UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA
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TESIS DOCTORAL

Nuevos mediadores de las acciones de p38alfa MAPK en la supervivencia y la migración-invasión celular: interacción con C3G.

New mediators of p38alpha MAPK actions on cell survival and migration-invasion : cross-talk with C3G

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directora

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Madrid, 2014

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Tesis Doctoral

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OPTA AL GRADO DE DOCTOR EUROPEO:

María Arechederra Calderón

A mis hermanos,

mis padres

A Kiri

ABBREVIATIONS

Akt	AK strain Transforming; v-Akt murine thymoma viral oncogene homolog 1
AP-1/2	Activator protein 1
ASK1	Apoptosis Signal Regulating Kinase 1
AT	Anaphylatoxin
ATF-1/2/6	Activating transcription factor 1/2/6
5A2dC	5-Aza-2´-deoxicytidine
Bax	BCL2-Associated X Protein
C/EBP	CCAAT-Enhancer binding protein
C3G	Crk SH3-domain-binding guanine-nucleotide-releasing factor
cbEGF	Calcium-binding EGF (Epidermal growth factor)
CCP	Complement Control Protein
Cdc7	Cell division cycle 7-related protein kinase
c-DNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CHOP	C/EBP homologous protein
CML	Chronic Myeloid Leukemia
CO	Carbon Monoxide
COX-2	Cyclooxigenase-2
$DCFH_2$	2',7'-diclorodihidrofluorescein
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMTs	DNA Methyl Transferases (DNMT1,-3a, -3b, -2, 3L)
DNRap-1	Dominant negative Rap-1
DTCs	Disseminated Tumour Cells
ECM	Extracellular Matrix
EC-SOD	Extracellular SOD
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic Elongation Factor 2
EFEMP-1	EGF-containing fibulin-like extracellular matrix protein 1
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N' tetraacetic acid
EGF	Epidermal Growth Factor
Elk-1	ETS domain-containing protein Elk-1
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
ERKs	Extracellular Signals Regulated Kinases
ES cells	Embryonic Stem cells
FA	Focal Adhesion

$FADH_2$	Reduced Flavin Adenine Dinucleotide
FBS	Fetal bovine serum
FC-domain	Fibulin-like domain
Fe	Ferrum
FGF2	Fibroblast Growth Factor 2
FoxM1	Forkhead box protein M1
FoxO/FOXO3a	Forkhead Box 0
GC-rich regions	Guanine-Cytosine-rich regions
GEF	Guanine Nucleotide Exchange Factor
GLUT4	Glucose Transporter Type 4
GPx	Glutathione Peroxidases
Grb2	Growth Factor Receptor-bound Protein 2
GSH	Glutathione
GSK3β	Glycogen Synthase Kinase 3β
GSTs	Glutathione-S-Transferases
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HDF	Human Diploid Fibroblasts
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia Inducible Factor
Hsp27	Heat-shock protein 27
HuR	Human antigen R
IGF1R	Insulin-like Growth Factor-1
IL-1/IL-3/ IL-6	Interleukin-1/3/6
JNK	c-Jun N-terminal Kinases
KDa	KiloDalton
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinases
MBDs	Methyl-binding domain proteins
MEFs	Mouse Embryonic Fibroblasts
Met	Met Tyrosine Kinase Receptor
MK2/MAPKAP-K2	MAPK-Activated Protein Kinases 2
MKK	MAP Kinase Kinase
MKKK	MAPK Kinase Kinase
MKP	MAP Kinase Phosphatases
MLK3	Mixed Lineage Kinase 3
MMPs	Matrix-Metalloproteases
Mn	Manganesum
MNK1	MAP Kinase Interaction Protein Kinase
mRNA	Messenger Ribonucleotic Acid
MSK	Mitogen and Stress activated Kinase
mTORC1	Mammalian Target Of Rapamycin Complex 1
NAC	N-Acetyl-cysteine
NADPH	Nicotinamide Adenine Dinucleoitide Phosphate
NF-kB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NOX	NADPH Oxidases

NP40	Nonidet P-40
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSAIDs	Non-Steroidal anti-inflammatory drug
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
OIS	Oncogene induced senescence
p70S6K	Ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-Kinase
PMSF	Phenylmethanesulfonylfluoride
PP2A	Protein Phosphatase 2A
PP2C	Ser/Thr phosphatase
PPARγ	Peroxisome proliferator-activated receptor gamma
Prx	Peroxiredoxins
PTP	Protein tyrosine phosphatase
Rac	Ras-Related C3 botulinum toxin
Rap-1	Ras-related protein 1
Ras	Rat Sarcoma virus
REM	Ras-Exchange-Motif
Rheb	Ras Homolog Enriched in Brain
Rho	Ras Homolog
ROS	Reactive Oxygen Species
RT-qPCR	Quantitative PCR
S1-5	Fibulin 3
SAP-1, SAP-90, SAP-97	Secreted Aspartyl Proteinase-1, -90, -97
SDS	Sodium Docecyl Sulphate
Ser	Serine
SH2/2/3	Src Homology 3
SOD	Superoxide dismutase
SOD1	Citosolyc Cu/Zn-SOD
SOD2	Mitochondrial Mn-SOD
TAB-1	TAK1-binding protein 1
TAK-1	Transforming growth factor-b-activated protein kinase 1
TCR	T-Cell Receptor
TGF-β1	Tumour Growth Factor beta-1
Thr	Threonine
TIMPs	Inhibitor of Metalloproteinsases
TNF-α	Tumour Necrosis Factor alpha
Trx	Thioredoxin
TrxR	Thioredoxin reductases
Tyr	Tyrosine
UCPs	Uncoupling Proteins

INDEX

INTRODUCTION

1. p38 MAPKs

1.1. 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 tyrosine and threonine residues in a conserved Thr-X-Try motif (where X is any amino acid) by a MAP kinase kinase (MKK), which in turn is activated when phosphorilated by a MAPK kinase kinase (MKKK) (Fig. 1). MAPK phosphatases reverse the phosphorylation and return the MAPK to their inactive state (Chang and Karin 2001;Dhillon *et al.* 2007).

In mammals, there are more than a dozen MAPK genes codifying for different MAPKs. The best known subfamilies of MAPKs are ERKs (Extracellular signal-regulated kinases: ERK-1 and ERK-2), mainly activated by growth factors, and the stress MAPKs, JNKs (c-Jun N-terminal kinases: JNK-1, JNK-2 and JNK-3)

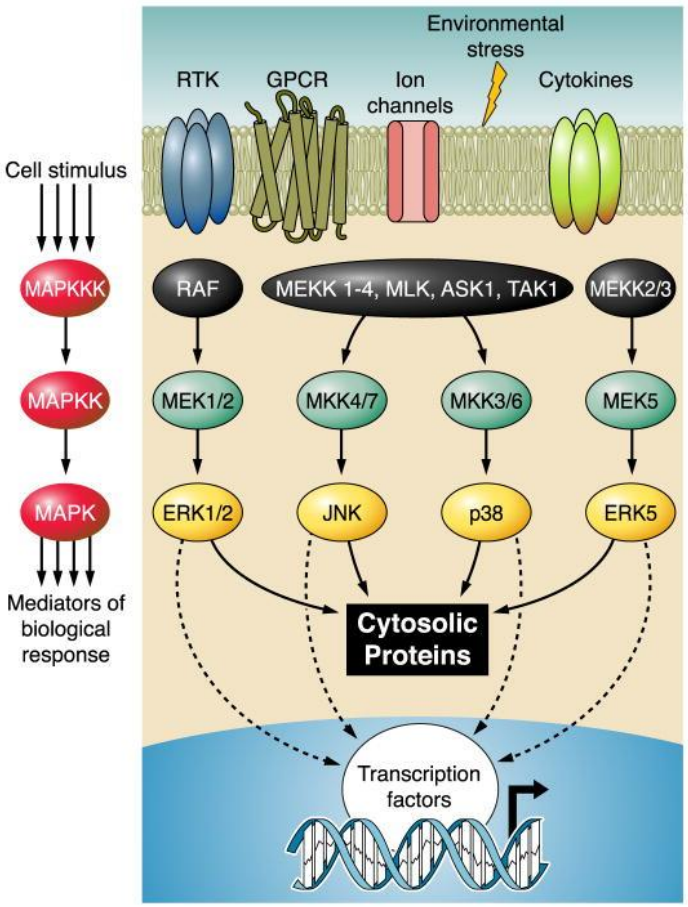

Figure 1: Schematic overview of MAPK pathway: Ilustration of the three-tiered MAPK cascades for ERK, JNK and p38 family members (Rose *et al.* 2010)

and p38 MAPKs, mainly activated by stress. Other MAPKs are ERK3, ERK5 and ERK7, which belong to the non-classical subfamily and have distinct regulation and functions (Raman *et al.* 2007).

There are four p38 MAPKs isoforms in mammals: α, β, γ and δ, which are 60% identical in their amino acid sequence. The four p38 MAPKs are encoded by different genes and have different tissue expression patterns. 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; Nebreda and Porras 2000).

The first member of the p38 MAPK family was independently identified by four groups. p38α was initially identified as a 38 KDa protein that was rapidly phosphorylated on tyrosine in response to LPS (lipopolysaccharide) stimulation (Han *et al.* 1994). In parallel, two other groups independently identified p38α as a kinase activated by stress (Rouse *et al.* 1994) and by IL-1 (Freshney *et al.* 1994;Kim *et al.* 2012a). Later on that year, another identification of p38α was reported as a polypeptide receptor for a class of pyridinyl imidazole anti-inflammatory drugs (CSAIDs) (Lee *et al.* 1994). A few years after the identification of p38α, three additional isoforms were described: p38β (Jiang *et al.* 1996), p38γ (Li *et al.* 1996) and p38δ (Goedert *et al.* 1997; Jiang *et al.* 1997).

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 testi, pancreas, kidney and small intestine (Cuenda and Rousseau 2007).

The canonical activation of p38 MAPKs occurs via dual phosphorylation of their Thr-Gly-Tyr motif by three dual-specificity MKKs. MKK6 can phosphorylate the four p38 MAPK isoforms, whereas MKK3 activates p38α, p38γ and p38δ, but not p38β. In addition, p38α can be also phosphorylated by MKK4, an activator of JNKs (Cuadrado and Nebreda 2010) (Fig. 2).

Moreover, three non-canonical mechanisms of p38α (and probably p38β) activation have also been described. One, apparently specific to antigen receptor [TCR (T-cell receptor)]-stimulated T-lymphocytes, involves p38α phosphorylation

on a non-canonical activating residue, Tyr323 (Salvador *et al.* 2005). A second mechanism of activation is mediated by the interaction of TAB-1 (Transforming growth factor-β-activated protein 1 (TAK1)-binding protein 1) with p38α, which induces p38α auto-phosphorylation (Ge *et al.* 2002;Li *et al.* 2005). Finally, down-regulation of the protein kinase Cdc7 induces an abortive S-phase leading to p38α-mediated apoptosis in HeLa cells (Im and Lee 2008).

