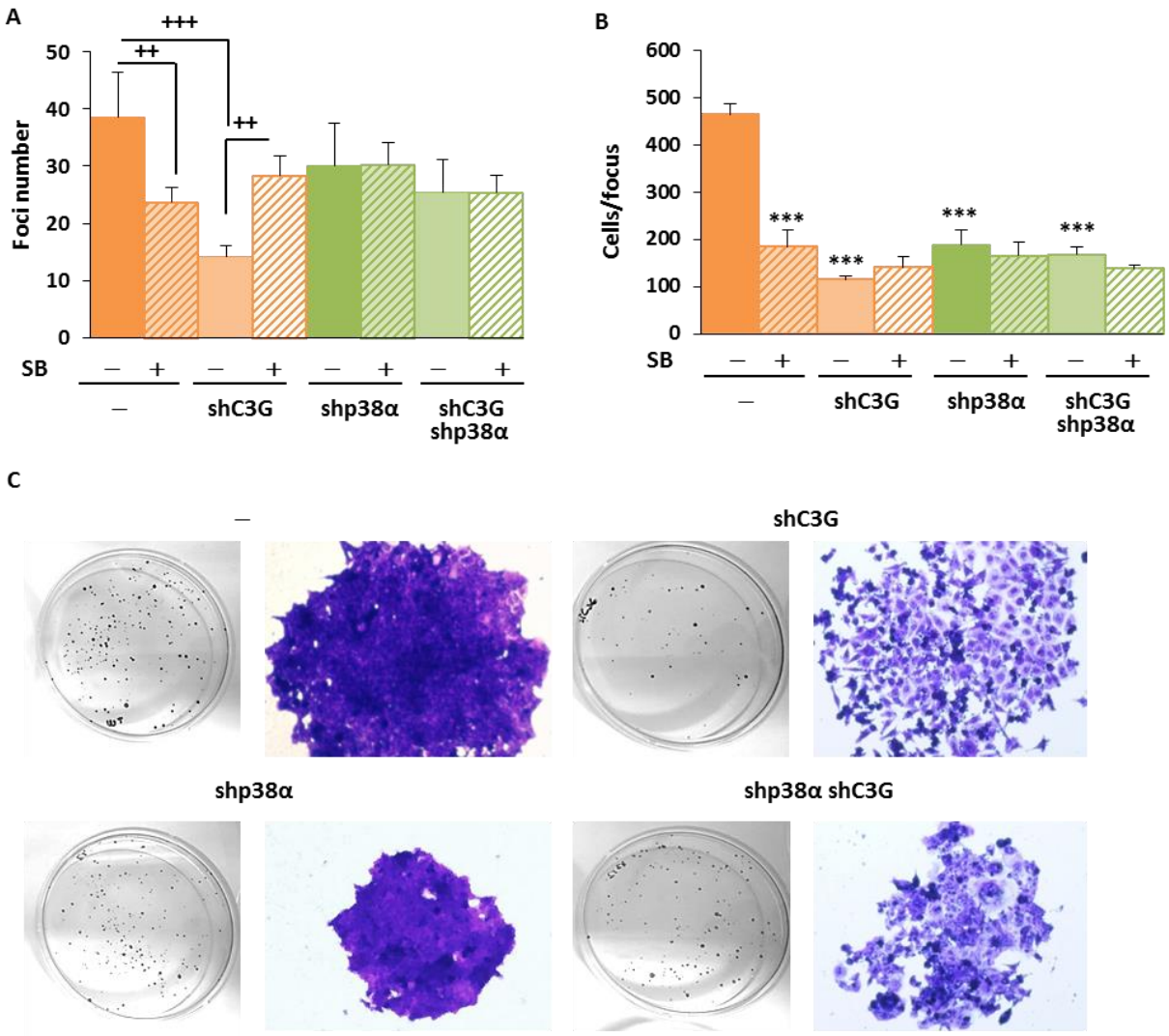

Figure 41-C3G and p38α MAPK promote anchorage dependent growth of HCT116 cells. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. When indicated, cells were treated with SB203580 (10μM) to inhibit p38α/β. Anchorage dependent growth assay at 10 days. (A and B) Histograms show the mean ± S.E.M. of foci number (A) or the number of cells per focus (B) (n=5). ***p<0.001 versus non-silenced cells; $^{++}$p<0.01, $^{+++}$p<0.001 compared as indicated. (C) Representative images of foci from a plate (left) and individual foci (right).

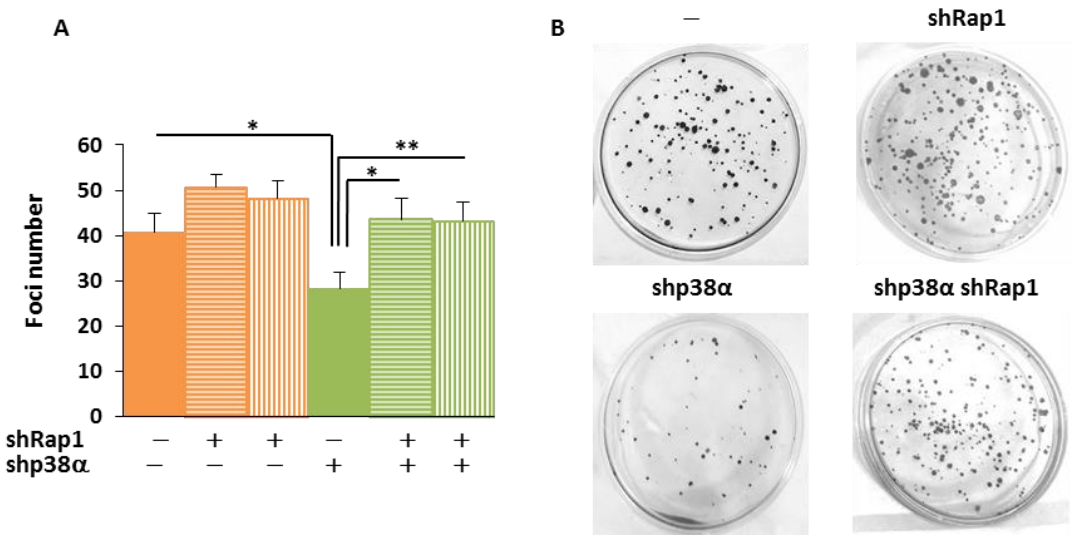

Figure 42-Rap1 inhibits anchorage dependent growth of HCT116 cells. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shRap1) or without Rap1 knock-down) were used. Anchorage dependent growth of HCT116 cells at 10 days. (A) Histogram showing the mean ± S.E.M. of foci number (n=3). *p<0.05, **p<0.01 compared as indicated. (B) Representative images of foci from a plate.

To further understand the role of C3G and p38α in tumor growth, we analyzed the effect of C3G and p38α knock-down on anchorage independent growth in soft-agar. As shown in figure 43A, the number of foci was highly reduced upon C3G Knock-down, p38α knock-down, double knock-down or p38α inhibition with SB203580. However, the largest reduction in the number of foci was observed in C3G depleted HCT116 cells expressing p38α. Moreover, foci generated by non-silenced HCT116 cells are larger in size and double knock-down cells foci seemed to have less cells and more disperse (Fig. 43B). Moreover, cells from double knock-down foci did not look to be healthy and tend to move out of the focus.

All these results suggest that C3G does not act through p38α to control anchorage independent growth in HCT116 cells.

As shown in figure 44, foci number was increased upon Rap1 knock-down, mainly in p38α knock-down HCT116 cells. This indicates that Rap1 is not mediating C3G effects, but rather counteracting them.

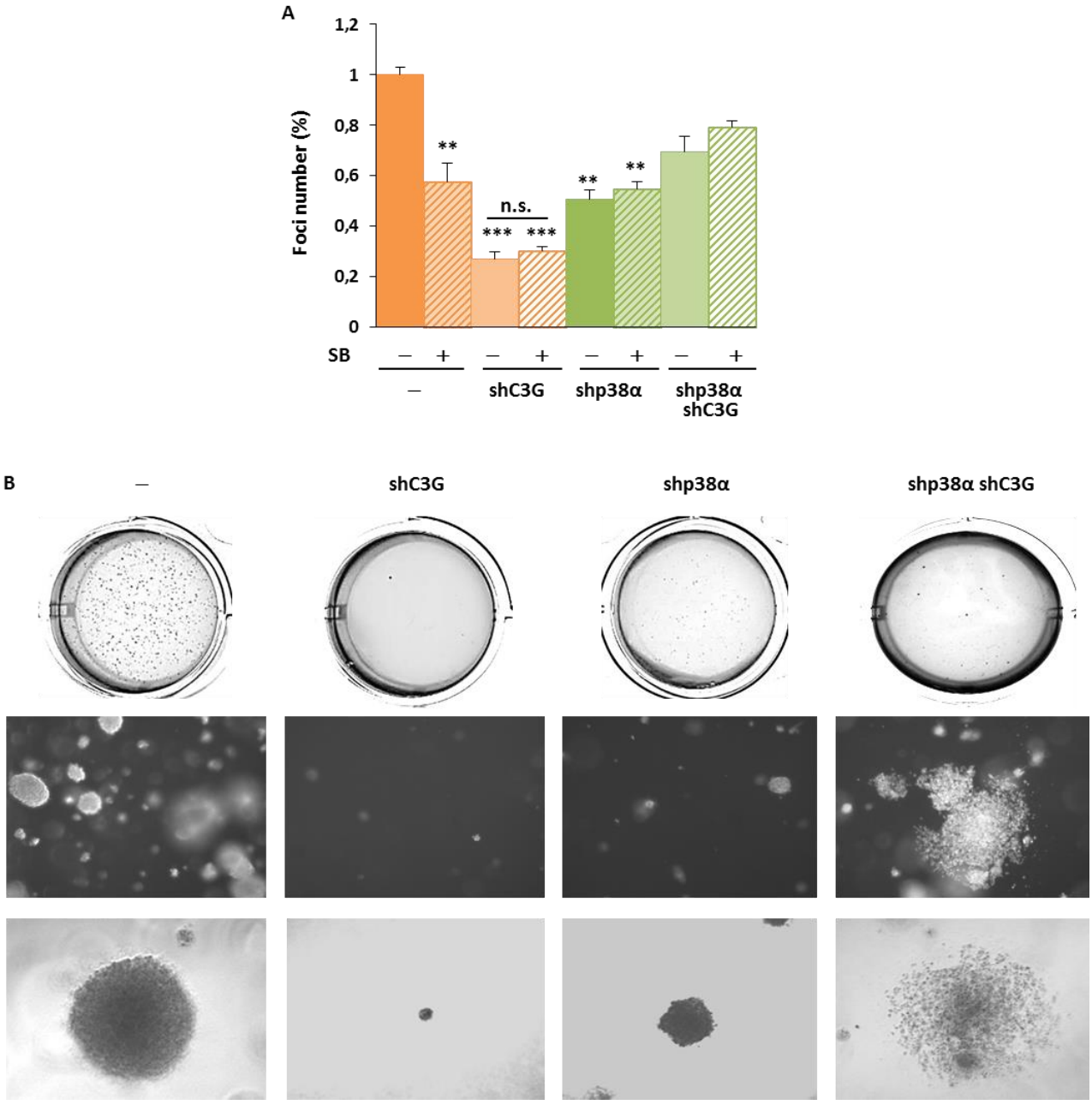

Figure 43-C3G and p38α MAPK promote tumor growth *in vitro* of HCT116 cells. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. Anchorage independent growth at 14 days, in the absence or presence of SB203580 (10μM) as indicated. (A) Histogram shows the mean value ± S.E.M. of the foci number expressed as a fold increase of non-silenced cells (n=4). **p<0.01, ***p<0.001 as compared with non-silenced cells. (B) Representative images of several (upper panel) or individual foci (lower panel).

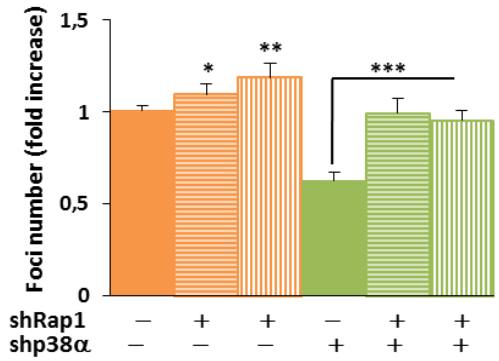

Figure 44- Rap1 does not promote tumor growth *in vitro* of HCT116 cells. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down; with (shRap1) or without Rap1 knock-down) were used. Anchorage independent growth of HCT116 cells at 14 days. Histogram showing the mean value ± S.E.M. of the foci number expressed as a fold increase of non-silenced cells (n=3). *p<0.05, **p<0.01, ***p<0.001 as compared with non-silenced cells, or as indicated.

Finally, we assessed the involvement of C3G on the tumorigenic activity of HCT116 cells in nude mice. Upon subcutaneous inoculation of control HCT116 cells, tumors were readily visible at 14 days and progressively grew over time (Fig. 45A and 45B). Tumor sizes were significantly reduced in cells subject to either C3G or p38α knock-down. Tumor growth was essentially undetectable in cells subject to double knock-down. This potential collaboration between C3G and p38α promoting tumor growth was not observed in anchorage independent growth assays, where double knock-down HCT116 cells grew a little bit more than either C3G or p38α knock-down cells.

All these results suggest that C3G and p38α MAPK are both promoting HCT116 cells foci formation and *in vivo* tumor growth, probably through independent mechanisms.