The magnitude and duration of p38 MAPK signal are critical determinants of its 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, grouped as the MAPK phosphatase family (MKP), can efficiently dephosphorylate p38α and p38β (but not p38γ and p38δ). In addition, other protein phosphatases, as PP2C (Ser/Thr phosphatase) and PTP (Tyr phosphatase), can inactivate all p38 MAPK isoforms (Cuenda and Rousseau 2007; Zarubin and Han 2005).

The *in vivo* functional redundancy of p38 isoforms, mainly p38α and p38β, has made the elucidation of their physiological functions a complicated process. The main tools used to identify p38 MAPKs physiological roles have been the chemical inhibitors and the knock-out mice. The inhibitors, SB203580 and SB202190 are selective for p38α and p38β, while BIRB796 can inhibit the four isoforms although at different doses (Kuma *et al.* 2005). p38α knock-out mouse was the first generated, but it is embryonic lethal, dying at midgestation due a placental defect (Adams *et al.* 2000;Mudgett *et al.* 2000). Tissue-specific p38α knock-outs have implicated this isoform in cardiomyocyte proliferation and survival (Engel *et al.* 2005). 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 and liver abnormalities (del Barco Barrantes *et al.* 2011). p38γ and p38δ and double p38γ/p38δ knock-out mice have also been generated, which are viable and fertile and have no obvious health problems (Sabio *et al.* 2005).

Although p38 MAPKs have overlapping substrate specificity, some substrates appear to be preferentially phosphorylated by one or more isoforms (Zarubin and Han 2005). The best known p38α and p38β substrates are some

transcription factors (ATF-1/2, CHOP or p53), protein kinases (MNK1, PRAK or MSK), cytoskeletal proteins, translational machinery components and other proteins such as metabolic enzymes, glycogen synthase or cytosolic phospholipase A2. p38γ and p38δ MAPK isoforms can phosphorylate typical p38 MAPK substrates such as the transcription factors ATF2, Elk-1 or SAP-1, but they have specific ones such as α1-syntrophin, SAP90/PSD95 and SAP97/Hd1g in the case of p38γ, and stathmin, tau and eEF2 (eukaryotic elongation factor 2) kinase in the case of p38δ (Fig. 2) (Cuadrado and Nebreda 2010; Cuenda and Rousseau 2007).

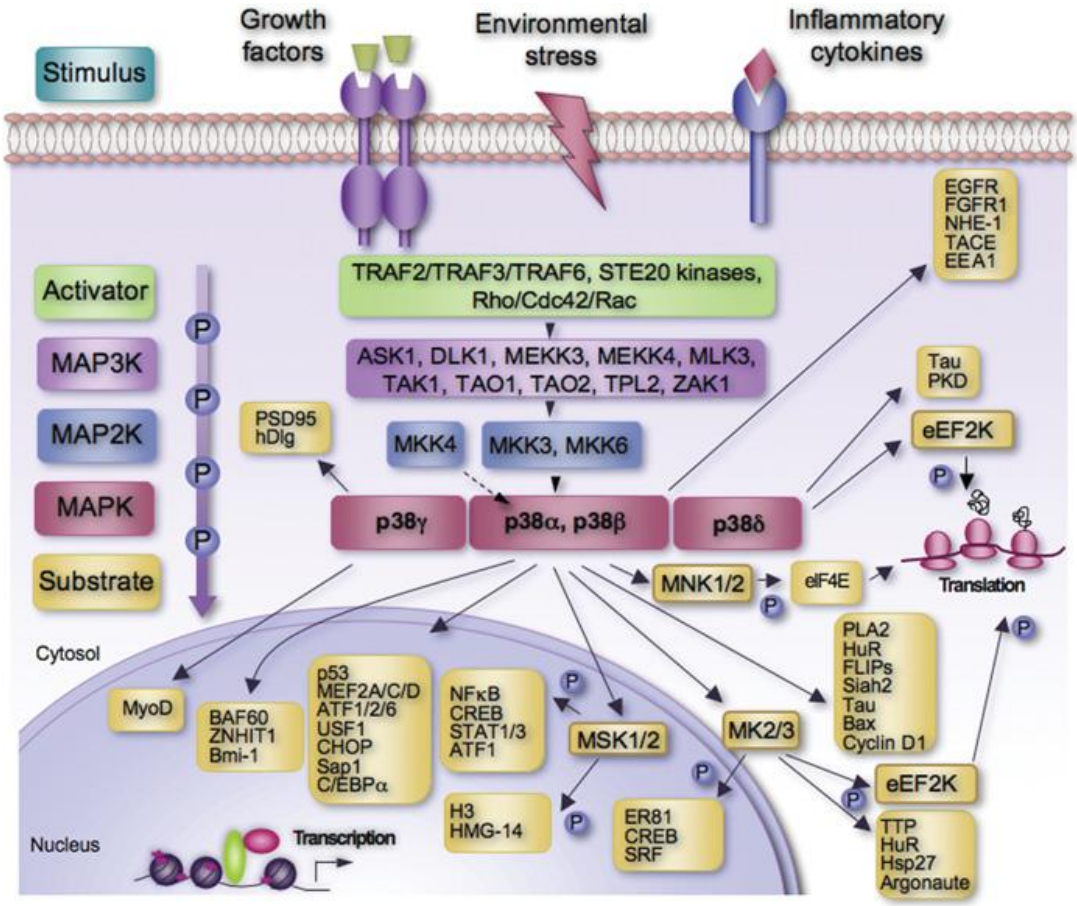

Figure 2: The p38 MAPK pathway. Different stimuli such as growth factors, inflammatory cytokines or environmental stresses can activate p38 MAPKs. A number of representative downstream targets, including protein kinases, cytosolic substrates, transcription factors and chromatin remodelers are shown (Cuadrado and Nebreda 2010).

1.2. p38 MAPKs FUNCTIONS

p38 MAPKs are important regulators of the cellular response to extracellular stimuli and therefore, they are implicated in a wide range of biological functions such as:

1. **Inflammation**: p38α plays essential roles in pro-inflammatory cytokines production such as IL-1, TNF-α and IL-6 (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) and colitis-associated colon cancer (Del Reino *et al.* 2014). By 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 adipocytic differentiation of 3T3-L1 fibroblasts, neuronal differentiation of PC12 cells or erythroid differentiation of SKT6 cells (Nebreda and Porras 2000). In this line, p38α promote skeletal muscle differentiation at different levels (Perdiguero *et al.* 2007), whereas p38γ blocks the premature differentiation of satellite cells (Gillespie *et al.* 2009).

3. **Development**: p38 MAPKs are involved in the regulation of wing morphogenesis and the correct asymmetric egg development in *Drosophila* (Nebreda and Porras 2000). In addition, p38α is essential for embryonic development. p38α Knock-out mice display embryonic lethality due to placental defects (Adams *et al.* 2000; Mudgett *et al.* 2000), whereas p38β, p38γ and p38δ knock-out animals are fully viable and fertile (Beardmore *et al.* 2005; Sabio *et al.* 2005).

4. **Cell cycle/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 negatively regulate cell cycle progression both at the G1/S and the G2/M transitions by several mechanisms (Ambrosino and Nebreda 2001; Thornton and Rincon 2009). p38γ and p38δ play also a role in proliferation. p38γ and p38δ-deficient MEFs have a slightly more rapid growth rate 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).

5. **Cell death**: p38α can play a dual role as a regulator of cell death, so it can either mediate cell survival or cell death through different mechanisms (Nebreda and Porras 2000; Wagner and Nebreda 2009), as it would be further explained.

6. **Migration/Invasion:** p38α mediates cell migration and invasion of several cell types, including tumour cells (Cuenda and Rousseau 2007; Wagner and Nebreda 2009), as it would be later explained.

7. **Adhesion:** p38α can negatively regulate cell adhesion as shown by the enhanced adhesion observed in p38α-deficient ES cells (Guo and Yang 2006) or in embryonic cardiomyocytes derived cell lines (Zuluaga *et al.* 2007b). However, in chronic myeloid leukemia (CML) cells, p38α despite inhibiting the expression and/or phosphorylation of some focal adhesion (FA) proteins, it increases cell adhesion (Maia *et al.* 2013).

8. **Senescence:** p38 MAPKs have been described to play a role in senescence induction (Bihani *et al.* 2004; Haq *et al.* 2002; McMullen *et al.* 2005). For example, in response to telomere shortening (Bihani *et al.* 2004; Haq *et al.* 2002) and in oncogene induced senescence (OIS) (Bulavin *et al.* 2003; Kwong *et al.* 2013; Kwong *et al.* 2009).

9. **Cancer:** p38α MAPK can function both, as a promoter of tumour growth and as a tumour suppressor. In the initial stages, p38α can act as a tumour suppressor. For example, in MEFs transformed by oncogenic H-Ras p38α promotes apoptosis, which in turn leads to tumour suppression (Dolado *et al.* 2007). According to this, p38α conditional deletion in adult mice favours unscheduled proliferation of progenitor cells and K-ras^{G12V} -induced tumourigenesis (Ventura *et al.* 2007). Similarly, p38α liver-specific deletion enhances tumour development through up-regulation of the JNK-c-Jun pathway (Hui et al. 2007). However, in colitis associated colon-cancer p38α MAPK plays a dual role, suppressing tumour initiation, while contributing to cell proliferation and survival later on (Gupta *et al.* 2014). In addition, it also favours colon cancer metastasis to the lung (Urosevic *et al.* 2014).

Furthermore, in head and neck cancer, p38α induces tumour dormancy, which favours survival of disseminated tumour cells (Aguirre-Ghiso 2007;Bragado *et al.* 2013). In addition, p38δ has also been shown to play an important role in skin carcinogenesis (Schindler *et al.* 2009) and p38γ/δ in colitis-associated colon cancer formation (Del *et al.* 2014). In contrast, another study revealed a tumour suppressor role for p38γ and p38δ (Cerezo-Guisado *et al.* 2011).

1.3. p38 MAPK CROSS-TALK WITH OTHER SIGNALING PATHWAYS

The cross-talk between different signaling pathways is a common theme in cell regulation. It usually depends on the cell context and plays an important role in fine-tuning biological responses (Cuadrado and Nebreda 2010). There are many examples of cross-talk between p38 MAPKs and other proteins. For example, p38α MAPK negatively regulates the activity of the ERKs pathway (Porras *et al.* 2004) likely through PP2A stimulation (Junttila *et al.* 2008).