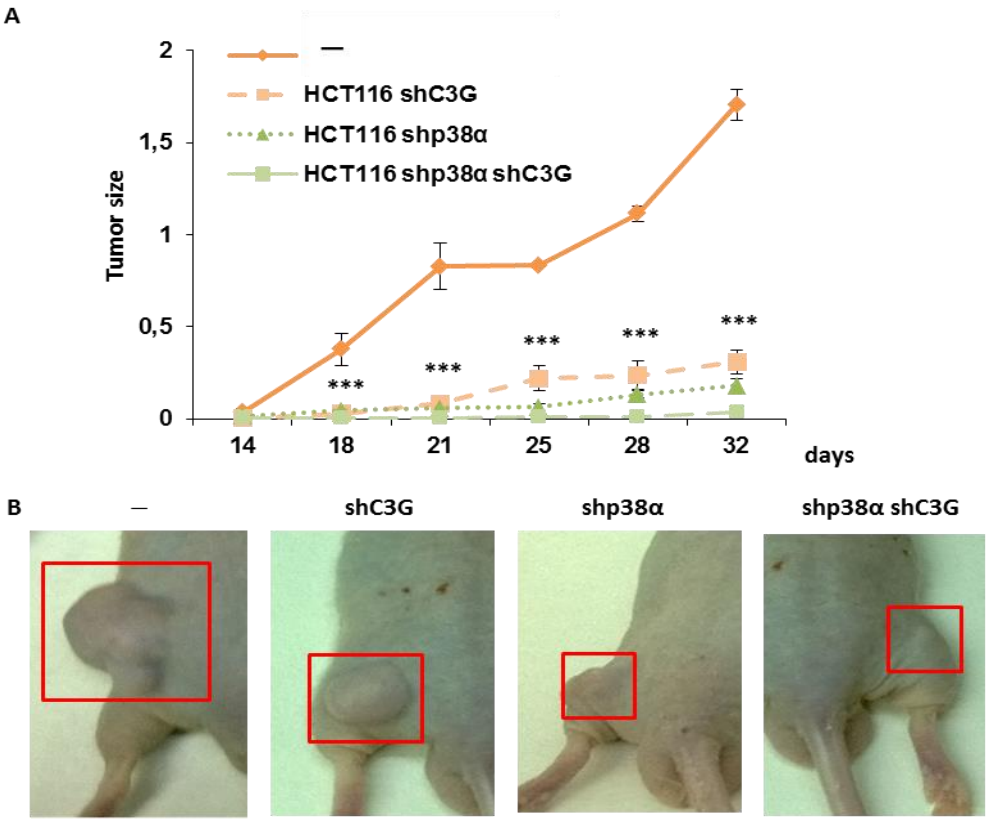

Figure 45- C3G and p38α MAPK promote tumor growth *in vivo* of HCT116 cells. Xenograft assay. Immunodeficient nude mice were injected subcutaneously with HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down). Tumor size was calculated by the formula ((L/2)x(W/2))xπ, where L and W are the longest and the shortest diameter in cm, respectively.
(A) Graph shows the mean value ± S.E.M. of tumor size at the indicated time points (n=6). ***p<0.001 versus non-silenced cells. (B) Representative images of tumors in nude mice.

2.5 MECHANISMS INVOLVED IN THE REGULATION OF TUMOR GROWTH BY C3G AND p38α

The potential collaboration between C3G and p38α promoting tumor growth was not observed in anchorage independent growth assays, where double knock-down HCT116 cells grew more than either C3G or p38α knock-down cells. To explain this different effect of C3G and p38α knock-down on tumor growth *in vitro* and *in vivo*, we first studied the viability of HCT116 cells. We did not find significant differences in cell viability under basal conditions and we subjected the cells to some stimuli that could mimic some aspects of the *in vivo* tumor microenvironment. First, taking into account the elevated rate of oxidative metabolism that leads to the generation of high levels of reactive oxygen species (ROS), cells were exposed to oxidative stress. As shown in figure 46A, treatment with H_2O_2 significantly reduces cell viability in all cases, but its effect was more noticeable in single C3G or p38α knock-down cells and even more in those with double silencing.

We also evaluated their ability to survive in suspension, and then, be able to attach. Cells suffer a particular type of cell death due to the absence of attachment to extracellular matrix (ECM) or upon cell adhesion to inappropriate location, called anoikis. Anoikis is a critical mechanism that prevents adherent-independent cell growth and attachment to an inappropriate matrix, thus avoiding the colonization of distant organs (Paoli et al. 2013). As shown in figure 46C, the number of adhered cells after 6 h in suspension significantly decreased in C3G knock-down cells, regardless of the presence of p38α. Moreover, the percentage of apoptotic cells under these conditions was increased, but mainly in double p38α and C3G knock-down cells (Fig. 46D). All this suggests that C3G-p38α depleted cells are more sensitive to the lack of attachment and they might dye under these conditions.

This reduction in cell viability might explain the differences between *in vitro* and *in vivo* assays of tumor growth. Thus, although there was no significant decrease in the number of foci generated by double shp38α-shC3G knock-down cells compared to non-silenced cells, their viability in response to different stimuli was significantly lower, which could explain the reduced *in vivo* tumor growth.

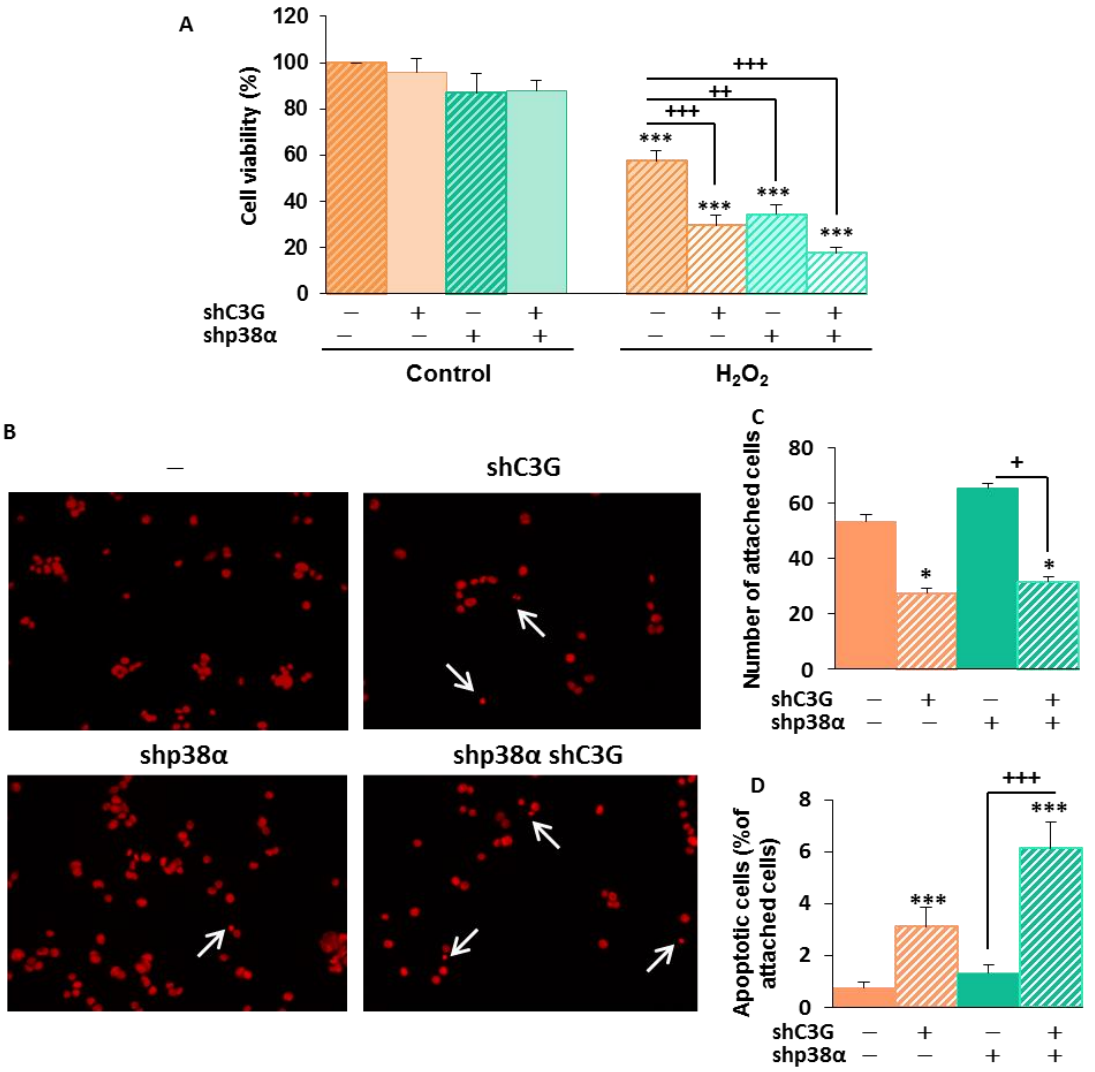

Figure 46-Effect of C3G and p38α knock-down on cell viability. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. (A) Viability assay. Cells were maintained in the absence of serum and treated with H_2O_2 2 mM for 4 h. Then, cells were fixed and stained with crystal violet. Histogram showing the mean value ± S.E.M. of the percentage of viable cells referred to untreated parental cells (100%) (n=4). ***p<0.001 versus non-silenced cells; ++p<0.01, +++p<0.001 compared as indicated. (B, C and D) Cell viability in suspension. Cells were maintained in suspension for 6 h and then, allowed to attach for 4 h. (B) Representative images of propidium iodide staining (fluorescence microscope), condensed nuclei are marked. (C) Histogram shows the mean value ± S.E.M. of the number of attached cells (n=3). *p<0.01 versus non silenced cells; +p<0.05 compared as indicated. (D) Histogram shows the mean value ± S.E.M. of the percentage of apoptotic cells (n=3). ***p<0.001 versus non-silenced cells; $^{+++}$p<0.001 compared as indicated.

To further understand the potential mechanisms involved in the *in vivo* regulation of tumor growth, we analyzed the tumors from xenografts assays. Different immunofluorescence and immunohistochemistry analyses were performed to assess the morphology of tumor cells and the expression of proteins from cell-cell interactions, as well as the presence of apoptotic cells and stromal cells within the tumors. Both E-cadherin (Fig. 47A) and ZO-1 (Fig. 47B) expression decreased in xenografts derived from C3G, p38α or C3G-p38α double silenced HCT116 cells. Additionally, E-cadherin was partially internalized in C3G knock-down cells expressing p38α (Fig. 47A), which would be in agreement with the migration and invasion data. These results suggest a disruption of adherent- and tight-junctions in cell-cell contacts upon C3G and/or p38α knock-down that might impair tumor formation.

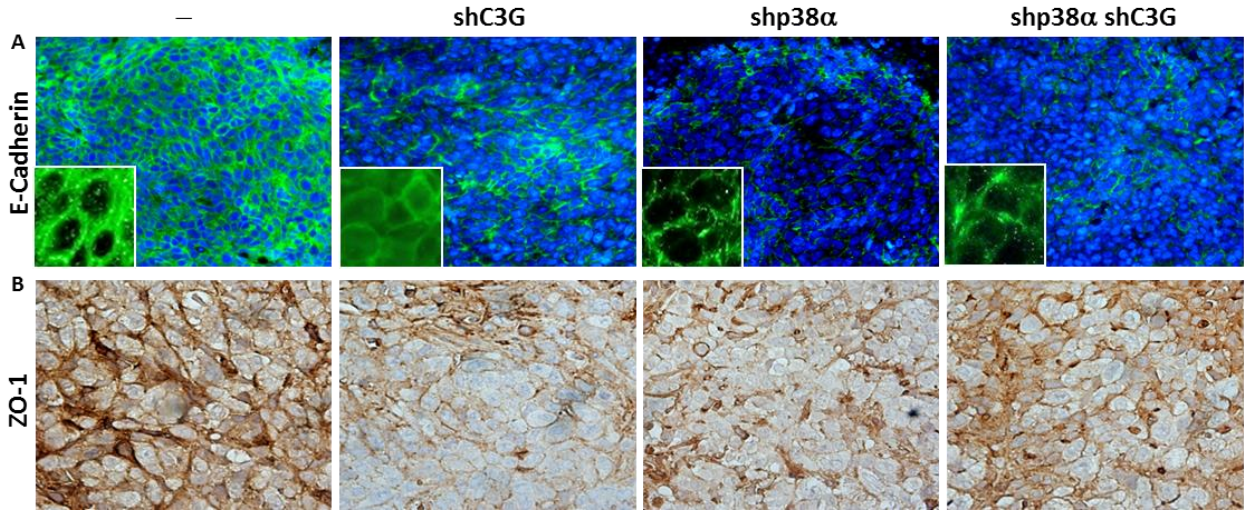

Figure 47-Analysis of E-cadherin and ZO-1 in tumors derived from C3G, p38α and C3G-p38α knock-down HCT116 cells. End point tumors generated from HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were analyzed. (A) Immunofluorescence analysis of E-Cadherin (green) and (B) immunohistochemistry analysis of ZO-1 (brown) staining using specific primary antibodies.