The interplay between the JNK and p38 MAPK pathways has been broadly documented. Although multiple stimuli can simultaneously activate both pathways, their activation have antagonistic effects in many cases. p38 MAPKs can negatively regulate JNK activity at the level of MAP3Ks, either by phosphorylating MLK3 or the TAK1 regulatory subunit TAB1 (Cuadrado and Nebreda 2010). Moreover, p38α controls myoblast proliferation by antagonizing the proliferation-promoting function of JNK (Perdiguero *et al.* 2007).

The interaction between p38α MAPK and Akt pathways at different levels is remarkable. Akt can inhibit the activation of p38α MAPK through phosphorylation of the upstream regulator, ASK1, on Ser83 (Yuan *et al.* 2003). On the other hand, p38α negatively modulates Akt activity through activation of PP2A in caveolae by regulating the interaction between caveolin-1 and PP2A (Zuluaga *et al.* 2007a). There are additional connections between the PI3K/Akt pathway and p38 MAPK. 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 stresses, amino acids and growth factors (Cully *et al.* 2010).

Moreover, in quiescent tumour cells, activation of ATF6 via p38α up-regulates mTORC1 and induces survival (Schewe and Aguirre-Ghiso 2008).

A link between the p38 MAPK and Wnt/β-catenin pathway has also been proposed. p38α MAPK inactivates GSK3β by phosphorylation, leading to β-catenin accumulation (Thornton *et al.* 2008).

The existence of a functional cross-talk between Rac and p38 MAPK has been largely reported in relation with different cellular functions. p38 can act either as an effector or an activator of Rac, as well as an inhibitor. 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.* 2007b). On the other hand, active Rac induces p38 activation in some cellular systems (Nobes and Hall 1995).

Some data from the literature point out to a regulation of p38 MAPK by Rap-1, which could be either positive or negative. Thus, Rap-1 can inhibit Ras-induced p38 MAPK activation in a thymoma cell line (Palsson *et al.* 2000;Stork and Dillon 2005), while FGF2 induced p38 MAPK activation in endothelial cells is mediated by Rap-1 (McDermott and O'Neill 2002). In addition, Rap-1 induces p38α activation in MEFs stimulated with H_2O_2 (Gutierrez-Uzquiza *et al.* 2010) and the combined function of p38 MAPK and Rap-1 is required for LPS (lipopolysaccharide)-induced chemotaxis breakdown (Yi *et al.* 2012).

Moreover, we have recently identified a new functional interaction between C3G, a GEF of Rap-1, and p38α MAPK involved in the regulation of apoptosis and cell adhesion. This cross-talk operates in MEFs (non-tumoural cells) (Gutierrez-Uzquiza *et al.* 2010) and in the K562 chronic myeloid leukemia cell line (Maia *et al.* 2009) and it would be later explained in detail in the group background section.

p38 MAPKs have been also linked with the expression and/or activity of some extracellular matrix proteins (Ivaska *et al.* 1999;Reddy *et al.* 2002). For instance, p38α/β inhibition has been demonstrated to inhibit TGF-β1 induced collagen expression in fibroblasts (Sato *et al.* 2002) and hepatic cells (Varela-Rey *et al.* 2002).

In summary, p38 MAPKs cross-talk with other signaling pathways is a

complex network that contributes to explain their involvement in several biological functions and their different context-dependent effects (Cuadrado and Nebreda 2010).

1.4. ROLE of p38 MAPKs IN CELL SURVIVAL/CELL DEATH

p38 MAPKs can play a dual role in the control of cell death, so it can either mediate cell survival or cell death through different mechanisms. The specific function of p38 MAPKs in apoptosis/survival depend on the cell type, the stimuli and/or the isoform (Nebreda and Porras 2000; Wagner and Nebreda 2009).

p38α is a mediator of apoptosis in response to a number of cellular stresses and other stimuli acting through transcriptional and post-transcriptional mechanisms in order to regulate apoptotic and/or survival pathways (Wagner and Nebreda 2009). For example, p38α sensitizes cardiomyocytes derived cell lines to apoptosis induced by different stimuli through both, up-regulation of the pro-apoptotic proteins 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 survival in embryonic stem (ES) cells (Guo and Yang, 2006) and contributes to mammary acinar morphogenesis by inducing anoikis of epithelial cells (Wen *et al.* 2011). The antitumoural effects of a number of chemotherapeutical drugs are also based on the activation of apoptosis through p38α. For example, it is essential for cisplatin-induced apoptosis in the colon carcinoma derived cell line, HCT116 (Bragado *et al.* 2007). However, in some tumours p38α is involved in chemotherapeutical resistance. Studies in head and neck cancer has shown that p38α/β upregulates the endoplasmic reticulum (ER) chaperone BiP/Grp78, which inhibits Bax activation and renders dormant HEp3 cells highly resistant to chemotherapy (Ranganathan *et al.* 2006).

On the other hand, p38α and β can have pro-survival roles (Cuenda and Rousseau 2007;Nebreda and Porras 2000;Wagner and Nebreda 2009). Anti-apoptotic roles of p38 MAPKs have been described in DNA-damaged fibroblasts (Heron-Milhavet and LeRoith 2002), differentiating neurons (Okamoto et al.,

2000) and activated macrophages (Park *et al.* 2002). In some cases, p38α has been identified as the isoform responsible for this survival effect. For example, in pulmonary arterial endothelial cells exposed to anoxia-reoxygenation during ischemia-reperfusion, carbon monoxide (CO) protects from apoptosis through p38α activation (Zhang *et al.* 2003;Zhang *et al.* 2005). In addition, in some tumour cells p38α has a pro-survival effect mediated by the up-regulation of the transcription factor ATF6, which favours survival of dormant tumour cells through activation of Rheb/mTOR pathway (Schewe and Aguirre-Ghiso 2008). In addition, p38α can also induce cell survival of colorectal cancer cells through inhibition of autophagy (Comes *et al.* 2007).

p38 MAPKs play also an important role in the coordination of cellular stress responses to reactive oxygen species (ROS), mediating cell death or survival, as it would be further explained.

However, the precise mechanisms by which p38 signaling achieves cell survival are poorly understood.

2. REACTIVE OXYGEN SPECIES

2.1. GENERALITIES

Oxidative stress is defined as a disturbance in the equilibrium between free radicals, reactive oxygen species (ROS) and endogenous antioxidant defenses. Therefore, each cell maintains a condition of homeostasis between the oxidant and antioxidant species (Dayem *et al.* 2010).

ROS is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH^-) (D'Autreaux and Toledano 2007).

There are numerous sources of ROS within the cell (Fig. 3). The majority of intracellular ROS production is derived from the mitochondria, where mitochondrial oxidants are formed predominantly at complex I (Kushnareva *et al.*

2002) or complex III (Chen *et al.* 2003) of the respiratory chain, when electrons derived from NADH or $FADH_2$ react with oxygen to produce superoxide anions. Peroxisomes are also known to produce H_2O_2 under physiologic conditions (Valko *et al.* 2004). Other important generators of intracellular ROS are the NADPH oxidases (NOX), which primary function is ROS production in the membrane (Bedard and Krause 2007). In addition, other intracellular enzymes such as xanthine oxidase, cyclooxygenases, cytochrome p450 and lipoxygenases produce ROS as part of their normal enzymatic function. ROS are also generated by exogenous substances, such as γ-ray or ultraviolet light irradiation (Kamata and Hirata 1999).

2.2. ROS FUNCTIONS

The intracellular redox state is tightly regulated because it is essential for the control of cell fate. Oxidative stress has been traditionally considered as a toxic by-product of cellular metabolism, but during the last years it has been established that ROS are actively involved in the regulation of signal-transduction pathways (Hancock *et al.* 2001).

Cells are capable of generating endogenously and constitutively ROS, which are necessary for many biochemical processes such as proliferation, differentiation, cell death, migration or inflammatory response (Finkel 2000; Valko *et al.* 2007a). Most cell types generate low concentrations of ROS in response to several cytokines, growth factors and hormones. Then, ROS can function as secondary messengers, reacting with transcription factors (AP-1, NF-kB or HIF-1) and proteins (protein tyrosine kinases or phosphatases) (Kamata and Hirata 1999; Valko *et al.* 2007a).

In contrast, high doses and/or inadequate removal of ROS can damage cellular constituents including proteins, lipids and DNA. Excessive generation of ROS has been linked to aging, neurodegenerative diseases such as Alzheimer, epilepsy and Parkinson, and vascular disease (Valko *et al.* 2007a). ROS and oxidative damage have been implicated in the induction of apoptosis or other types of cell death. Moderate levels of oxidative stress usually induce apoptotic

cell death, whereas a higher dose of ROS leads to necrosis (Kamata and Hirata 1999). For example, H_2O_2 at a low concentration is able to induce apoptosis (Pierce *et al.* 1991) and some extracellular signals, such as TNF-α, can also induce apoptosis through a mechanism dependent on ROS generation (Talley *et al.* 1995).

Different signaling pathways have been involved in mediating cell death induced by ROS such as p38 MAPKs, JNK and Akt. For instance, ASK-1 activates JNK and p38 MAPK in response to ROS, inducing apoptosis (Tobiume *et al.* 2001). Moreover, ROS generated by oncogenic H-Ras induces apoptosis through p38α activation, inhibiting tumour initiation (Dolado *et al.* 2007), whereas p38α inhibition results in ROS up-regulation, which in turn activates the JNK pathway sensitizing human tumour cells to cisplatin-induced apoptosis (Pereira *et al.* 2013). Although Akt generally inhibits apoptosis induced by multiple apoptotic stimuli, it has been shown that Akt activation can mediate ROS-induced apoptosis. Hence, Akt would elevate ROS levels by two mechanisms: (i) increasing oxygen consumption, (ii) and impairing ROS scavenging through inhibition of FoxO transcription factors (Nogueira *et al.* 2008). Additionally, the PI3K/Akt signaling pathway would play important roles in NOX activation (Nakanishi *et al.* 2014).

ROS can cooperate with oncogenic signaling in cellular transformation and cancer. The carcinogenic effects of ROS accumulation have been proposed to operate at different levels, including changes in gene expression (Allen and Tresini 2000), increased proliferation and DNA-mutation, as well as generation of genomic instability (Woo and Poon 2004). Furthermore, high levels of ROS have been detected in several human cancer cell lines (Szatrowski and Nathan 1991), as well as in human tumours from different tissues (Toyokuni *et al.* 1995). According to this, the levels of ROS scavenger enzymes, such as superoxide dismutase (SOD), glutathione peroxidase and peroxiredoxin, have been shown to be significantly decreased in cancer cells (Dayem *et al.* 2010).