We also studied cell death within the tumors. As shown in figure 48A, tunel assay revealed the existence of a high number of dead cells in the inner part of all tumors. However, we found a significant increase in the number of tunel positive cells in tumors generated from C3G and p38α double knock-down HCT116 cells as compared with those produced by parental cells (Fig. 48B). This suggests that the double depletion of C3G and p38α induces apoptosis, resulting in the inhibition of tumor formation, which could explain the reduced size of the tumors generated by these cells.

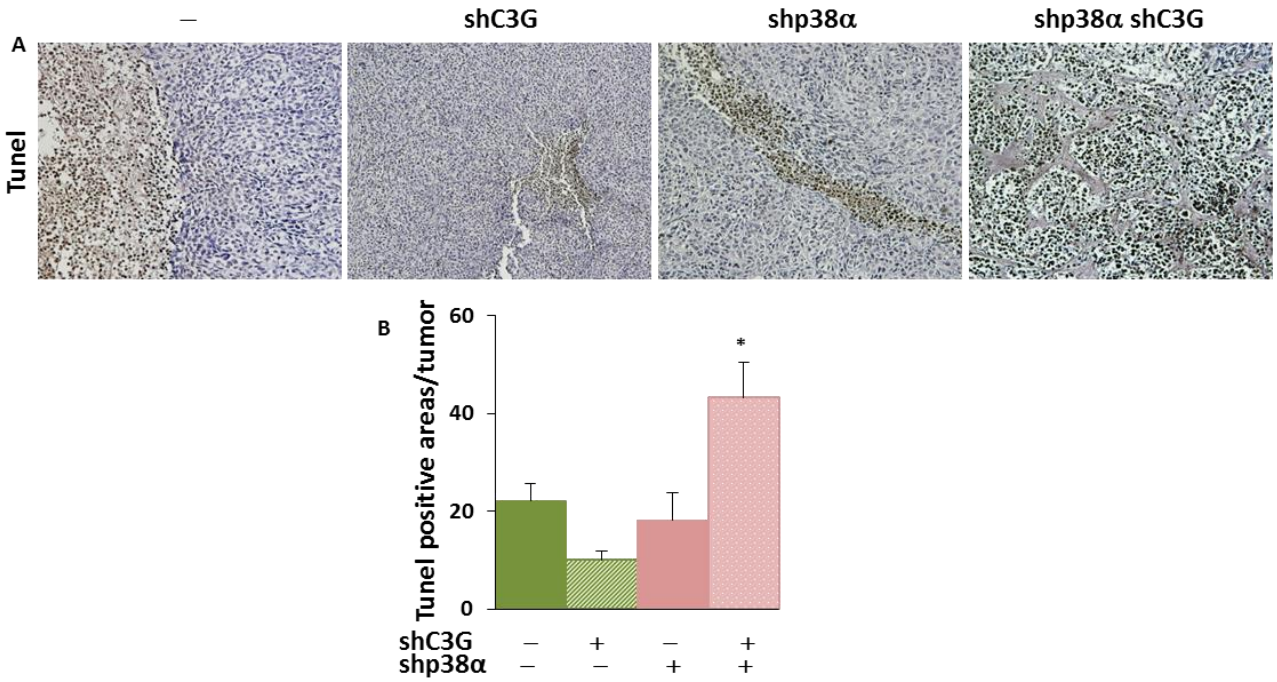

Figure 48-Analysis of cell death in tumors derived from C3G, p38α and C3G-p38αknock-down HCT116 cells. End point tumors generated from HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were analyzed. (A) Dead cells detected by tunel assay (black). (B) Histogram showing the mean value ± S.E.M. of the percentage of tunel positive areas per tumor. *p<0.05 versus non-silenced cells.

To complete the analysis of the xenografts, we studied the presence of stromal cells within the tumors. As shown in figure 49A, fibroblast infiltration (α-SMA positive cells) was increased in the tumors with either C3G and/or p38α silencing, meanwhile, macrophage infiltration (CD14 cells) was decreased in tumors generated by these cells (Fig. 49B). Moreover, the presence of vessels (MECA 32 positive cells) was increased upon silencing of p38α and this effect was independent of C3G in all tumors (Fig. 49C).

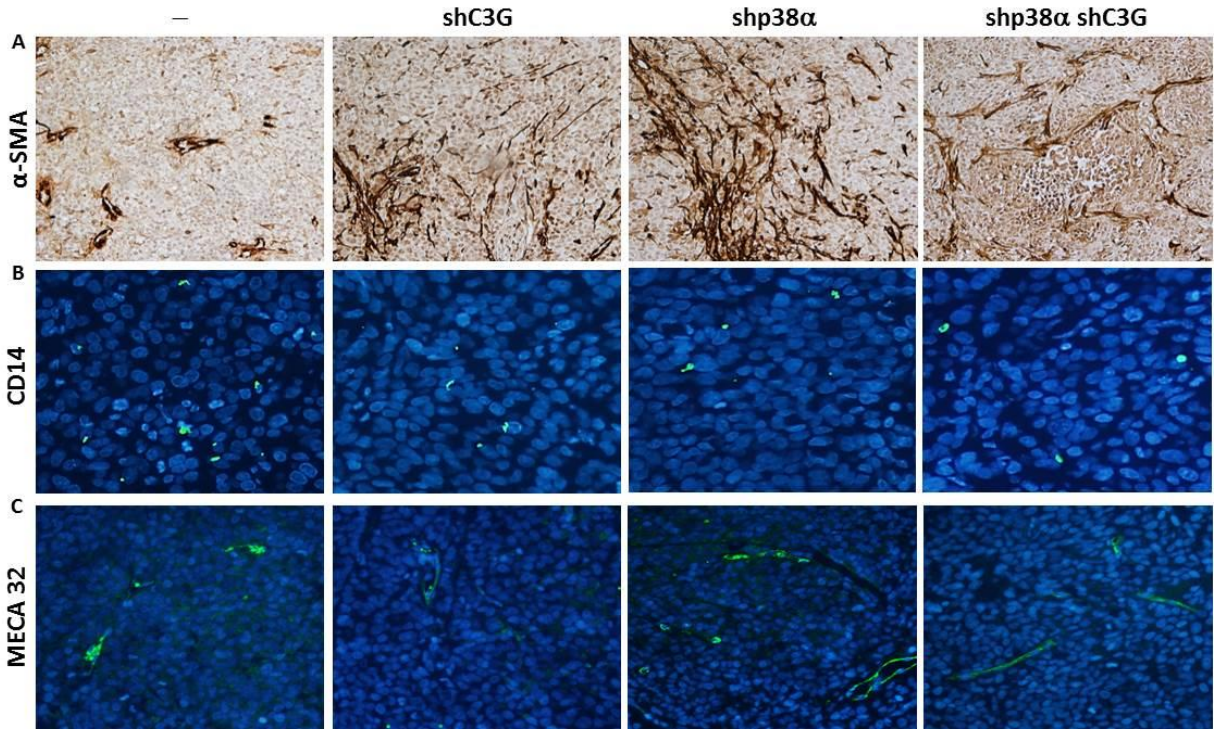

Figure 49- Analysis of infiltrated cells in tumors in tumors derived from C3G, p38α and C3G-p38α knock-down HCT116 cells. End point tumors generated from HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were analyzed. (A) Immunohistochemistry analysis of αSMA (brown) and immunofluorescence analysis of (B) CD14 (green) and (C) MECA32 (green) staining using specific primary antibodies.

In the tumor niche, colorectal cancer cells interact in a synergistic cross-talk with the host stromal microenvironment that supports tumor growth and metastasis (Gout and Huot 2008). So, differences between *in vivo* and *in vitro* assays could be also explained based on the interactions between stroma and tumor cells. TGFβ has a relevant role enhancing the effect of cancer-associated fibroblasts (CAFs) promoting colorectal cancer initiation (Calon et al. 2015). Other cytokines such as TNFα or chemokines such as CXCL12 (SDF-1) also have a key role in stromal regulation (Kim 2012, Katanov et al. 2015). Thus, we next explored the effect of either C3G and/or p38α knock-down on TGFβ, TGFβ receptor 1 (TGFβR1), CXCL12, its receptor, CXCR4, and TNFα mRNA expression. We found that TGFβ1 mRNA level was significantly reduced upon C3G, p38α or double knock-down in HCT116 cells (Fig. 50), which correlates with the reduction in tumor sizes. TNFα, CXCL12, TGFβR1 and CXCR4 mRNA levels were decreased in C3G-p38α double knock-down cells. Moreover, CXCL12 mRNA levels were also reduced upon single p38α silencing (Fig. 50). These results suggest that CXCL12 could play a role in p38α-mediated pro-tumorigenic effect and TNFα and CXCL12 in the concerted effect of C3G and p38α on tumor growth.

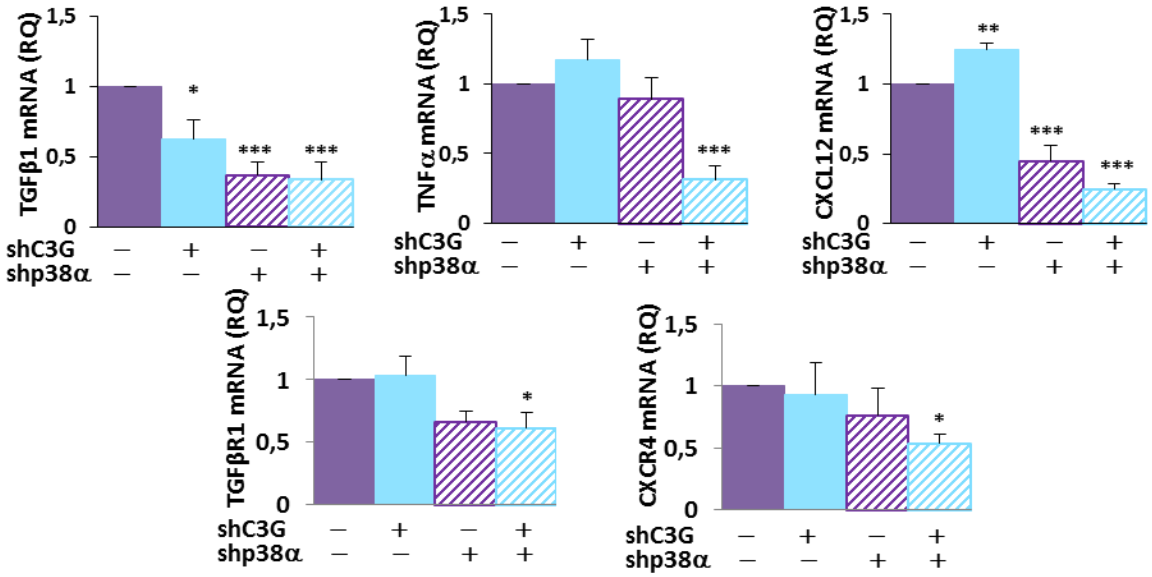

Figure 50-Effect of C3G and p38α knock-down on different stromal regulation factors expression. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. Analysis by RT-qPCR of TGFβ1, TGFβ1R, TNFα, CXCL12 and CXCR4 mRNA levels in HCT116 cells. Results represent the mean ± S.E.M. of RQ values (n=4). *p<0.05, **p<0.01, ***p<0.001 as compared with non-silenced cells.

As p38α and C3G seemed to have an important function controlling tumor growth, we studied the role of p38α and C3G in the regulation of the sphere formation capacity of HCT116 cells. As shown in figure 51, the number of spheres formed by p38α knock-down HCT116 cells was significantly higher than that of parental or C3G silenced cells. Curiously, when both C3G and p38α were silenced, there was almost no sphere formation (Fig. 51), which suggests that C3G knock-down impairs the effect of p38α depletion. Therefore, while p38α inhibits sphere formation, C3G would promote it.

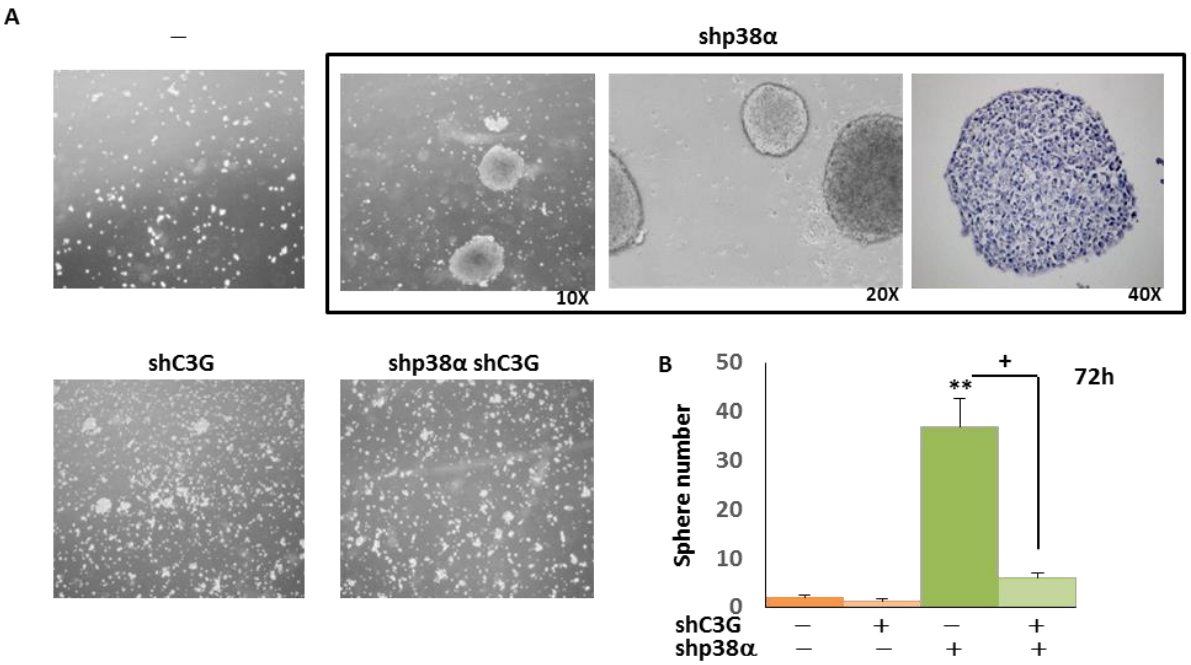

Figure 51-Effect of C3G and p38α knock-down on sphere formation. HCT116 cells (non-silenced (-) and p38α knock-down (shp38α); with (shC3G) or without C3G knock-down) were used. Sphere like growth assays. (A) Representative images of spheres taken with a phase contrast microscope and hematoxylin/eosin staining of the inside of a sphere formed by p38α knock-down HCT116 cells. (B) Histogram shows the mean value ± S.E.M. of the number of spheres at 72 h (n=3). **p<0.01 compared with non-silenced cells; $^{+}$p<0.05 compared as indicated.