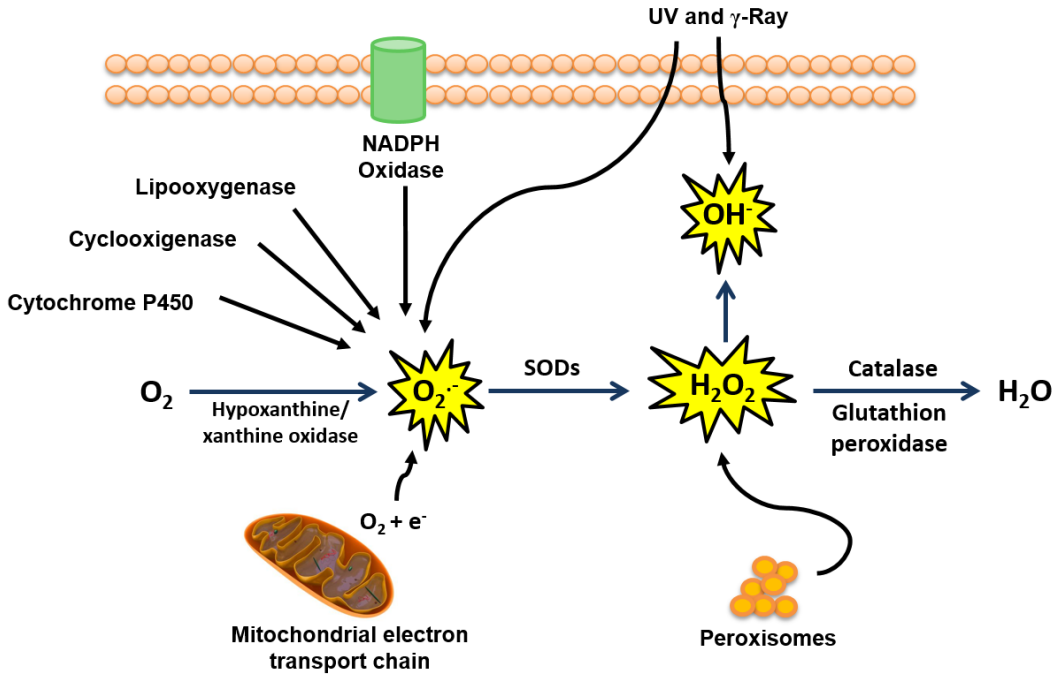

Figure 3: ROS sources and antioxidant defenses. There are numerous sources of ROS within the cell: Mitochondria, peroxisomes, NADPH oxidases (NOX), xanthine oxidase, cyclooxygenases, cytochrome p450 and lipoxygenases or exogenous molecules such as UV or γ-Ray. Cellular enzymatic antioxidant defenses include superoxide dismutases (SODs), catalase or glutathione peroxidases (GPx), among others.

2.3. ANTIOXIDANT SYSTEMS

To prevent excessive accumulation of ROS and the subsequent cell damage, aerobic organisms have developed efficient defense systems of enzymatic and non-enzymatic antioxidants (Kamata and Hirata 1999; Mates *et al.* 1999; Valko *et al.* 2007b). Exposure to oxidative stress results in the up-regulation of the enzymatic activity of the antioxidant enzymes and/or their levels (Fig. 3).

Cellular enzymatic antioxidant defenses include superoxide dismutases (SODs), catalase, glutathione peroxidases (GPx), glutathione-S-transferases (GSTs), glutathione reductase, heme oxigenase, peroxiredoxins (Prx), thioredoxin (Trx), thioredoxin reductases (TrxR) and the uncoupling proteins (UCPs), which participate in the prevention of ROS accumulation (Monsalve *et al.* 2007) (Fig. 3).

The balance between ROS production and antioxidant defenses

determines the degree of oxidative stress. Regulation of these proteins plays a pivotal role in balancing the concentration of ROS. The Nrf2 transcription factor is a relevant regulator of the expression of host antioxidants and detoxifying genes through binding to promoter sequences containing a consensus antioxidant response element (Singh *et al.* 2010).

The main antioxidant enzymes are:

- Superoxide dismutases (SODs), which catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide. In humans, there are three isoforms of SOD: cytosolic Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and extracellular SOD (EC-SOD). These isoforms elicit a similar function, but their protein structure characteristics, chromosome localization, metal cofactor requirements, gene distribution, and cellular compartmentalization are distinctly different (Kamata and Hirata 1999; Valko *et al.* 2006). They can be regulated at a transcriptional, post-transcriptional and post-translational (activity) level. Many transcriptional regulatory elements in the proximal promoter regions of the *sod* genes are binding sites for several transcription factors, such as NF-κB, AP-1, AP-2, Sp-1, FOXO3a or C/EBP. In addition, Sod-1 and sod-2 promoters have GC-rich regions, susceptible to methylation (Miao and St Clair 2009).

  Among the three SOD isoforms, MnSOD is the only SOD that is essential for the survival of aerobic organisms (Carlioz and Touati 1986). Thus, MnSOD knock-out mice showed dilated cardiomyopathy and neurodegeneration and die shortly after birth (Lebovitz *et al.* 1996;Li *et al.* 1995).

- Catalase is a tetrameric hemoprotein containing Fe (III) at its active site. It is responsible for the degradation of the H_2O_2 into water and molecular oxygen. It is mainly localized in the peroxisomes (Kamata and Hirata 1999; Valko *et al.* 2007b; Valko *et al.* 2006).

  Catalase can also be regulated at a transcriptional, post-transcriptional and post-translational (activity) level, as well as in a tissue-specific manner. A few transcription factors have been reported to directly bind to the human catalase promoter: Sp1 (Nenoi *et al.* 2001), PPARγ (Okuno *et al.* 2010), FoxM1 (Park *et al.* 2009) or Oct-1 (Quan *et al.* 2011). NFκB and AP-1 have been

associated with catalase gene expression in mouse muscle cells (Zhou *et al.* 2001). An increase in catalase mRNA stability has been also shown to up-regulate catalase levels (Clerch *et al.* 1991; Sen *et al.* 2005). On the other hand, hypermethylation of a CpG island in the promoter leads to down-regulation of catalase expression upon prolonged ROS exposure (Min *et al.* 2010).

- Glutathione peroxidases (GPx) catalyze the reduction of peroxides by using reduced glutathione (GSH). Their active sites contain selenium ions in the form of selenocysteine. There are five different isoforms, which present a differential tissue distribution and localization inside the cell (Kamata and Hirata 1999; Valko *et al.* 2006).
- Non-enzymatic antioxidants are represented by GSH, ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids or flavonoids (Valko *et al.* 2006).

2.4. p38α MAPK AND ROS

p38 MAPKs play an important role in the coordination of cellular stress responses to reactive oxygen species (ROS), mediating cell death or survival.

Years ago, it was described that ASK-1 is activated in response to ROS, activating JNK and p38 MAPK, which induces apoptosis (Tobiume *et al.* 2001). According to this, ROS-induced activation of p38α in the initial stages of tumour transformation promotes apoptosis and prevents ROS accumulation and their carcinogenic effects (Dolado *et al.* 2007). Moreover, p38α is necessary for cisplatin-induced apoptosis upon activation by p53-mediated ROS production (Bragado *et al.* 2007). Accordingly, highly tumourigenic cancer cell lines have developed mechanisms to uncouple p38α activation from ROS production, so that they can survive (Dolado *et al.* 2007).

In contrast, a newly identified important function for the p38α pathway is to facilitate cell survival in response to stress (Gutierrez-Uzquiza *et al.* 2010; Thornton and Rincon 2009). For instance, p38 allows survival of cells exposed to osmotic shock or oxidative stress by inducing cell cycle arrest (Joaquin *et al.*

2012). According to this, p38α (and to a lesser extend p38β) inhibition results in ROS up-regulation, which in turn activates the JNK pathway sensitizing some human tumour cells to cisplatin-induced apoptosis (Pereira *et al.* 2013).

Initially, stress signaling mechanisms are pro-survival systems as they tend to repair damage before committing cells to death or senescence. In this line, p38α has been proposed to be a positive regulator of antioxidant enzymes expression. p38α up-regulates catalase levels in response to low doses of H_2O_2 through enhancement of mRNA stability (Sen *et al.* 2005) and heme oxygenase-1 mRNA levels (Aggeli *et al.* 2006).

3. C3G

3.1. GENERALITIES

C3G (Crk SH3-domain-binding guanine-nucleotide-releasing factor) is a guanine nucleotide exchange factor (GEF) for Rap-1 (Gotoh *et al.* 1995) and R-Ras (Gotoh *et al.* 1997), two members of the Ras family of small GTPases. In addition, C3G can activate other Ras family members, such as TC21 and the Rho family member TC10 (Ehrhardt *et al.* 2002). Nevertheless, C3G can also act through a GEF independent mechanism (Guerrero *et al.* 1998; Guerrero *et al.* 2004; Shivakrupa *et al.* 2003). Alternate names of C3G are Rap GEF1, GRF2 and DKFZ p781p1719.

C3G was the first Rap GEF identified in 1994, originally isolated as an interacting partner of Crk (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, and antibodies against C3G have been shown to detect a protein of 130-140 kDa (Tanaka *et al.* 1994). The human C3G gene comprises 24 exons spanning 163kb on chromosome 9q34.3 (Takai *et al.* 1994).

Although C3G is ubiquitously expressed, some tissue-specific differences in expression levels have been seen. C3G transcripts are subject to alternative splicing and variant isoforms have been cloned from different species. In humans, C3G levels are higher in adult skeletal muscle, placenta, fetal heart and brain than

in 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.

Sequence comparison revealed 88% nucleotide sequence identity between mouse and human C3G c-DNA (Zhai *et al.* 2001). Human C3G has two predominant isoforms, 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 (lacking N-terminal 305 resiudes), named p87C3G, is abundantly expressed in chronic myeloid leukemia (CML) cells and in primary cells from CML patients. p87C3G showed differences in expression levels depending on disease remission during treatment, suggesting a role for C3G in CML (Gutierrez-Berzal *et al.* 2006). In mice, two transcripts with or without a 114 bp insertion in the N-terminal are expressed in most tissues (Zhai *et al.* 2001). C3G is built up with three modular domains clearly differentiated, both structurally and functionally (Fig. 4). The C-terminal C3G catalytic domain is homologous to CDC25 and, 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 SH3 domains of Crk, p130Cas, Grb2, c-Abl and Hck (Radha *et al.* 2011). In the N-terminal region (from amino acids 144 to 230) there is a binding site for the cytoplasmic domain of E-cadherin (Hogan *et al.* 2004). The N-terminal region negatively regulates its GEF activity, since deletion of the amino terminus results in constitutive catalytic activity (Ichiba *et al.* 1999).

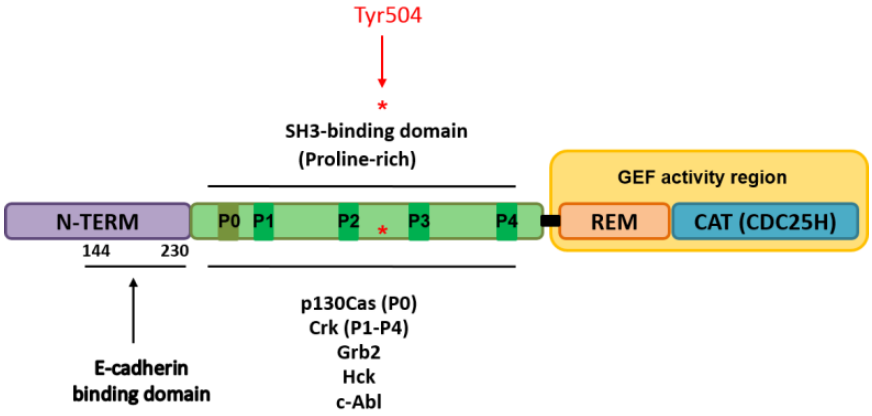

Figure 4: Schematic diagram showing the domain organization of C3G protein as well as its interaction with other proteins.

Currently, very little information is available on regulation of C3G expression and activation. It is known that a variety of stimuli such as T cell receptor stimulation (Reedquist *et al.* 1996), hepatocyte growth factor (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.

The major C3G activating mechanism in response to these stimuli is the multimolecular complex formation. It primarily occurs through membrane recruitment of Crk-C3G complex, which facilitates its interaction with the substrate Rap-1 (Gotoh *et al.* 1995; van den Berghe *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). Moreover, C3G activation has been shown to be regulated by tyrosine phosphorylation at Y504 and membrane targeting, enabled by its interaction with Crk (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 is phosphorylated in other tyrosine residues, but its contribution to C3G regulation remains unknown (Radha *et al.* 2011). Recently, it has been also described a C3G serine-phosphorylation (Utreras *et al.* 2013).

3.2. C3G FUNCTIONS

Over the last 15 years, several studies have thrown light on the involvement of C3G in multiple signaling pathways and its role in the regulation of diverse cellular functions (summarized in Fig. 5). In addition, it is important to highlight that C3G actions are not always dependent on its GEF activity and it can also be mediated by protein-protein interactions (Guerrero *et al.* 1998; Shivakrupa *et al.* 2003).

C3G-/- homozygous mice died before embryonic day 7.5 due to its function in integrin-mediated cellular adhesion and migration. Moreover, C3G-dependent activation of Rap1 is required for adhesion and spreading of

embryonic fibroblasts derived from C3G knock-out mice (Ohba *et al.* 2001).

C3G inhibits cell proliferation in some cell types such as neuroblastoma cells (Radha *et al.* 2008). In agreement with this, C3G-deficient neuroepithelial cells show an over-proliferation of the cortical neuroepithelium (Voss *et al.* 2006).

Vesicle traffic is also regulated by C3G. Insulin-stimulated GLUT4 (glucose transporter type 4) translocation in muscle and adipose tissue is dependent on C3G mediated TC10 activation (Chiang *et al.* 2001).

C3G has also been shown to play a role in cell death regulation. It can induce apoptosis though Hck (Shivakrupa *et al.* 2003) or c-Abl (Radha *et al.* 2008), 2008). Moreover, C3G through the negative regulation of p38α activity mediates cell survival or death depending on the stimulus and cell type (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009).

C3G also plays a relevant role as a mediator of cell adhesion, spreading, migration and cell-cell junction assembly and disassembly (Radha *et al.* 2011), as it would be explained later on.

The function of C3G in human cancer is controversial. C3G can act as a tumour suppressor gene, preventing malignant transformation induced by several oncogenes (Guerrero *et al.* 1998;Guerrero *et al.* 2004;Martin-Encabo *et al.* 2007). According to this, C3G expression was shown to be reduced in cervical squamous cell carcinoma (Okino *et al.* 2006). In contrast, an increase in C3G expression was observed in human non-small-cell lung cancers (Hirata *et al.* 2004), which is in agreement with the involvement of the Crk-C3G-Rap1 pathway, downstream RET, 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).

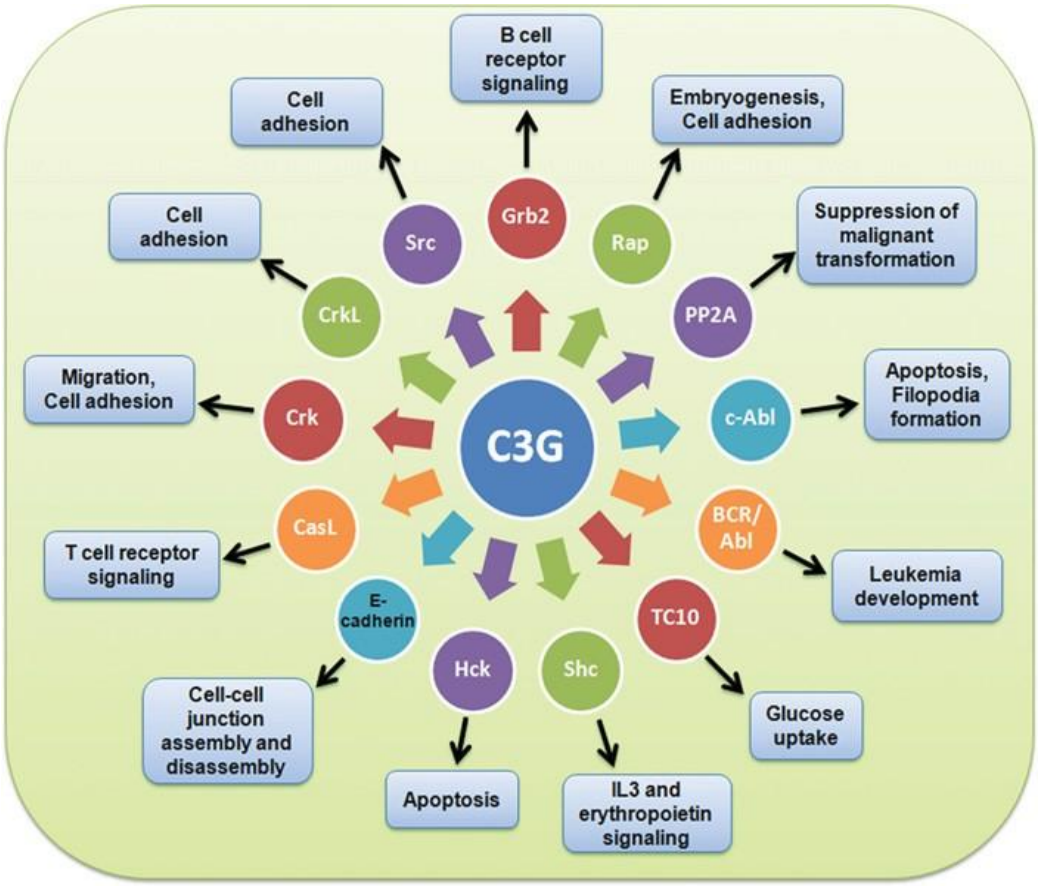

Figure 5: Interacting partners of C3G and their involvement in pathways leading to specific functions (Radha *et al.* 2011). C3G acting through a wide range of effectors regulates several cellular functions and biological processes, such as embryogenesis, cell adhesion, migration, apoptosis or leukemia development.

4. CELL MIGRATION AND INVASION

4.1. GENERALITIES

Cell migration is a complex biological process playing a key role in physiological and pathological processes. During embryogenesis, complex patterns of cell migration are essential for proper tissue formation (Franz *et al.* 2002). In the process of renewal of skin and intestine, new epithelial cells migrate from the basal layer or the crypts, respectively, to the top layer. Migration is also a prominent component of tissue repair and immune response, in which

leukocytes from the circulation migrate into the surrounding tissue to destroy invading microorganisms and infected cells (Ridley *et al.* 2003). On the other hand, migration also contributes to several important pathological processes, including vascular disease, osteoporosis, chronic inflammatory diseases or cancer (Ridley *et al.* 2003). In metastasis, tumour cells migrate from the initial tumour mass into the circulatory system and finally, to a new site, where they form a secondary tumour (van Zijl *et al.* 2011).

There are different types of cell migration. Cells can migrate as individual cells, referred to as "individual cell" migration or expand in solid cell strand, sheets, files or clusters, called "collective" migration (Friedl and Wolf 2003; Rorth 2009).

Cell migration through the tissues results from a continuous cycle of coordinated and interdependent steps that involve the cytoskeletal machinery (Friedl and Brocker 2000). Cell motility is a complex and highly coordinated process, and it is likely that changes in the expression of several genes are required for the cell to become motile (Ridley *et al.* 2003).

Several studies have demonstrated that stromal cells play a role in the control of tumour cells migration and invasion (Sameni *et al.* 2003). For example, fibroblasts and macrophages produce matrix-metalloproteases (MMPs) in many tumours, which allows extracellular matrix (ECM) degradation, promoting cancer cell motility and invasion (Wels *et al.* 2008).

4.2. MATRIX METALLOPROTEASES (MMPs)

The extracellular matrix (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 (Frantz *et al.* 2010). It is composed of a large collection of biochemically distinct components including collagens, laminins, fibronectin, elastin, fibrillins, tenascin, nidogen, entactin, fibulins, fibrinogen or thrombospondins (Halper and Kjaer 2014).

During cell migration and invasion, ECM is degraded preparing the path for cells to migrate, invade and spread to distant areas. Matrix metalloproteinases (MMPs) play a crucial role in this process. MMPs belong to a family of zinc-dependent endopeptidases (Bourboulia and Stetler-Stevenson 2010). There are 23 human MMPs (24 in mouse) which differ from each other in their structural domain architecture, in the substrate specificity, and their temporal and tissue specific expression patterns (Radisky and Radisky 2010). Originally, they were divided based on their cellular localization (17 soluble, secreted enzymes and 6 membrane-associated enzymes), or according to their substrate specificity: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), and MMPs that degrade a broad spectrum of ECM proteins called stromelysins (MMP-3, -10 and -11) or matrilysins (MMP-7). 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 (Fig. 6). 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. Additional domains are unique for a number of MMP members (Kessenbrock *et al.* 2011; Tallant *et al.* 2010).