DISCUSSION

1. p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH DNA METHYLATION. ROLE IN MIGRATION, INVASION AND TUMOR GROWTH

p38α MAPK can play a dual role in cancer acting either as a tumor suppressor or tumor promoter depending on the tumor stage and type of cancer (Wagner and Nebreda 2009). However, the function of p38 MAPKs in cancer has not been fully characterized yet. At early stages of cancer progression, p38α MAPK can inhibit tumor initiation, for example, acting as a sensor of oxidative stress (Dolado et al. 2007), but at later stages, it can promote survival (Aguirre-Ghiso 2007, Schewe and Aguirre-Ghiso 2008), as well as migration and invasion leading to metastasis through regulation of cell motility, MMPs expression and/or activity (Wagner and Nebreda 2009, del Barco Barrantes and Nebreda 2012). Furthermore, in non-tumor cells, p38α promotes migration processes under different physiological and pathological conditions (Rousseau et al. 2006), which might be relevant during embryonic development or tissue regeneration.

Our data uncover Fibulin 3 as a new target of p38α MAPK, which participates in the regulation of migration and invasion in the human HCT116 colon carcinoma cell line. Furthermore, we demonstrate that p38α down-regulates Fibulin 3 expression through DNA methylation. p38α might do so through the upregulation of DNMT3A protein levels, as DNMT3A protein levels highly decrease upon p38α knock-down, which correlates with a higher expression of Fibulin 3. In addition, the re-expresion of DNMT3A in p38α-silenced HCT116 cells decreases Fibulin 3 levels, rescuing the effect of p38α. Moreover, HuR would be involved in the p38α-mediated up-regulation of DNMT3A through dnmt3a mRNA stabilization as it was demonstrated in MEFs by our group (M. Arechederra´s Thesis). This p38α-HuR mediated upregulation of DNMT3A might be also operating in HCT116 cells based on the reduction of HuR protein levels upon p38α knock-down. This would be also in agreement with the previously established stabilization of dnmt3b mRNA by the binding of HuR protein to its 3´UTR (Lopez de Silanes et al. 2009). Therefore, as dnmt3b is highly homologous to dnmt3a mRNA, it is reasonable that dnmt3a mRNA can be regulated in a similar way. Our data also agree with the reported function of p38α as a regulator of HuR. Thus, p38α was shown to stabilize p21 mRNA in response to γ-radiation through HuR phosphorylation, which leads to cytoplasmic accumulation of HuR and enhancement of its binding to the 3´UTR of the mRNA (Lafarga et al. 2009). Similarly, p38 MAPK mediated cytoplasmic accumulation of HuR stabilizes survival motor neuron mRNA (Farooq et al. 2009). Other studies also support a role for this p38α/HuR cascade in the control of the stability of other mRNAs, but they involve the participation of additional proteins. For example, p38 MAPK via MK2 also regulates the stability of TNF mRNA through regulation of HuR and tristetraprolin (TTP) (Tiedje et al. 2012). Hence, MK2 phosphorylates the destabilizing factor, TTP, decreasing its affinity to the AU-rich element and its ability to replace HuR, which allows HuR-mediated initiation of TNF mRNA translation. Hyperphosphorylation of TTP via p38 MAPK is also involved in the up-regulation of IL-8 and VEGF in malignant gliomas (Suswam et al. 2008).

It is important to highlight the relevance of fibulin 3 gene silencing induced by hypermethylation of its regulatory sequences in cancer (Tong et al. 2011, Kimet al. 2012). The down-regulation of fibulin 3 by hypermethylation of the promoter region is associated with poor prognosis in various tumors such as non-small cell lung carcinoma (Kim et al. 2012, 2013). However, it remains unknown which are the mechanisms controlling fibulin 3 gene hypermethylation. Therefore, the finding of p38α MAPK as a novel regulator of fibulin 3 expression in a colorectal cancer model opens new possibilities to fully characterize the mechanisms underlying fibulin 3 actions in cancer.

The group has also demonstrated this regulation in MEFs, where it was proved that p38α-induced hyper-methylation of fibulin 3 gene regulatory sequences, leads to fibulin 3 down-regulation (M. Arechederra´s Thesis). Hence, p38α MAPK down-regulates Fibulin 3 in HCT116 cells in a similar way as it does in MEFs (M. Arechederra´s Thesis). However, the role played by Fibulin 3 regulating migration and invasion is different in the two cell types. In HCT116 cells, Fibulin 3 promotes migration and invasion, while it inhibits these processes in MEFs. This agrees with previous data from the literature where Fibulin 3 was shown to act as either an activator or an inhibitor of migration and invasion. For example, in glioma and pancreatic adenocarcinoma, Fibulin 3 is overexpressed and promotes cell migration and invasion (Hu et al. 2009, Seeliger et al. 2009). In contrast, in non-small cell lung cancer cell lines Fibulin 3 is a negative regulator of invasiveness, so those cell lines where fibulin 3 is silenced by promoter methylation, became highly invasive (Kim et al. 2012). Activation of Wnt/β-catenin signaling and MMP7 expression (Chen et al. 2014), as well as epithelial to mesenchymal transition (EMT) and shelf-renewal of lung cancer stem cells contributes to lung cancer invasion and metastasis induced by this down-regulation of Fibulin 3 (Kim et al, 2013). In addition, Fibulin 3 is a negative regulator of invasiveness in nasopharyngeal carcinomas (Hwang et al. 2010). On the other hand, although fibulin-3 down-regulation in colorectal cancer was correlated with lymph node metastasis and poor survival in a high percentage of patients (Tong et al. 2011), in the HCT116 colon carcinoma cell line we have demonstrated that fibulin 3 silencing inhibits migration, invasion and tumor growth. This raises the possibility that Fibulin 3 could play different roles in colon carcinoma depending on the tumor stage.

During cell migration and invasion, MMPs are relevant for extracellular matrix degradation (Kessenbrock et al. 2011). We have found that MMPs are mediators of p38α and Fibulin 3 effects on invasion in HCT116 cells. In particular, there is a good correlation between MMP2/9 activities and the invasive capacity suggesting their involvement, which was further supported by the impairment of invasion by a broad spectrum MMP inhibitor. Moreover, p38α is a positive regulator of these MMPs, in agreement with data from the literature (Xu et al. 2006, Hou et al. 2009, Loesch et al. 2010, Kessenbrock et al. 2011, del Barco Barrantes and Nebreda 2012, Ren et al. 2013).

Curiously, in our model, Fibulin 3 activates p38α/β and its effect on migration and invasion appears to be dependent on this activation. Therefore, in HCT116 cells, Fibulin 3 favors cell migration and invasion mainly in cells expressing p38α. So, the low level of Fibulin 3 produced by non-silenced HCT116 cells is enough to promote

migration and invasion acting through p38α. In the A549 lung carcinoma cell line, p38 is also activated by Fibulin 3, but its function has not been characterized (Xu et al. 2014). In contrast, in MEFs, Fibulin 3 down-regulates p38α and p38β activation, which limits migration and invasion (M. Arechederra´s Thesis). Although there is not a straight forward explanation to the distinct regulation of p38 activation by Fibulin 3 in MEFs and HCT116 cells, there are a great number of differences between these two cell models that might account for this discrepancy. MEFs are non-tumor embryonic mesenchymal cells from a murine origin, while HCT116 cells are epithelial tumor cells from a human origin. These differences can support an opposite response mainly based on the following reasons: (i) epithelial and mesenchymal cells express different proteins (i.e. E-cadherin and N-cadherin, respectively) and (ii) HCT116, as other tumor cells, present several genetic alterations. Thus, it should be highlighted that the colon carcinoma HCT116 cell line bears a mutation in codon 13 of the ras gene, which leads to the up-regulation of a number of signaling pathways such as Ras/ERKs, PI3K/Akt or even p38 MAPKs. In addition, TGFβ1 and β2 are expressed by these cells, which would dysregulate additional pathways and genes expression.

It is also noticeable the role played by p38α promoting tumor growth of HCT116 cells *in vitro* and *in vivo*. This is in contrast with its pro-apoptotic function, previously identified in HCT116 cells treated with cisplatin (Bragado et al. 2007) and with its tumor suppressor role in other tumor cell lines. For example, there is good evidence that p38α can function as a tumor suppressor in several mouse models of cancer (Hui et al. 2007, Ventura et al. 2007, Sakurai et al. 2008, Wakeman, Schneider et al. 2012), but it should be noted that all these studies have been performed by genetic deletion of p38α before tumor initiation (Gupta et al. 2015). In other tumors and in agreement with our results, increased levels of phosphorylated p38α have been correlated with malignancy (Wagner and Nebreda, 2009) such as in head and neck carcinoma (Leelahavanichkul et al. 2014), where p38α promotes tumor growth *in vitro* and *in vivo*. In addition, in a mouse model of colitis-associated tumor induction, p38α deficiency decreases cell proliferation and survival of colon tumors (Gupta el al. 2014). According to this, pharmacological p38α inhibition reduces tumor growth in patient-derived xenografts (Gupta el al., 205). However, initially, p38α suppresses colitis-associated tumor initiation maintaining an intact epithelial barrier (Gupta el al. 2014).

In conclusion, we have described that p38α down-regulates fibulin 3 expression in HCT116 cells through DNA methylation. p38α might do so through the up-regulation of DNMT3A. In addition, we have identified Fibulin 3 as a positive regulator of cell migration, invasion and tumor growth acting through a mechanism dependent on p38α, which acts as a potent inducer of tumor growth.

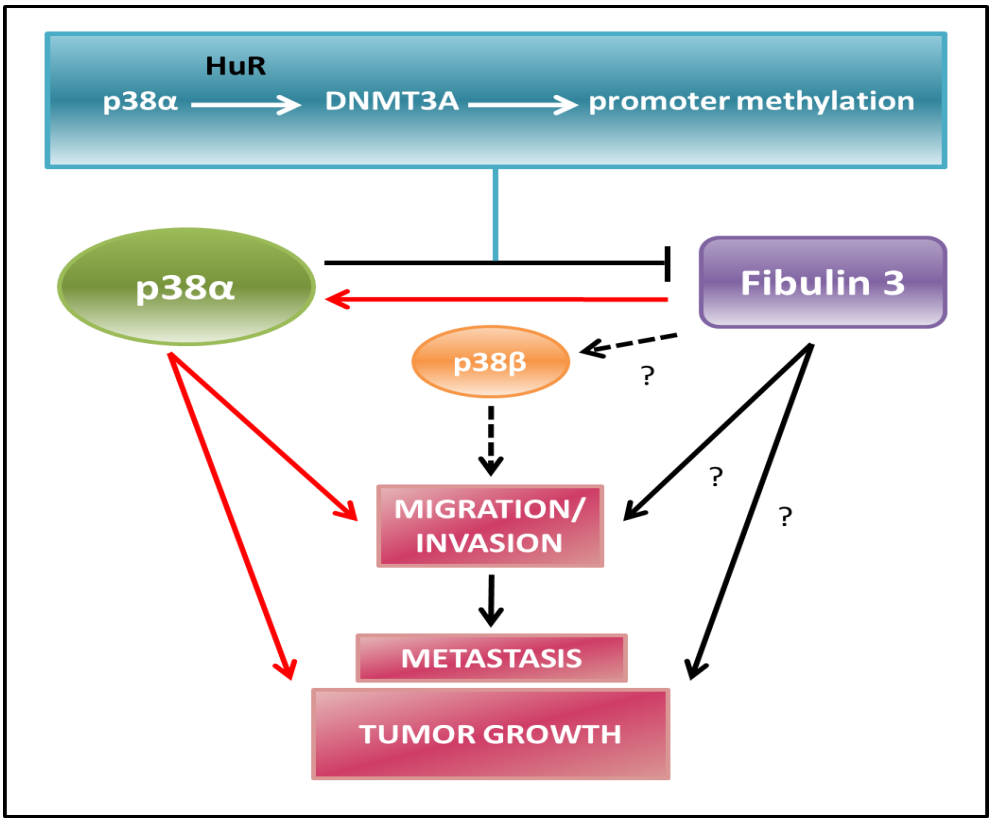

Figure 52-p38α MAPK down-regulates Fibulin 3 expression regulating cell migration, invasion and tumor growth in HCT116 cells. Scheme showing that p38α decreases Fibulin 3 expression in HCT116 cells, which contributes to the regulation of migration, invasion and tumor growth. p38α acting through HuR would up-regulate DNMT3A, leading to fibulin 3 promoter methylation and silencing of the gene. Fibulin 3 promotes migration, invasion and tumor growth through p38α/β activation, so that, its down-regulation by p38α would represent a negative feed-back loop.

2. C3G REGULATES CELL ADHESION, MIGRATION AND INVASION THROUGH DOWN-REGULATION OF p38α MAPK ACTIVITY BY A MECHANISM NOT MEDIATED BY RAP1. INVOLVEMENT IN TUMOR GROWTH

Our group has previously described in MEFs and CML cells a new functional cross-talk between C3G and p38α MAPK that regulates apoptosis, so that, C3G through down-regulation of p38α MAPK activity controls cell death (Maia et al. 2009, Gutierrez-Uzquiza et al. 2010). Depending on the cell type, Rap1 is a mediator of C3G actions (Maia et al. 2009) or not (Gutierrez-Uzquiza et al. 2010). Moreover, in CML both proteins participate in the same signaling pathway regulating cell adhesion (Maia et al. 2013). In the present work, we have explored whether C3G and p38α could also act in a common pathway to regulate cell migration, invasion and tumor growth of HCT116 cells and if C3G effects could be mediated by Rap1.

Previous data from the literature showed that p38α MAPK plays a role in different aspects of cell migration and invasion (Rousseau et al. 2006, Wagner and Nebreda 2009, del Barco Barrantes and Nebreda 2012). C3G plays also a relevant role in cell migration, although its precise function has not been well characterized. C3G deficiency in MEFs enhances migration (Ohba et al. 2001) and accordingly, C3G overexpression decreased cell migration of highly invasive breast carcinoma cells (Dayma and Radha 2011). In contrast, its overexpression results in increased migration of glomerulal epithelial cells in glomerulonephritis (Rufanova et al. 2009). In agreement with this, C3G is required for a correct migration of sympathetic preganglionic (Yip et al. 2012) and cortical neurons (Voss et al. 2008) and C3G/Rap1 pathway also mediates IGF-1-induced migration of MCF-7 breast cancer cells (Guvakova et al. 2014). Therefore, all these data indicate that C3G can play opposite functions in the control of cell motility, which might be dependent on the cell type, stimulus and/or other factors.

In this study, we have demonstrated that in HCT116 colon carcinoma cells, C3G inhibits migration and invasion through down-regulation of p38α activity. In fact, C3G silencing enhanced migration/invasion in cells expressing p38α, but not in those where p38α is silenced. Therefore, the previously identified C3G/p38α cascade that regulates cell death (Maia et al. 2009, Gutierrez-Uzquiza et al. 2010) would be also regulating cell migration and invasion in HCT116 cells. Data from our group indicates that this cascade would be also controlling these processes in MEFs (M. Arechederra´s Thesis).

Our results also indicate that Rap1 does not mediate C3G actions on migration and invasion in HCT116 cells, which fits well with previous data showing that Rap1 basal activity remains unaltered in C3G knock-out cells (Ohba et al. 2001). This also agrees with our previous results showing that C3G regulated apoptosis in MEFs acting through p38α, but independently of Rap1 (Gutierrez-Uzquiza et al. 2010). In contrast, in K562 Rap1 is a component of the C3G/p38α cascade controlling cell death in CML cells (Maia et al. 2009). Indeed, C3G effects are not necessarily dependent on its GEF activity (Guerrero et al. 1998, Shivakrupaet al. 2003, Guerrero et al. 2004, Martin-Encabo et al. 2007), but rather rely on specific protein-protein interactions in some contexts.

In relation to this, it is important to highlight that there are several Rap1 GEFs, different from C3G, able to activate Rap1 (Hattori and Minato 2003, Schultess et al. 2005, Stork and Dillon 2005, Pannekoek et al. 2009). Hence, depending on the cell type and the stimulus, specific GEFs are activated leading to Rap1 activation.

Our findings indicate that Rap1 contributes to cell migration and invasion, which agrees with data from the literature, where Rap1 promotes these processes in different models. For example, Rap1 mediates migration, invasiveness and in vivo dissemination of B-cell lymphoma cells (Lin et al. 2010), invasion of renal (Kim et al. 2012) and prostate cancer cells (Bailey et al. 2009) and migration of breast cancer cells (McSherry et al. 2011). Accordingly, optimal cell migration has been associated with cycles of Rap1 activation (Takahashi et al. 2013).

Rap1 activates p38α in MEFs in response to different stimuli (Gutierrez-Uzquiza et al. 2010). We have seen here that in HCT116 cells, the promigratory effect of p38α is impaired upon Rap1 knock-down. Thus, it is likely that Rap1 could act through p38α in the context of migration. Although Rap1 can regulate p38 MAPK, there are contradictory results in the literature. For example, Rap1 is known to activate p38 in response to FGF-2 in endothelial cells (Yan et al. 2008). However, Rap1 has an inhibitory effect on p38 MAPK activation upon IL-1 stimulation (Palsson et al. 2000, McDermott and O'Neill 2002) and it acts in parallel with p38 MAPK to induce chemotaxis in monocytes treated with LPS (lipopolysaccharide) (Yi et al. 2012).

Our results support a role for p38α in the actions of C3G on migration and invasion, as C3G knock-down enhances these processes acting through p38α. Our group (M. Arechederra´s Thesis) and other authors (del Barco Barrantes and Nebreda 2012) have previously shown that p38α plays a role in different aspects of cell migration, invasion and metastasis, favoring tumor progression (del Barco Barrantes and Nebreda 2012). p38α mediates migration in HeLa cells and MEFs through the regulation of the actin cytoskeleton by MK2 (Rousseau et al. 2006). In addition, in HGF/Met-activated cortical neurons, the Rac1/p38 cascade is crucial for migration (Segarra et al. 2006) and activation of p38α can also induce cytoskeletal remodeling and a migratory response in tumor cells by increasing Hsp27 phosphorylation (Laferriere et al. 2001). In this line, our results indicate that C3G acting through p38α regulate actin organization in HCT116 cells. Thus, in C3G silenced cells p38α promotes the formation of filopodia and other migratory structures. In addition, the induced internalization of ZO-1 and the partial loss of E-cadherin would disrupt cell-cell interactions, which would favor cell migration. This is also supported by *in vivo* data derived from xenografts analysis, which showed a reduction in ZO-1 and E-cadherin expression and a partial internalization of E-cadherin.

According to our results, many studies have shown an overall decrease in the expression of E-cadherin in colorectal tumor cells (Schuhmacher et al. 1999, Rosivatz et al. 2004). Moreover, loss of E-cadherin has been associated with an invasive and aggressive phenotype of colorectal cancer cells (Tsanou et al. 2008). ZO-1 is also frequently down-regulated in primary CRC, although re-expressed in liver-metastases (Kaihara et al. 2003). Moreover, low expression of E-cadherin and high expression of N-cadherin are correlated with local infiltration, vascular invasion and tumor

dedifferentiation (Ye et al. 2015). This increased expression of N-cadherin, together with the low E-cadherin expression was observed in C3G knock-down cell. Additionally, data derived from the analysis of transcription factors involved in EMT suggest a possible induction of EMT upon C3G silencing that might promote invasion. Nevertheless, some of these changes in N-cadherin, E-cadherin and EMT transcription factors were also observed in single p38α and double C3G-p38α silenced cells, even though they are less migratory and invasive. This might be explained by their reduced capacity to form filopodia and other migratory structures.

Several studies have shown the implication of MMP2 and/or MMP9 in cell migration and invasion (Kessenbrock et al. 2011). Moreover, p38 MAPK promotes these processes in a number of tumors (Wagner and Nebreda 2009). In melanoma cells (Estrada et al. 2009) or ovarian cancer cells (Zhou et al. 2007), p38 MAPK does so through regulation of MMP9. p38α also mediates tumor cell invasion in hepatocellular carcinoma, head and neck squamous carcinoma, glioblastoma and pancreatic cancer cell lines (Wagner and Nebreda 2009). Accordingly, p38α induces MMP1, MMP2, MMP9 and MMP13 expression in bladder, breast, liver, skin, keratinocytes and prostate cancer (del Barco Barrantes and Nebreda 2012). Our results also indicate that C3G, acting through p38α regulates MMP2 and MMP9 activities in HCT116 colon carcinoma cells, which correlates with its effect on cell migration and invasion. Similarly, in the previous section of this memory, MMP2 and MMP9 were shown to be regulated by p38α/Fibulin 3 and mediated invasion of HCT116 cells. This fits with our previous observations that p38α is a positive regulator of MMP2 and MMP9 activities in MEFs (M. Arechederra´s Thesis).

The role of Rap1 in the control of MMP2/9 activities is different from that of C3G, which indicate that Rap1 is not the mediator of C3G actions on cell invasion. Results from our group (M. Arechederra´s Thesis) also indicate that in MEFs, Rap1, in the presence of p38α, leads to MMP2 and MMP9 activation, which agrees with the role of Rap1 inducing MMP9 secretion and invasion in head and neck squamous carcinoma cells (Mitra et al. 2008). In contrast to our results, recent published data suggest that C3G, acting through Rap1, might promote invasion of epithelial ovarian cancer cells through the induction of MMP2 and 9 secretion (Che et al. 2015).

Our studies using HCT116 colon carcinoma cells also revealed a positive role for both p38α and C3G in promoting anchorage-independent growth in soft agar as well as tumor growth in nude mice. Our data support the concept that C3G does not regulate tumor growth through down-regulation of p38α activity, which differs from the mechanism used to regulate the migratory and invasive responses. Additionally, our data indicate that Rap1 does not mediate these C3G-driven effects. Accordingly, results derived from our *in vivo* studies revealed that C3G and p38α double knock-down led to a larger reduction in tumor size than each individual knock-down. However, this was not observed in anchorage dependent or independent growth assays, where the number of foci was higher in the double knock-down than in that of C3G knock-down, although there were less cells per focus. One plausible explanation for this discrepancy may be that in an *in vivo* context, other mechanisms may influence the C3G response. In fact, an enhanced cell death is produced in these tumors, which might impair tumor development. This possibility is also supported by the low viability

detected *in vitro* when silenced cells are submitted to oxidative stress or to a lack of attachment. Therefore, it is very likely that C3G, p38α or double C3G-p38α silencing can inhibit tumor growth through the reduction of cell viability. The low adhesion of single C3G silenced cells and the lack of interaction between them might also impair tumor growth *in vivo* and *in vitro* (anchorage-dependent growth). In fact, the low adhesion of C3G knock-down cells correlates with a low number of foci and p38α inhibition with SB203580 reverts both effects. It is also important to mention that p38α was previously shown to promote *in vivo* cell proliferation and survival of human colon carcinoma cells (Gupta et al. 2015), leading to tumor growth. This is in agreement with our results that support a role for p38α in cell viability in response to oxidative stress.

Additionally, the tumor stroma could be influencing *in vivo* tumor growth. Tumor stroma plays an essential role in the development of colorectal cancer (Kalluri and Zeisberg 2006), regulating both the initiation and progression of cancer through mechanisms that involved the secretion of cytokines and growth factors and/or the expression of their receptors (Gout and Huot 2008). In particular, TGFβ generated by colon cancer cells acts on cancer associated fibroblasts, present in the stroma, which contributes to tumor growth by increasing the frequency of tumor initiation cells (Calon et al. 2015). Notably, we found a remarkable reduction in the expression of TGFβ mRNA in C3G, p38α or double knock-down HCT116 cells, which suggests that TGFβ might play a role in C3G and/or p38α-mediated-colon carcinoma tumor growth, as well as its receptor. We also found a significant decreased in TNFα mRNA levels in tumors generated by double C3G-p38α knock-down HCT116 cells, which correlates with a decrease in tumor size. This is in agreement with other studies supporting a pro-tumoral role of TNFα in CRC (De Simone et al. 2015). Moreover, CXCL12 (SDF-1)/CXCR4 pathway seems to play an important role in the progression of CRC and generation of liver metastasis (Kim et al. 2012, Kollmar et al. 2007). In this line, our studies has shown a decreased expression of CXCL12 and CXCR4 in double C3G-p38α knock-down cells, associated with the generation of small tumors in nude mice and a low invasive capacity. Furthermore, the reduction in CXCL12 mRNA levels in p38α knock-down cells, agrees with the p38α-mediated SDF-1 expression in human lung stem cells (Ruiz et al. 2014).

It may be also possible that other cytokines, chemokines and growth factors could be contributing to this response as well.

Cancer stem cells play critical roles in CRC initiation and progression, including the generation of metastasis (Huang and Wicha 2008, Cherciu et al. 2014). We have found that p38α knock-down in HCT116 cells promotes sphere formation, which suggests that these cells would be able to initiate tumor growth acting as cancer stem cells (CSCs). Hence, although we have also demonstrated here that p38α depletion initially inhibits *in vivo* and *in vitro* tumor growth, the potential generation of CSCs might allow tumor initiation and progression over time. In contrast, C3G knock-down reversed this effect of p38α, suggesting that C3G silencing impairs the generation of CSC. Future studies are required to further understand the precise role played by C3G and p38α in the regulation of stemness and cancer stem cells.