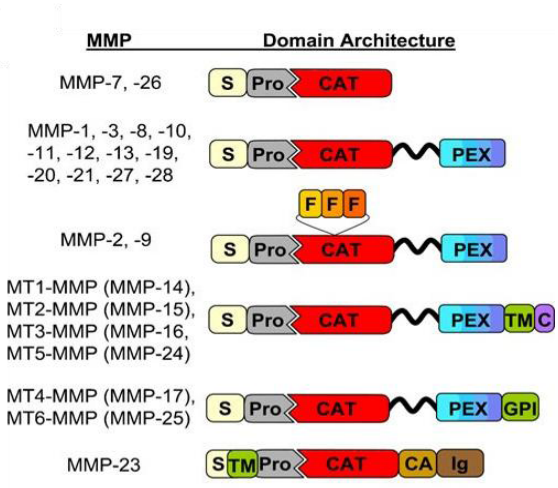

Figure 6: MMP domain structure: The various domain organizations of human MMPs. S, signal peptide; Pro, propeptide; CAT, catalytic domain; F, fibronectin repeats; PEX, hemopexin domain; TM, transmembrane domain; GPI, glycophosphatidylinositol membrane anchor;C, cytoplasmic domain; CA, cysteine array; Ig, immunoglobulin-like domain (Radisky and Radisky 2010).

MMPs are mainly regulated via modulation of gene expression, compartmentalization and inhibition of its activity by endogenous inhibitors, named tissue inhibitor of metalloproteinases (TIMPs). There are four TIMP family members (TIMP-1, -2,-3 and -4) that can inhibit all active MMPs, however, not with the same efficacy (Bourboulia and Stetler-Stevenson 2010).

Most MMPs are not constitutively transcribed, but are expressed after external induction by cytokines and growth factors (Tallant *et al.* 2010). MMPs are initially expressed in an enzymatically inactive state (pro-enzymes) due to the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site. Only after disruption of this interaction by a mechanism called cysteine switch, the enzymes become proteolytically active (Sternlicht and Werb 2001).

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 tumour progression, cancer cells participate in several interactions with the microenvironment involving extracellular matrix (ECM), growth factors and cytokines, as well as surrounding cells. The proteolytic activity of MMPs is required for a cancer cells to degrade physical barriers during local expansion and for intravasation at nearby blood vessels, extravasation and invasion at a distant location (Gialeli *et al.* 2011). Cancer cells stimulate host cells, such as fibroblast that constitute an important source of MMPs (Wels *et al.* 2008).

Increased MMP2 and MMP9 expression and activity has been described in hundreds of publications related to malignant diseases ranging from breast, urogenital, brain, lung, skin, colorectal and many more (Klein and Bischoff 2011).

4.3. ROLE of p38 MAPKs IN MIGRATION/INVASION AND IN THE GENERATION OF METASTASIS

p38 MAPKs also play a role in different aspects of cell migration, invasion and metastasis, favouring tumour progression (del Barco Barrantes and Nebreda 2012). p38α MAPK can mediate cell migration in HeLa cells and MEFs through the regulation of the actin cytoskeleton by MK2 (del, I and Nebreda 2012;Rousseau *et al.* 2006). In addition, in HGF/Met-triggered cortical neurons, Rac1/p38 cascade is important for migration (del, I and Nebreda 2012;Segarra *et al.* 2006). The activation of p38α can also induce cell migration and cytoskeletal remodeling in tumour cells by increasing the phosphorylation of Hsp27 (heat-shock protein 27) (Laferriere *et al.* 2001).

p38 MAPKs are also relevant for invasion. For example, in melanoma cells (Estrada *et al.* 2009) or ovarian cancer cells, where EGF, HGF and p38 MAPK promote cell migration and invasion through regulation of MMP-9 (Zhou *et al.* 2007). In addition, p38α induces the expression of MMP-1, MMP-2, MMP-9 and MMP-13 in bladder, breast, liver, skin, keratinocytes and prostate cancer (del Barco Barrantes and Nebreda 2012). A role for p38α mediating invasion of tumour cell has been observed in hepatocellular carcinoma, head and neck squamous carcinoma, pancreatic o glioma cell lines (del Barco Barrantes and Nebreda 2012; Wagner and Nebreda 2009).

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

In addition to p38α, p38γ and p38δ also regulate these processes. p38δ isoform increases head and neck squamous cell carcinoma invasion, which correlates with an increase of MMP-1 and MMP-13 expression (Junttila *et al.* 2007). p38γ also regulates invasion and metastasis acting as an activator and a cofactor for c-Jun-induced MMP-9 expression (Loesch *et al.* 2010).

In summary, p38 MAPKs contribute to the different steps (epithelial-mesenchymal transition (EMT), migration/invasion, anoikis resistance,

extravasation, tumour dormancy, etc) required for cancer cells to form metastatic tumours (Fig. 7) (del Barco Barrantes and Nebreda 2012).

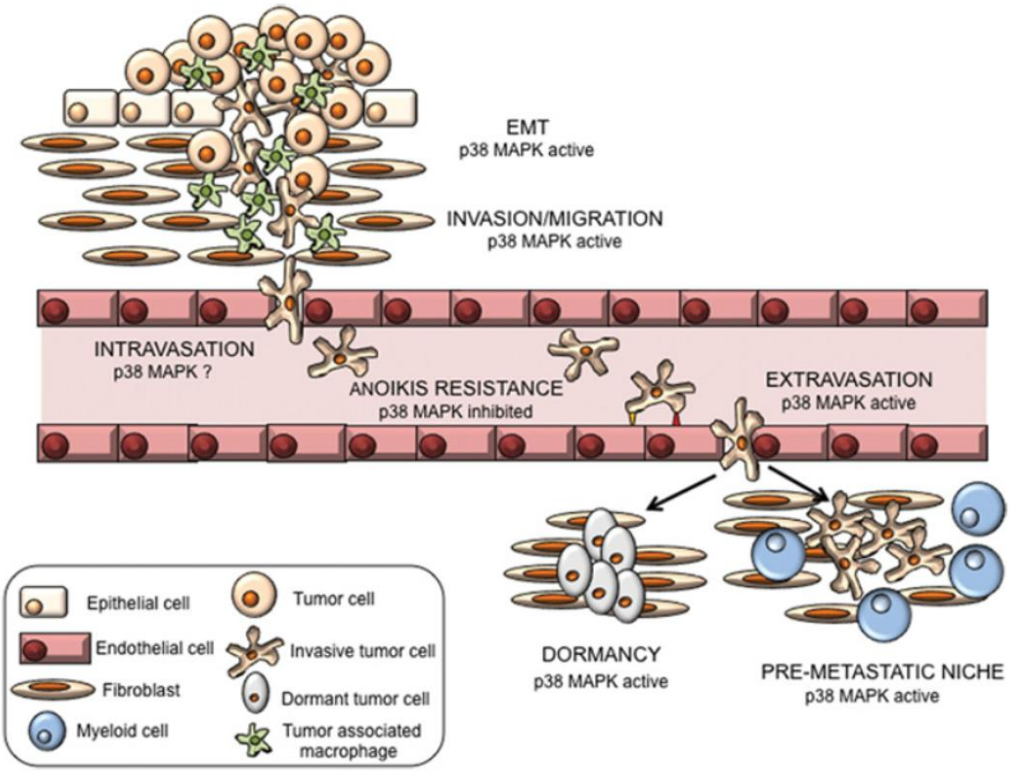

Figure 7: Roles of p38 MAPKs in tumour progression: The activation of p38 MAPKs has been reported to contribute to the EMT of cells from the primary tumour, acquisition of invasion and migrating capabilities resistance to anoikis, extravasation of migrating cells, pre-metastatic niche formation and tumour cell dormancy (del Barco Barrantes and Nebreda, 2012).

4.4. ROLE OF C3G IN ADHESION AND MIGRATION

C3G plays a relevant role as a mediator of cell adhesion and spreading activated by integrins. Hence, C3G deficient MEFs shows impaired cell adhesion, delayed cell spreading and accelerated cell migration (Ohba *et al.* 2001; Voss *et al.* 2003). This function of C3G is, at least, partially mediated by its main target, Rap-1 (Huang *et al.* 2008; Ohba *et al.* 2001; Pannekoek *et al.* 2009), although

overexpression of C3G can also promote c-Abl-induced filopodia formation during cell spreading through mechanisms independent of its catalytic activity (Radha *et al.* 2007). C3G and/or Rap-1, as well as other proteins such as Crk or p130Cas, are involved in the multiprotein complex formation during integrin-linked focal adhesions (Bos 2005).

In addition, Dr. Guerrero´s group in collaboration with our group has recently shown that C3G and p38α are acting through a common pathway promoting cell adhesion in K562 chronic myeloid leukemia cell line, where both proteins interact forming a complex (Maia *et al.* 2013). However, despite they positively regulate cell adhesion, they display antagonistic roles in the regulation of the levels of focal adhesion proteins. Thus, whereas C3G silencing inhibits their expression, p38α knock-down leads to an increase of some of these proteins (Maia *et al.* 2013).

The role of C3G in cell migration has not been well characterized, although a number of studies indicate that it can play specific roles in different contexts. On one side, C3G deficient MEFs present an enhanced migration (Ohba *et al.* 2001), while sympathetic preganglionic neurons migrate abnormally in a C3G mutant mouse (Yip *et al.* 2012). On the other side, C3G overexpression results in opposite outcomes in different cell types: increase cell migration in glomerulonephritis (Rufanova *et al.* 2009) or decrease cell migration in highly invasive breast carcinoma cells (Dayma and Radha 2011).

C3G also regulates cell-cell interactions (Kooistra *et al.* 2007; Pannekoek *et al.* 2009), where E-cadherin plays a key role (Pannekoek *et al.* 2009). Mechanistically, C3G binds intracellular E-cadherin to activate Rap-1 and induces E-cadherin translocation (Pannekoek *et al.* 2009). Moreover, as 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. All this is relevant for cell migration.

5. FIBULINS

5.1. GENERALITIES

Fibulins are a family of ECM secreted glycoproteins in the 50-200 KDa range. In 1989, Argraves described fibulin-1 (Argraves *et al.* 1989) as an ECM protein (Argraves *et al.* 1990). Since then, six more members have been identified in mammals (de Vega *et al.* 2009).

As shown in figure 8, structurally, fibulins are organized in three domains. All fibulins share a globular C-terminal domain referred to as “fibulin-like” or “FC domain” (domain III). Functionally, these fibulin-like modules have been implicated in mediating a number of protein-protein interactions. 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 varies among the family members (Obaya *et al.* 2012).

The members of the fibulin family have been classified into two subgroups according to their domain architectures. The first subgroup includes fibulin-1 (100kDa) and fibulin-2 (195kDa), which are larger than the other members, as 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 contains the rest of the members, from fibulin-3 to fibulin-7. They are smaller proteins of around 50-60 kDa, except fibulin-6 or hemicentin-1, which is the largest member of the family (615kDa) due to the presence of 44 tandem of immunoglobulin C-2 and 6 thrombospondins type-I modules within their domain I. Fibulin-7 has a unique N-terminal domain I, containing a sushi domain, also known as complement control protein (CCP) domain (de Vega *et al.* 2009).