To conclude, our data demonstrate that C3G down-regulation promotes migration and invasion in HCT116 colon carcinoma cells through a mechanism that requires p38α activation, but not mediated by Rap1. In contrast, down-regulation of Rap1 impairs migration and invasion. On the other hand, C3G and p38α promote growth of HCT116 cells *in vitro* and *in vivo* most likely through different mechanisms. The effect of C3G might be dependent on its pro-adhesive and pro-survival role that would allow cell attachment and subsequent proliferation and/or survival of the cells. p38α might promote cell survival and/or proliferation as previously demonstrated (Gupta et al. 2014). Furthermore, the regulation of the interactions of tumor cells with the stroma by C3G and p38α might contribute to induce tumor growth *in vivo*.

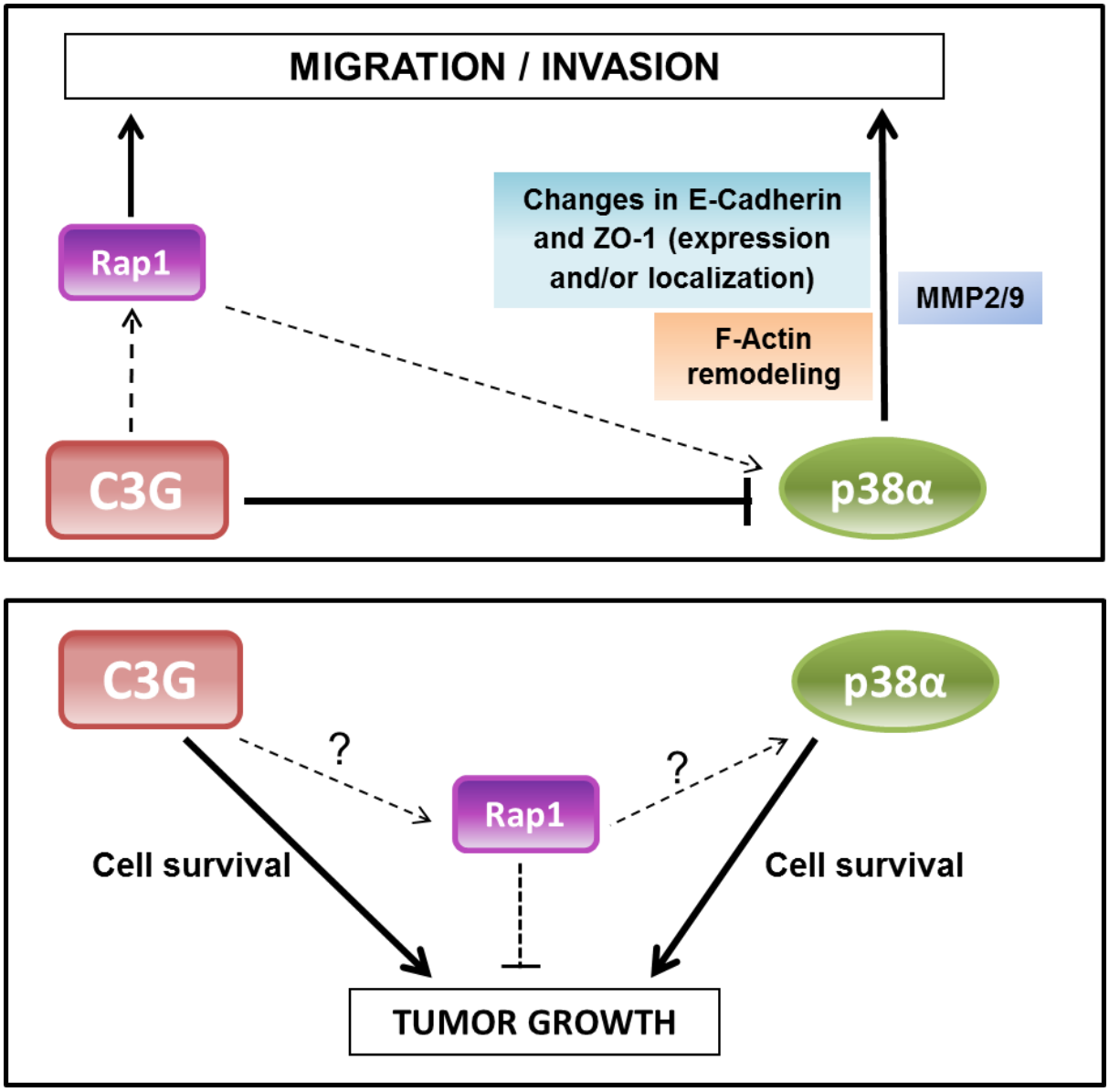

Figure 53-Role of C3G/p38α cascade in the control of cell migration, invasion and tumor growth of HCT116 cells. Diagram showing that C3G inhibits cell migration and invasion through down-regulation of p38α activity by a mechanism not mediated by Rap1, which promotes these processes. Partial loss of E-cadherin, ZO-1 internalization and F-actin cytoskeleton remodeling may mediate p38α and/or C3G actions on migration and invasion. MMP2/9 contribute to p38α-induced invasion. In contrast, both C3G and p38α promote tumor growth probably through independent pathways, inducing cell survival.

3. GENERAL DISCUSSION

In the two parts of this Thesis, and in agreement with other groups (Gupta et al. 2014; Gupta et al. 2015), our results strongly indicate that p38α acts as a promoter of tumor growth in colon carcinoma. We have also described that p38α induces migration/invasion of human colon carcinoma HCT116 cells, identifying Fibulin 3 as a new effector of p38α. Additionally, our results uncover C3G as a negative regulator of migration and invasion that acts through p38α. In contrast, both C3G and p38α promote tumor growth.

In the first part of this study, we have shown that Fibulin 3 is a new target of p38α in human colon carcinoma HCT116 cells, which acts as a positive regulator of migration, invasion and tumor growth through a mechanism dependent on p38α. Curiously, p38α down-regulates Fibulin 3 expression through DNA methylation, which would lead to a decrease in cell migration, invasion and tumor growth. This might represent a negative feed-back loop to limit p38α effects. Therefore, our findings support a pro-tumorigenic role of Fibulin 3 in colorectal cancer, which requires p38α, but it is also regulated by it. Based on the partial reduction in tumor size upon fibulin 3 knock-down, it might collaborate with other genes to induce tumor growth as it happens in lung carcinoma (Kim et al. 2012, Kim et al. 2014). Fibulin 3 would represent a potential new therapeutical target, opening new perspectives for colon cancer treatment. However, as previous data from the literature has associated fibulin-3 down-regulation in CRC with lymph node metastasis and poor survival (Tong et al. 2011), it would be important to further characterize how Fibulin 3 expression is regulated during CRC progression and its function in each step.

In the second part of the study, we have demonstrated that C3G inhibits cell migration and invasion through down-regulation of p38α activity in HCT116 cells. Hence, the C3G/p38α cascade, previously identified by our group as a regulator of cell death (Maia et al. 2009, Gutierrez-Uzquiza et al. 2010), is also involved in the control of cell migration and invasion in HCT116 cells, as well as in MEFs (M. Arechederra´s Thesis). These results are in agreement with the increased migration observed in C3G-/- MEFs (Ohba et al. 2001) and with the reduced migration of breast carcinoma cells overexpressing C3G (Dayma and Radha 2011). Furthermore, we have found that Rap1 does mediate the effects of C3G on migration and invasion of HCT116 cells, but rather icounteracts them. F-acting cytoskeleton remodeling is involved in the regulation of migration and invasion by C3G, as it has been also demonstrated in other studies (Sasi et al. 2015). However, we have additionally demonstrated that this function of C3G is dependent on p38α and is associated with a decrease in E-cadherin and ZO-1 internalization.

Our results have also uncovered a pro-tumoral role of C3G in colorectal cancer that appears to be dependent on its pro-adhesive and pro-survival function, but it is not mediated by down-regulation of p38α activity.

Therefore, we have demonstrated that C3G/p38α cascade controls cell migration and invasion in colorectal cancer, characterizing some of the mechanisms involved. p38α and C3G also regulate tumor growth in colorectal cancer by independent mechanisms. Furthermore, we have identified Fibulin 3 as a new mediator of p38α MAPK actions in this model. In consequence, our results open new perspectives for future studies to find new targets for cancer therapies.

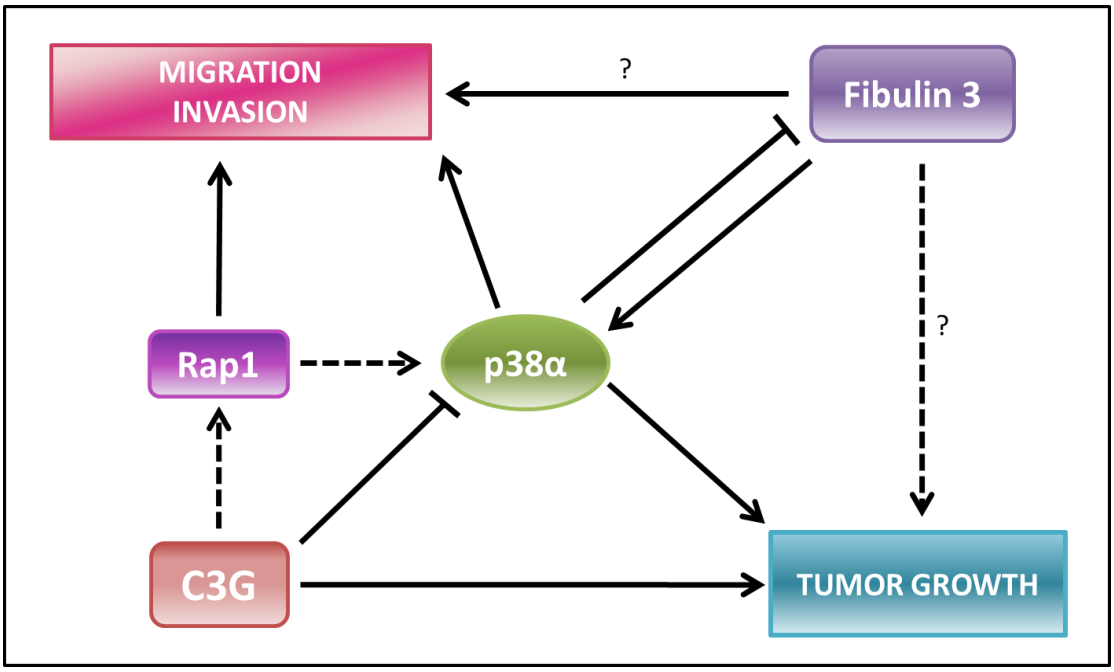

Figure 54- Crosstalk between p38α, Fibulin 3 and C3G in the regulation of migration, invasion and tumor growth in HCT116 cells. p38α MAPK promotes cell migration, invasion and tumor growth. Fibulin 3 also regulates these processes through p38α activation, which in term leads to down-regulation of fibulin 3 expression, generating a negative feed-back loop. On the other hand, C3G inhibits migration and invasion through down-regulation of p38α MAPK activity through a Rap1 independent mechanism

CONCLUSIONS

1. p38α down-regulates Fibulin 3 expression by promoting DNA methylation through up-regulation of DNMT3A protein levels in the human colon carcinoma HCT116 cell line.

2. Fibulin 3 promotes cell migration and invasion through mechanisms involving p38α/β, as well as MMP2 and MMP9 activation.

3. Fibulin 3 contributes to *in vitro* and *in vivo* tumor growth of HCT116 cells when p38α is expressed.

4. p38α mediates migration and invasion, which is correlated with MMP2 and MMP9 activation. Moreover, inhibition of MMPs impairs invasion.

5. p38α promotes *in vitro* and *in vivo* tumor growth of HCT116 cells.

6. C3G protein levels are inversely correlated with the invasive capacity of different human colon carcinoma cell lines.

7. C3G inhibits migration and invasion of HCT116 cells through down-regulation of p38α MAPK activity.

8. Rap1 does not mediate, but rather counteracts, C3G inhibitory effect on HCT116 cells migration and invasion.

9. C3G promotes cell adhesion, while p38α and Rap1 negatively regulate it.

10. C3G knock-down enhances migration acting through p38α, which involves ZO-1 internalization, loss of E-cadherin and F-actin cytoskeleton re-organization.

11. C3G promotes *in vitro* and *in vivo* tumor growth of HCT116 cells through a mechanism that does not involve down-regulation of p38α activity. In contrast, both proteins contribute to tumor growth by favouring cell survival.

12. TFGβ1 mRNA levels decrease upon C3G, p38α and double C3G-p38α knock-down in HCT116 cells, which correlates with inhibition of *in vivo* tumor growth.

13. p38α knock-down enhances sphere formation in HCT116 cells.

CONCLUDING REMARKS

Fibulin 3 acting through p38α promotes migration, invasion and tumor growth. However, p38α limits these effects of Fibulin 3 inducing its silencing by promoting DNA methylation, which generates a negative feed-back loop that would limit Fibulin 3 effects. On the other hand, C3G inhibits cell migration and invasion through the negative regulation of p38α activity via Rap1 independent mechanisms. In contrast, C3G does not regulate tumor growth through inhibition of p38α, but rather collaborate with it to induce tumor growth, probably acting through different pathways.