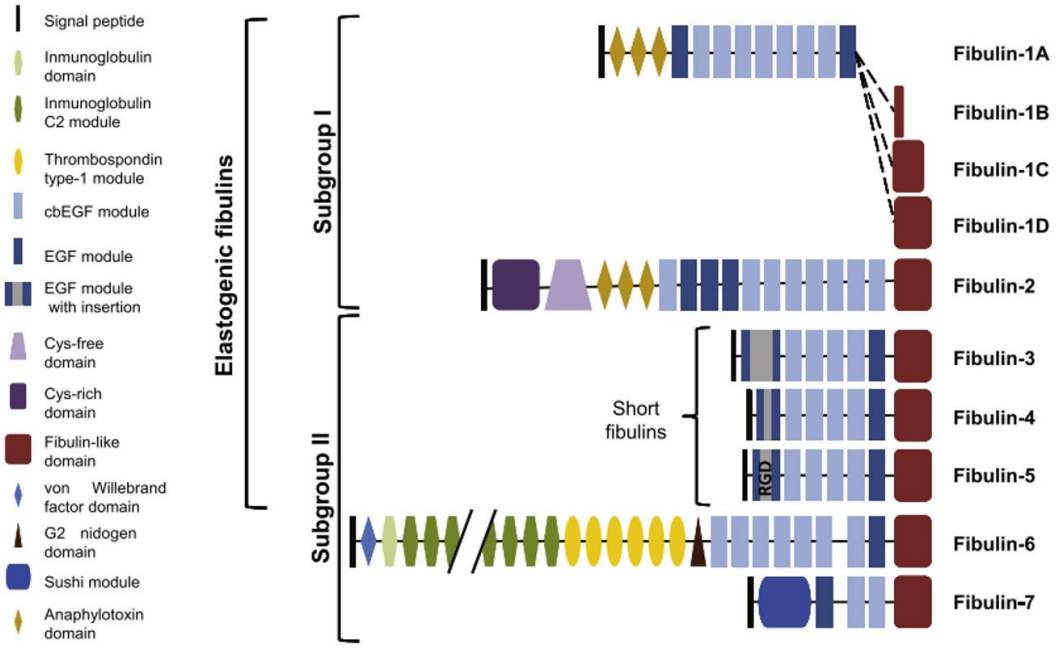

Figure 8: Schematic representation of Fibulin family members (Obaya *et al.* 2012).

The different fibulins have a distinct tissue distribution, although some of them are expressed in the same places.

The functional relevance of fibulins is reinforzed when performing the phenotypic characterization of mouse models deficient in a specific fibulin gene. 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 deficient mice evidence alterations associated with premature aging and reduced lifespan, reproductive difficulties, loss of body mass and generalized atrophy in tissues such as fat or muscle (McLaughlin *et al.* 2007). Fibulin 4 knock-out mice die perinatally and exhibit lung and vascular disorders (McLaughlin *et al.* 2006). Finally, mice lacking Fibulin 5 survive to adulthood, but display highly disorganized elastic fibers (Yanagisawa *et al.* 2002).

Fibulins exhibit an extensive array of protein-protein interactions, particularly, with other extracellular matrix proteins. Indeed, it is thought that fibulins act as intramolecular bridges within the ECM, connecting various

supramolecular structures, and mediate certain cell signaling events (Gallagher *et al.* 2005). Fibulins have been shown to modulate cell morphology, growth, adhesion and motility. The dysregulation of certain fibulins occurs in a range of human disorders, including cancer. So far, different studies conclude that both tumour suppressive functions and oncogenic activities can be elicited by fibulins (Obaya *et al.* 2012).

5.2. 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 was overexpressed in Werner syndrome and senescent normal HDFs. It is induced by growth arrest of young normal cells, but significantly decreases by high serum (Lecka-Czernik *et al.* 1995).

Fibulin 3 is detected in bone and cartilage structures during the development and is also highly expressed in retina, as well as in epithelial and endothelial cells throughout the body (de Vega *et al.* 2009). The R345W mutation in fibulin-3 causes age-related macular degeneration and is associated with Malattia Leventinese (Marmorstein *et al.* 2007; Stone *et al.* 1999).

Fibulin 3 exhibits both antitumour and oncogenic activities towards human cancers. Fibulin 3 is simultaneously overexpressed with MMP-2, MMP-9 and ADAMTS-5 in glioma cells promoting tumour invasion and survival (Hu *et al.* 2009). A pro-angiogenic role for Fibulin 3 in gliomas through the activation of Notch signaling has also been described (Nandhu *et al.* 2014). Moreover, Fibulin 3 has been associated with progression of pancreatic adenocarcinomas through interaction with epithelial growth factor (EGF) receptor, which triggers Akt pathway activation (Camaj *et al.* 2009).

However, there are also a considerable number of studies that support that Fibulin 3 behaves as an anti-tumour protein. In particular, in non-small cell lung cancer, Fibulin 3 down-regulates MMP-2 and MMP-7 and consequently, decreases the invasiveness of these cells (Kim *et al.* 2012a). Furthermore, Fibulin

3 suppresses both EMT and self-renewal of the lung cancer stem cells by modulating the IGF1R/PI3K/Akt/GSK3β pathway (Kim *et al.* 2014). Down-regulation of Fibulin 3 also contributes to lung cancer invasion and metastasis by activating Wnt/β-catenin signaling (Chen *et al.* 2014). Moreover, Fibulin 3 is down-regulated in nasopharyngeal carcinomas and it has the ability to suppress cell migration and invasion by decreasing Akt activity (Hwang *et al.* 2010). This Fibulin 3 down-regulation is mostly correlated with epigenetic silencing through promoter methylation in different types of cancer, including lung cancer (Kim *et al.* 2012a), hepatocellular carcinoma (Nomoto *et al.* 2010), colorectal carcinoma (Tong *et al.* 2011) and prostate cancer (Kim *et al.* 2011).

6. EPIGENETIC REGULATION AND DNA-METHYLASES

6.1. EPIGENETICS. DNA METHYLATION

The term epigenetics has been defined as the inheritance of changes in gene function without any change in the DNA nucleotide sequence (Fraga and Esteller 2007).The epigenome refers to the overall state of the cell. Epigenetic modifications can be grouped into three main categories: DNA methylations, post-translational histone modifications and chromatin remodeling (Portela and Esteller 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 (Portela and Esteller 2010). DNA methylation is essential for proper mammalian development, crucial for imprinting and plays a role in maintaining genomic stability, as well as in dosage compensation. DNA methylation patterns are susceptible to change in response to environmental factors such as diet, inflammation, aging or toxins. Aberrant DNA methylation changes have been detected in several diseases, particularly in cancer (Tost 2010).

Several studies have led to the generalization that DNA methylation

functions to silence promoter activity and is associated with repressed chromatin structure (Bird and Wolffe 1999; Esteller 2002). There are two general mechanisms by which DNA methylation inhibits gene expression. First, modification of cytosine bases can inhibit the association of some transcription factors and second, methyl-binding domain proteins (MBDs) directly recognize methylated DNA and recruit repressive chromatin-modifying complexes (Fig. 9) (Portela and Esteller 2010).

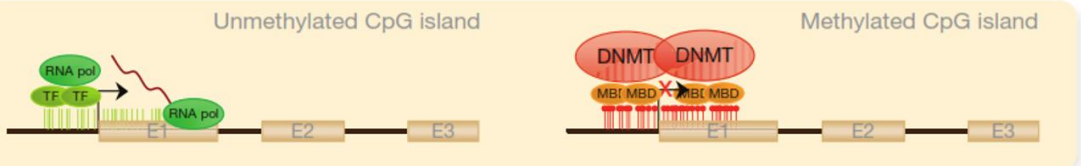

Figure 9: DNA methylation. CpG island at promoters of genes are normally unmethylated, allowing transcription. Aberrant hypermethylation leads to transcriptional inactivation (Portela and Esteller 2010).

6.2. DNA METHYLTRANSFERASES (DNMTs)

DNA methylation results from the activity of a family of DNA methyl transferases (DNMTs) that catalyze the addition of a methyl group to cytosine residues at CpG (Robertson 2001).

In mammals, five members of the DNMTs have been identified, but only three of them have been shown to possess methyltransferase activity. DNMT1 has a preference toward hemimethylated DNA and is responsible for maintaining the methylation patterns following DNA replication. DNMT3 functions as a *novo* methyltransferase (equal preference for unmethylated and hemimethylated DNA) and consists of two related proteins encoded by distinct genes, dnmt3a and dnmt3b. DNMT2 and DNMT3L, do not show methyltransferase activity (Newell-Price *et al.* 2000;Subramaniam *et al.* 2014).

The DNMTs are comprised of two regions: a C-terminal catalytic domain and a N-terminal region with a large multi-domain of variable size, which encodes regulatory functions (Subramaniam *et al.* 2014).

Moreover, DNMT3 (a and b) levels can be regulated, being of relevance its post-transcriptional regulation (Denis *et al.* 2011). In particular, binding of HuR protein (an RNA-binding protein that stabilizes and/or modulates the translation of target mRNAs) to the 3´UTR of DNMT3B mRNA enhances its stability and increases its protein levels in colorectal RKO cells (Lopez de Silanes *et al.* 2009).

The levels of DNMTs, especially those of DNMT3A and DNMT3B, are often increased in various cancer tissues and cell lines, including colon, prostate, breast, liver and in leukemia (Subramaniam *et al.* 2014).

GROUP BACKGROUND

1. ROLE OF p38α MAPK IN THE CONTROL OF CELL DEATH. CROSS-TALK WITH C3G.

p38 MAPKs regulate several cellular functions, including cell death. Initially, a number of studies indicated that p38 MAPKs promoted cell death and a few suggested that they could also mediate cell survival depending on the cell type, stimulus and p38 isoform (Nebreda and Porras 2000). However, the role played by the p38α isoform was unclear. Thus, our group focused their studies on the characterization of the function of p38α MAPK pathway in the regulation of cell death and its interactions with other routes. To do it, cardiomyocytes and MEFs-derived cell lines deficient in p38α were used. Additionally, some studies were also carried out in some tumour cell lines, where p38α was permanently knocked-down. Data from those studies demonstrated that p38α plays an important role as a mediator of apoptosis in response to different stimuli through both, the up-regulation of the pro-apoptotic proteins, Bax and Fas (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.* 2007a). In addition, p38α was shown to be relevant for the cisplatin-induced apoptosis in colon carcinoma HCT116 cells, where the p53/ROS/p38α MAPK cascade mediated this process (Bragado *et al.* 2007).