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ABSTRACT

ROLE OF C3G AND p38α IN THE REGULATION OF MIGRATION, INVASION AND TUMORAL GROWTH IN COLON CARCINOMA. FUNCTION OF FIBULIN 3

1. INTRODUCTION

Colorectal cancer (CRC) represents the third most common cancer type and the second leading cause of cancer-related death in the western world. CRC results from the accumulation of both acquired genetic and epigenetic changes that transform normal glandular epithelium into adenocarcinoma (Lao and Grady 2011), affecting several genes such as Apc, K-ras, dcc/Smad4 and p53 or DNA mismatch repair genes (Pancione et al. 2012).

p38 MAPKs are a subfamily of Serine-Threonine kinases activated by different stimuli that control fundamental cellular processes such as cell growth, proliferation, differentiation, migration and apoptosis (Dhillon et al. 2007, Nebreda and Porras 2000, Wagner and Nebreda 2009). There are four p38 MAPKs isoforms in mammals: α, β, δ and γ. p38α MAPK is ubiquitously expressed and is the most abundant isoform (Cuenda and Rousseau 2007). p38α is involved in the regulation of many cellular functions, among them, cell migration and invasion. In cancer, it can act as either a promoter or a suppressor of tumor growth, playing different roles during tumor progression (del Barco Barrantes and Nebreda 2012).

C3G is a guanine nucleotide exchange factor (GEF) mainly for the Ras family members: Rap1 (Gotoh et al. 1995) and R-Ras (Gotoh et al. 1997), but it can also act through GEF independent mechanisms. C3G regulates several cellular functions such as cell death, adhesion, migration and invasion (Radha et al. 2011).

In collaboration with Dr. Carmen Guerrero's group (Centro del Investigación del Cáncer de Salamanca), our group has found a new functional relationship between C3G and p38α MAPK involved in the regulation of cell death in MEFs (Gutierrez-Uzquiza et al. 2010) and in the chronic myeloid leukemia (CML) K562 cell line (Maia et al. 2009). Moreover, C3G and p38α act through a common regulatory pathway to control cell adhesion in K562 cells regulating focal adhesion proteins (Maia et al. 2013).

Fibulins are a family of extracellular matrix secreted glycoproteins with at least seven members (Argraves et al. 1990, de Vega et al. 2009). Fibulins have both anti- and pro-oncogenic effects and their expression can be deregulated in some human cancers, correlating with tumor progression (Obaya et al. 2012). In particular, Fibulin 3 is involved in the regulation of cell migration and invasion during tumor progression and can have a pro- or anti-tumoral effects. For example, Fibulin 3 plays a pro-tumoral role in glioma (Hu et al. 2009, Nandhu et al. 2014) and pancreatic adenocarcinomas (Camaj et al. 2009). In contrast, Fibulin 3 acts as a tumor suppressor in lung cancer

(Kim et al. 2012, Chen et al. 2014) and nasopharyngeal carcinoma (Hwang et al. 2010).

2. OBJECTIVES/AIMS

1. To determine if p38α MAPK regulates fibulin 3 expression, how it does it and the role of Fibulin 3 as a potential mediator of p38α actions on cell migration, invasion and tumor growth.

2. To analyze whether C3G could act through p38α MAPK to regulate migration, invasion and tumor growth, identifying the mechanisms involved.

3. RESULTS

1. p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH METHYLATION OF DNA REGULATORY SEQUENCES. ROLE IN MIGRATION, INVASION AND TUMOR GROWTH

p38 MAPK down-regulates fibulin 3 expression

Based on our group data derived from Affymetrix microarrays showing that fibulin 3 mRNA expression was down-regulated by p38α in MEFs, we have characterized the role played by p38α in the regulation of Fibulin 3 in the human colon carcinoma HCT116 cell line.

We found that p38α MAPK is a negative regulator of Fibulin 3 expression in HCT116 cells. It was known that Fibulin 3 expression was mainly regulated at the transcriptional level through methylation of regulatory sequences (Blackburnet al. 2003, Kim et al. 2012). Thus, we studied whether p38α might down-regulate Fibulin 3 expression through DNA methylation. Treatment with 5-aza-2´-deoxicytidine (5A2dC) suggests that p38α represses fibulin 3 expression through hypermethylation of DNA. Furthermore, we have found that the DNA methylase, DNMT3A, which is up-regulated in cells expressing p38α MAPK, might be mediating this methylation. The p38α-mediated up-regulation of the RNA-binding protein, HuR, would stabilize dnmt3a mRNA leading to an increase in DNMT3A protein levels. .

Fibulin-3 knock-down inhibits migration and invasion of HCT116 cells

Our results derived from the analysis of the effect of fibulin 3 knock-down indicate that Fibulin 3 promotes migration and invasion of human colon carcinoma HCT116 cells through a mechanism that appears to require p38α/α activation. As expected, p38α mediates migration and invasion in these cells. Our data also suggest that MMP2 and 9 might be involved in the regulation of cell invasion by Fibulin 3/p38α.

Role of Fibulin 3 in the tumorigenic capacity of HCT116 cells

We have also studied the involvement of Fibulin 3 and p38α in the regulation of tumor growth by *in vitro* and *in vivo* (xenografts in nude mice) assays. We have found that Fibulin 3 promotes tumor growth of HCT116 cells through a mechanism dependent on p38α. Moreover, p38α is a potent positive regulator of tumor growth in this tumor model.

2. C3G REGULATES CELL ADHESION, MIGRATION AND INVASION THROUGH DOWN-REGULATION OF p38α MAPK ACTIVITY BY A MECHANISM NOT MEDIATED BY RAP1. INVOLVEMENT IN TUMOR GROWTH

Our group has previously described that C3G through down-regulation of p38α MAPK activity was able to control apoptosis (Gutierrez-Uzquiza et al. 2010). Thus, we have also explored if the C3G/p38α cascade might be involved in the regulation of migration and invasion processes in HCT116 cells.

C3G inhibits cell migration and invasion of HCT116 cells through down-regulation of p38α activity by a mechanism not mediated by Rap1. Function in cell adhesion

Based on Oncomine database, C3G expression can be deregulated in colon carcinoma. Hence, we have analyzed C3G protein expression in different colorectal cancer lines with distinct invasive capacities and we have found that there was an inverse correlation between C3G protein levels and their invasive capacity. We have also demonstrated that C3G knock-down promotes cell migration and invasion through the up-regulation of p38α activity by a mechanism not mediated by Rap1 in the human colon carcinoma HCT116 cell line. Moreover, C3G promotes adhesion through down-regulation of p38α activity, while Rap1 exerts an opposite effect.

MMP2/9 might be involved in the regulation of migration and invasion processes by C3G/p38α. Furthermore, changes in the expression of E-Cadherin and ZO-1 and their subcellular localization, together with F-actin re-organization might be responsible for the effects of C3G and p38α on these processes.

Role of C3G in the regulation of the tumorigenic capacity of HCT116 cells

Our results also indicate that both C3G and p38α MAPK promote *in vitro* and *in vivo* tumor growth of HCT116 cells, probably through independent mechanisms. Based on the reduction in cell viability in the absence of C3G and/or p38α expression, C3G and/or p38α would be favoring survival leading to tumor growth. TGFβ and other cytokines and/or chemokines might contribute to mediate the actions of C3G and/or p38α on tumor growth.

Finally, we have found that p38α knock-down enhances sphere formation in HCT116 cells.

4. DISCUSSION

In the first part of this Thesis project, we uncover Fibulin 3 as a new target of p38α MAPK in the human colon carcinoma HCT116 cell line. Furthermore, we demonstrate that p38α down-regulates fibulin 3 expression through induction of DNA methylation by DNMT3A. p38α might do so through the upregulation of HuR, which would stabilize dnmt3a mRNA, leading to an increase in DNMT3A protein levels. The down-regulation of fibulin 3 by hypermethylation of the promoter region has been previously found in non-small cell lung carcinoma and colorectal cancer (Kim et al. 2012, Tong et al. 2011). However, the mechanisms underlying Fibulin 3 actions in cancer are still poorly understood and the finding of p38α MAPK as a novel regulator of fibulin 3 expression in a colorectal cancer model is important to further understand its role in tumor progression. In addition, Fibulin 3 was shown to act as either an activator of migration and invasion (Hu et al. 2009, Seeliger et al. 2009) or an inhibitor of these processes (Kim et al. 2012). In our study, we have identified Fibulin 3 as a positive regulator of cell migration, invasion and tumor growth acting through a mechanism dependent on p38α, which also acts enhancing tumor growth of HCT116 cells.

In the second part of our study, we show that C3G regulates migration and invasion in HCT116 cells through down-regulation of p38α activity in a similar way it controls cell death in MEFs and chronic myeloid leukemia cells (Gutierrez-Uzquiza et al. 2010, Maia et al. 2009). However, these actions of C3G are not mediated by Rap1. In fact, Rap1 plays an opposite effect. This is in agreement with the increased migration observed in C3G-/- MEFs (Ohba et al. 2001) and the reduced migration found in breast carcinoma cells with C3G over-expression (Dayma and Radha 2011). Rap1 might be promoting cell migration and invasion through p38α activation. This positive role of Rap1 in invasion and migration was previously shown in other studies (Kim et al. 2012, Lin et al. 2010, McSherry et al. 2011). Our data also indicate that C3G acting through p38α regulates F-actin remodeling, which together with changes in E-cadherin expression and ZO-1 subcellular localization would mediate their effects on migration and invasion. Moreover, C3G and p38α promote tumor growth of HCT116 cells *in vitro* and *in vivo* most likely through different mechanisms. This effect of C3G might be dependent on its role favoring cell adhesion and survival. p38α, in agreement with previous findings (Gupta et al. 2015), might also induce cell survival. Additionally, C3G and p38α through regulation of the stroma, might also contribute to induce *in vivo* tumor growth.

5. MAIN CONCLUSION

p38α silences fibulin 3 expression through the induction of DNA methylation in human colon carcinoma HCT116 cells. This would represent a negative feed-back loop to limit its pro-migratory and invasive effect that is mediated by mechanisms involving p38αactivation. Moreover, Fibulin 3 promotes tumor growth acting through p38α, which plays a pro-tumorigenic role. On the other hand, C3G inhibits cell migration and invasion through the negative regulation of p38α activity by a mechanism not dependent on Rap1. However, both C3G and p38α collaborate to induce tumor growth, probably acting through different pathways.

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RESUMEN

PAPEL DE C3G Y p38α EN LA REGULACIÓN DE LA MIGRACIÓN, INVASIÓN Y CRECIMIENTO TUMORAL EN CARCINOMA DE COLON. FUNCIÓN DE FIBULINA 3

1. INTRODUCCIÓN

El cáncer colorectal (CCR) es el tercer tipo de cáncer más común y la segunda causa de muerte por cáncer en el mundo occidental. El CCR aparece como resultado de la acumulación de alteraciones genéticas y epigenéticas que transforman el epitelio glandular normal en un adenocarcinoma (Lao and Grady 2011), afectando a diversos genes como Apc, K-ras, dcc/Smad4 y p53 o genes reparadores de apareamientos incorrectos en el DNA (Pancione et al. 2012).

Las p38 MAPKs son una subfamilia de Serina/Treonina quinasas activadas por distintos estímulos que controlan procesos celulares fundamentales como el crecimiento celular, la proliferación celular, la diferenciación celular, la migración celular o la apoptosis (Dhillon et al. 2007, Nebreda and Porras 2000, Wagner and Nebreda 2009). Existen cuatro isoformas de p38 MAPKs en humanos: α, β, δ y γ. p38α MAPK se expresa de forma ubicua y es la isoforma más abundante (Cuenda and Rousseau 2007). p38α está implicada en la regulación de muchas funciones celulares, entre ellas, cabe destacar la regulación de la migración e invasión celular en distintos tipos celulares. En cáncer puede actuar, tanto promoviendo como inhibiendo el crecimiento tumoral, desempeñando distintos papeles durante la progresión tumoral (del Barco Barrantes and Nebreda 2012).

C3G es un factor activador del intercambio de nucleótidos de guanina (GEF), principalmente, para los miembros de la familia de Ras: Rap1 (Gotoh et al. 1995) y R-Ras (Gotoh et al. 1997). Además, puede actuar por mecanismos independientes de su actividad catalítica como GEF. C3G regula diversas funciones celulares como por ejemplo la muerte celular, la adhesión, la migración y la invasión (Radha et al. 2011).

En colaboración con el grupo de la Doctora Carmen Guerrero (Centro de Investigación del Cáncer de Salamanca), nuestro grupo ha descrito una nueva interacción funcional entre C3G y p38α MAPK que está implicada en la regulación de la muerte celular en MEFs (Gutierrez-Uzquiza et al. 2010) y en las células de leucemia mieloide crónica K562 (Maia et al. 2009). Así mismo, C3G y p38α actúan a través de una cascada común favoreciendo la adhesión celular en células K562, regulando proteínas de las adhesiones focales (Maia et al. 2013).

Las fibulinas son una familia de proteínas de la matriz extracelular que son secretadas como glicoproteínas al medio. Consta de, al menos, siete miembros descritos (Argraves et al. 1990, de Vega et al. 2009). Las fibulinas desempeñan papeles tanto pro- como anti-tumorales y su expresión está desregulada en algunos cánceres humanos, lo que se correlaciona con la progresión tumoral y la generación de metástasis (Obaya et al. 2012). En particular, la Fibulina 3 está implicada en la

regulación de la migración e invasión durante la progresión tumoral y puede ejercer un papel pro- o anti-tumoral. Por ejemplo, la Fibulina 3 ejerce una función pro-tumoral en glioma (Hu et al. 2009, Nandhu et al. 2014) y adenocarcinoma pancreático (Camaj et al. 2009), mientras que desempeña una función como supresor tumoral en el cáncer de pulmón (Kim et al. 2012, Chen et al. 2014) y el carcinoma nasofaríngeo (Hwang et al. 2010).

2. OBJETIVOS

1. Determinar si la p38α MAPK regula la expresión de fibulina 3, cómo lo hace y el papel de Fibulina 3 como potencial mediador de los efectos de p38α sobre la migración, la invasión y el crecimiento tumoral.

2. Analizar si C3G podría actuar a través de p38α MAPK para regular la migración, la invasión y el crecimiento tumoral.

3. RESULTADOS

1. p38α MAPK REGULA NEGATIVAMENTE LA EXPRESIÓN DE FIBULINA 3 MEDIANTE METILACIÓN DE SECUENCIAS REGULATORIAS DEL ADN. PAPEL EN LA MIGRACION CELULAR, LA INVASIÓN CELULAR Y EL CRECIMIENTO TUMORAL

p38α MAPK regula negativamente la expresión de fibulina 3

Basándonos en datos previos del grupo derivados de análisis de expresión génica mediante *microarrays* de Affymetrix que mostraban que la expresión de fibulina 3 estaba reprimida por p38α en MEFs, hemos caracterizado el papel de p38α en la regulación de la expresión de fibulina 3 en la línea celular humana de carcinoma de colon HCT116.

Hemos visto que p38α MAPK regula negativamente la expresión de fibulina 3 en estas células. Se había descrito que la expresión de fibulina 3 se regulaba principalmente a nivel transcripcional mediante metilación de secuencias reguladoras (Blackburn et al. 2003, Kim et al. 2012). Por ello, estudiamos si p38α podría estar inhibiendo la expresión de fibulina 3 mediante metilación del DNA. Los resultados obtenidos tras el tratamiento de las células con 5-aza-2´-deoxicytidine (5A2dC) sugieren que p38α reprime la expresión de fibulina 3 mediante hipermetilación del DNA. Además, encontramos que la DNA metilasa, DNMT3A, cuyos niveles están incrementados cuando se expresa p38α, podría estar mediando esta metilación. El aumento de los niveles de la proteína de unión a RNA, HuR, inducido por p38αMAPK podría favorecer la estabilización del RNAm de dnmt3a, lo que daría lugar a un incremento en los niveles proteicos de la DNMT3A.

El silenciamiento de fibulina 3 inhibe la migración y la invasión en células HCT116

Nuestros resultados derivados del análisis del efecto del silenciamiento de fibulina 3 indican que la Fibulina 3 promueve la migración y la invasión en la línea celular humana de cáncer colorectal HCT116 mediante un mecanismo que parece requerir la activación de p38α y/o p38β. Como cabía esperar, p38α media la migración y la invasión en estas células. Nuestros resultados también sugieren que las metaloproteasas MMP2 y 9 podrían estar implicadas en la regulación de la invasión celular por Fibulina 3/p38α.

Papel de Fibulina 3 en la capacidad tumorigénica de las células HCT116

Además hemos estudiado la implicación de Fibulina 3 y de p38α en la regulación del crecimiento tumoral mediante ensayos *in vitro* e *in vivo* (xenotransplantes). Hemos visto que la Fibulina 3 promueve el crecimiento tumoral de las células HCT116 a través de un mecanismo dependiente de p38α. Además, en este modelo tumoral, p38α es un potente activador del crecimiento tumoral.

2. C3G REGULA LA ADHESIÓN, MIGRACIÓN E INVASIÓN CELULAR A TRAVÉS DE LA REGULACIÓN NEGATIVA DE LA ACTIVIDAD DE p38α MAPK MEDIANTE UN MECANISMO NO MEDIADO POR RAP1. IMPLICACIÓN EN EL CRECIMIENTO TUMORAL

Nuestro grupo ha demostrado previamente que C3G a través de la regulación negativa de la actividad de p38α MAPK controla la apoptosis (Gutierrez-Uzquiza et al. 2010). En base a esto, hemos estudiado si esta cascada C3G/p38α podría estar también implicada en la regulación de los procesos de migración e invasión celular en las células HCT116.

C3G inhibe la migración y la invasión de las células HCT116 mediante la regulación negativa de la actividad de p38α en un mecanismo no mediado por Rap1. Función en la regulación de la adhesión celular

De acuerdo a la información de la base de datos Oncomine, la expresión de C3G puede estar desregulada en carcinoma de colon. Basándonos en ello, hemos analizado la expresión de C3G en diferentes líneas de cáncer colorectal con diferente capacidad invasiva y hemos visto que existe una correlación inversa entre los niveles de C3G y la capacidad invasiva de estas líneas celulares. Además, hemos demostrado que el silenciamiento de C3G promueve la migración y la invasión a través del aumento de la actividad de p38α mediante un mecanismo no mediado por Rap1 en la línea humana de carcinoma de colon HCT116. Así mismo, C3G promueve la adhesión celular mediante la inhibición de la actividad de p38α, mientras que Rap1 ejerce un efecto opuesto.

MMP2 y 9 podrían estar implicadas en la regulación de la migración e invasión por C3G/p38α. Además, los cambios en la expresión de E-Cadherina y ZO-1, así como de su localización subcelular, junto con la reorganización del citoesqueleto de F-actina, podrían ser responsables de los efectos de C3G y p38α en estos procesos celulares.

Papel de C3G en la regulación de la capacidad tumorigénica de las células HCT116

Nuestros resultados sugieren que tanto C3G como p38α MAPK promueven el crecimiento tumoral de las células HCT116 *in vitro* e *in vivo*, probablemente mediante mecanismos independientes. En base a la reducción de la viabilidad celular al silenciar la expresión de C3G y/o p38α, C3G y/o p38α favorecerían la supervivencia de estas células, lo que daría lugar al crecimiento tumoral. El TGFβ y otras citoquinas y/o quimoquinas podrían contribuir a mediar las acciones de C3G y/o p38α sobre el crecimiento tumoral.

Por último, hemos encontrado que el silenciamiento de p38α aumenta la formación de esferas en la línea celular HCT116.

4. DISCUSIÓN

En la primera parte de esta Tesis, hemos identificado a la Fibulina 3 como una nueva diana de p38α MAPK en la línea celular humana de cáncer colorectal HCT116. Además, hemos demostrado que p38α regula negativamente la expresión de fibulina 3 mediante la inducción de la metilación del DNA por la DNA metilasa, DNMT3A. p38α ejercería dicho efecto aumentando los niveles de HuR, lo que daría lugar a la estabilización del RNAm de la dnmt3a. La regulación negativa de la expresión de fibulina 3 mediante hipermetilación de la región promotora ha sido previamente descrita en cáncer de pulmón de célula no pequeña y cáncer colorectal (Kim et al. 2012, Tong et al. 2011). Sin embargo, los mecanismos que subyacen a las acciones de la Fibulina 3 en cáncer, aún no se conocen bien. Por tanto, el hecho de identificar a la p38α MAPK como un nuevo regulador de la expresión de fibulina 3 en el modelo de cáncer colorectal es importante para entender mejor el papel que juega la Fibulina 3 en la progresión tumoral. Además, se había demostrado que la Fibulina 3 puede actuar tanto como activador de los procesos de migración e invasión (Hu et al. 2009, Seeliger et al. 2009) o como inhibidor (Kim et al. 2012). En nuestro estudio, hemos identificado a la Fibulina 3 como un regulador positivo de la migración, la invasión y el crecimiento tumoral actuando a través de mecanismos dependientes de p38α, que, a su vez, actúa favoreciendo el crecimiento tumoral de las células HCT116.

En la segunda parte de este estudio, hemos demostrado que C3G regula la migración y la invasión celular en las células HCT116 a través de la inhibición de la p38α MAPK, al igual que controla la muerte celular en MEFs y en células de leucemia mieloide crónica (Gutierrez-Uzquiza et al. 2010, Maia et al. 2009). Sin embargo, estas acciones de C3G no están mediadas por Rap1. De hecho, Rap1 juega un papel opuesto. Estos resultados están de acuerdo con el aumento de migración descrito en MEFs C3G-/- (Ohba et al. 2001) y la reducción en la migración encontrada en células de carcinoma de mama con sobre-expresión de C3G (Dayma and Radha 2011). Rap1 favorecería la migración y la invasión celular a través de la activación de p38α. Este papel de Rap1 como promotor de estos procesos, también ha sido descrito en otros estudios (Kim et al. 2012, Lin et al. 2010, McSherry et al. 2011). Nuestros datos también indican que C3G actuando a través de p38α regula la remodelación del citoesqueleto de F-actina.

Esto unido a los que cambios que induce en la expresión de E-Cadherina y la localización subcelular de ZO-1 mediarían sus efectos sobre la migración y la invasión. Además, C3G y p38α promueven el crecimiento tumoral de las células HCT116 *in vitro* e *in vivo*, probablemente a través de mecanismos distintos. Este efecto de C3G sería dependiente de su papel como activador de la adhesión y la supervivencia celular. p38α, también induciría la supervivencia de estas células, lo que estaría de acuerdo con datos previos de la literatura (Gupta et al. 2015). Adicionalmente, C3G y p38α podrían estar implicados en la regulación del estroma en el nicho tumoral, lo que contribuiría a la inducción del crecimiento tumoral *in vivo.*

5. CONCLUSIÓN FINAL

La p38α MAPK reprime la expresión de fibulina 3 a través de la inducción de la metilación del DNA en las células humanas de carcinoma de colon HCT116. Esto podría representar un mecanismo de retro-inhibición para limitar su efecto pro-migratorio e invasivo, que está mediado por mecanismos que implican la activación de p38α. Además, la Fibulina 3 promueve el crecimiento tumoral actuando a través de la p38α, la cual, juega un papel pro-tumoral. Por otro lado, C3G inhibe la migración y la invasión a través de la regulación negativa de la actividad de p38α por un mecanismo no mediado por Rap1. Sin embargo, tanto C3G como p38α colaboran para inducir el crecimiento tumoral, probablemente actuando a través de diferentes rutas.

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APPENDIX

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p38 MAPK Down-regulates Fibulin 3 Expression through Methylation of Gene Regulatory Sequences

*ROLE IN MIGRATION AND INVASION**

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Background: p38α MAPK regulates migration/invasion.
Results: p38α induces hypermethylation of Fibulin 3 gene regulatory sequences leading to Fibulin 3 down-regulation. This contributes to regulate migration and invasion in MEFs and HCT116 cells.
Conclusion: p38α down-regulates fibulin 3 expression through promoter methylation to control p38α-mediated migration and invasion.
Significance: To understand the function of new p38α targets in migration/invasion and tumorigenesis.

p38 MAPKs regulate migration and invasion. However, the mechanisms involved are only partially known. We had previously identified fibulin 3, which plays a role in migration, invasion, and tumorigenesis, as a gene regulated by p38α. We have characterized in detail how p38 MAPK regulates fibulin 3 expression and its role. We describe here for the first time that p38α, p38γ, and p38δ down-regulate fibulin 3 expression. p38α has a stronger effect, and it does so through hypermethylation of CpG sites in the regulatory sequences of the gene. This would be mediated by the DNA methylase, DNMT3A, which is down-regulated in cells lacking p38α, but once re-introduced represses Fibulin 3 expression. p38α through HuR stabilizes dnmt3a mRNA leading to an increase in DNMT3A protein levels. Moreover, by knocking-down fibulin 3, we have found that Fibulin 3 inhibits migration and invasion in MEFs by mechanisms involving p38α/β inhibition. Hence, p38α pro-migratory/invasive effect might be, at least in part, mediated by fibulin 3 down-regulation in MEFs. In contrast, in HCT116 cells, Fibulin 3 promotes migration and invasion through a mechanism dependent on p38α and/or p38β activation. Furthermore, Fibulin 3 promotes *in vitro* and *in vivo* tumor growth of HCT116 cells through a mechanism dependent on p38α, which surprisingly acts as a potent inducer of tumor growth. At the same time, p38α limits fibulin 3 expression, which might represent a negative feed-back loop.

p38 MAPKs[5] are a subfamily of MAPKs activated by several stimuli, which are involved in the regulation of the main cellular functions, including migration and invasion (1–2). There are four isoforms of p38 MAPKs: p38α, p38β, p38γ, and p38δ, which can have both overlapping and specific functions (1). p38α and p38β show a high grade of homology and are ubiquitously expressed, while p38γ and p38δ have more restricted expression patterns and some specialized functions (2).

p38α is essential for embryonic development (3–4), being expressed at high levels (5). It regulates different cellular functions. For example, it can inhibit proliferation (6–8) and adhesion (9) and promote differentiation (7, 10), apoptosis (11–13), migration (14), and invasion (15–16). In addition, it can also activate proliferation (17–18) or survival (19–21). In fact, it is now clear that p38α can play dual roles depending on the stimulus, cellular context, or other additional factors (8). This is also true for cancer, where p38α behaves as either a tumor suppressor or promoter depending on the type of cancer and the tumor stage (8). In a number of tumors, p38α inhibits tumor initiation because of its role in cell cycle arrest and in the induction of apoptosis (13). However, at later stages, it can promote survival (19–20), migration, and invasion leading to metastasis (8, 16).

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[5] The abbreviations used are: MAPKs, mitogen-activated protein kinase; ECM, extracellular matrix; DNMT, DNA methylase; MMPs, matrix metalloproteases.