During the last years, in collaboration with Dr. Carmen Guerrero's group (Centro del Cáncer de Salamanca), we have found a new functional relationship between C3G and p38α MAPK that regulates apoptosis. This cross-talk operates in MEFs (Gutierrez-Uzquiza *et al.* 2010) and in the K562 chronic myeloid leukemia (CML) cell line (Maia *et al.* 2009). In both cell types exposed to apoptotic stimuli, C3G regulated cell death through down-regulation of p38α activity. In K562 CML cells, C3G silencing enhanced STI-571-induced apoptosis through the up-regulation of p38α activity mediated by the decrease in Rap-1 activation (Maia *et al.* 2009). In MEFs, C3G knock-down also through the up-regulation of p38α activity either promoted cell survival in response to oxidative stress or cell death upon serum-deprivation, but through a Rap-1 independent mechanism (Fig. 10) (Gutierrez-Uzquiza *et al.* 2010). Therefore, in MEFs lacking p38α treated with a moderate dose of H_2O_2, C3G silencing reduced survival.

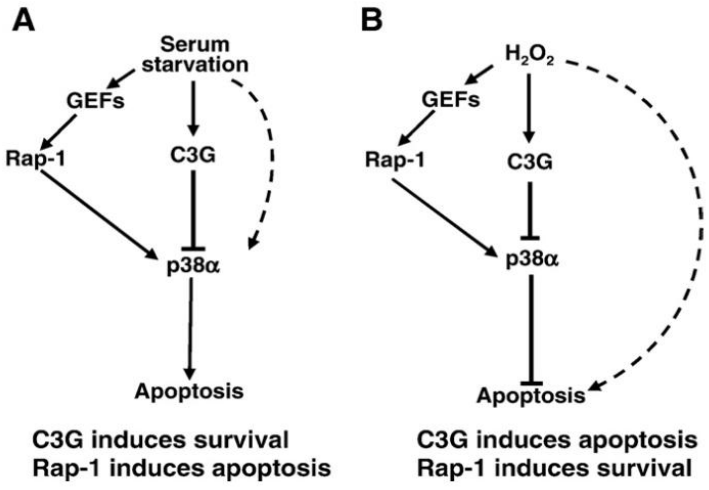

Figure 10: Regulation of apoptosis by C3G and Rap-1 through p38α MAPK. Model showing the regulation of p38α by C3G and Rap-1 in MEFs and their effects on the balance between apoptosis and cell survival., A) Upon serum deprivation C3G through the negative regulation of p38α activity mediates cell survival, while activation of Rap-1 by other GEFs would lead to p38α activation inducing apoptosis. B) Upon treatment with H_2O_2, C3G mediates apoptosis through inhibition of p38α activity and Rap-1 induces cell survival through p38α activation (Gutierrez-Uzquiza *et al.* 2010).

This prompted us to investigate this pro-survival role of p38α under mild levels of oxidative stress. First, it was confirmed by p38α reconstitution in p38α-/- cells that p38α rescue increased cell viability in response to H_2O_2 up to the levels observed in wt cells (Fig. 11)

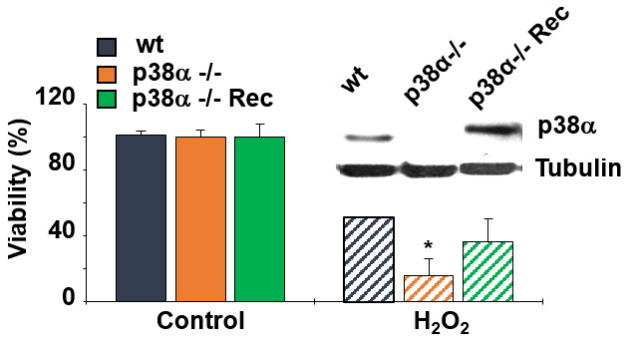

Figure 11: p38α protects from H_2O_2-induced cell death. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 1 mM H_2O_2 for 6h when indicated. p38α expression increases cell viability in cells treated with H_2O_2. p38α reconstitution in p38α -/- cells (Rec) rescues cells from cell death upon treatment with H_2O_2. Data correspond to cell viability expressed as percentage, and p38α expression determined by Western-blot and normalized with tubulin. ((Gutierrez-Uzquiza *et al.* 2010) and A. Gutierrez´s Thesis).

As Akt activity is up-regulated in p38α-/- cells (Zuluaga *et al.* 2007a) and it could play a role sensitizing cells to oxidative stress (Nogueira *et al.* 2008), the activation of the Akt/mTORC1/p70S6K pathway was analyzed in response to H_2O_2. It was observed that H_2O_2-induced Akt activation in p38α-deficient MEFs was higher than in wt cells, but p70S6K was not activated (Fig. 12: A. Gutierrez´s Thesis). Additionally, preliminary data from the group (A. Gutiérrez´s Thesis) suggested that the mTORC1 pathway was not relevant in this process of cell death.

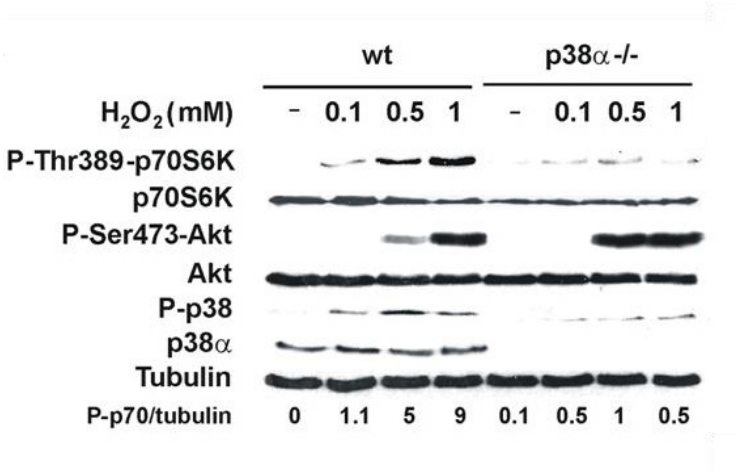

Figure 12: p38α MAPK positively regulates p70S6K through an Akt independent mechanism. MEFs (wt and p38α -/-) maintained in the presence of serum were treated with H_2O_2 (0.1-1mM in A) for 20min when indicated. Western-blot analysis of the levels of P-Thr389-p70S6K, P-Ser939-TSC-2, P-Ser473- Akt, as well as total levels of p70S6K, Akt and p38α normalized with tubulin.

Therefore, a more detail study was required: (i) to fully characterize the role played by this Akt/mTORC1/p70S6K pathway and (ii) to identify other potential mechanisms involved in this pro-survival role of p38α in response to oxidative stress. In this way, we could understand how p38α regulates cell death in this context.

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

p38 MAPKs play also a relevant role regulating cell adhesion and migration. Our group has previously shown that p38α was a negative regulator of cell adhesion in embryonic cardiomyocytes derived cell lines through a mechanism involving down-regulation of Rac-1 activity (Zuluaga *et al.* 2007b). Additionally, in collaboration with Dr. Carmen Guerrero's group, we found that C3G/p38α pathway also regulated cell adhesion in the K562 chronic myeloid leukemia (CML) cell line (Maia *et al.* 2013). Both proteins promoted K562 cells adhesion, acting through a common regulatory pathway. However, C3G and p38α displayed antagonistic roles in the regulation of focal adhesion complex formation in K562 cells, where p38α regulated C3G expression and both proteins interacted forming a complex (Maia *et al.* 2013).

To uncover novel mechanisms of action of p38 MAPKs in cell adhesion and migration/invasion, DNA microarray analyses were performed using wt and p38α knock-out MEFs (A. Gutiérrez´s Thesis). Changes in the pattern of expression of different genes were found. Some of these genes encoded proteins involved in the regulation of cell adhesion and/or migration, including the fibulin family. Among them, fibulin 3 was highly up-regulated in p38α knock-out MEFs. Therefore, we decided to focus our research on the study of fibulin 3 as a target of p38α. Thus, these results were validated by RT-PCR and RT-qPCR and it was confirmed that p38α negatively regulated fibulin 3 expression, but not that of other fibulins (Fig. 13). Therefore, a more detail study was required: (i) to characterize how p38α MAPK regulates Fibulin 3 expression and (ii) the implication of this p38α/Fibulin 3 pathway in the regulation of cell migration and invasion.

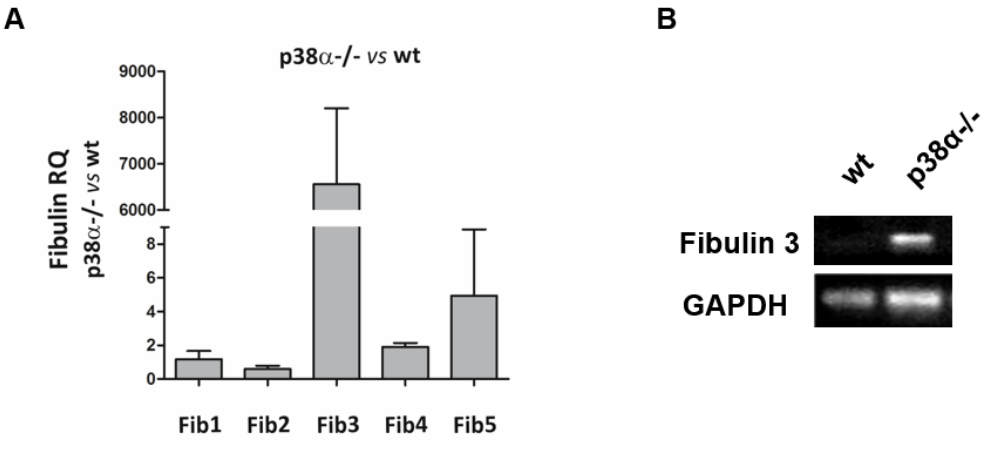

Figure 13: Preliminary results showing fibulins mRNA expression in MEFs. Total RNA was isolated from MEFs (wt and p38α-/-) maintained in 10% FBS. A) Fibulin 1, 2, 3, 4 and 5 mRNA levels determined by RT-qPCR. B) Fibulin 3 levels were determined by RT-PCR.

AIMS

General Aim:

The main objective of this research project is to further characterize the role played by p38α MAPK in different cellular processes and its functional interaction with C3G pathway. In particular, we aim to investigate how p38α MAPK controls cell survival and migration/invasion and to identify new targets and regulators.

Specific Aims:

1. To characterize the pro-survival function of p38α MAPK in response to oxidative stress and the potential implication of the Akt/mTORC1/p70S6K pathway.

2. To identify the mechanisms involved in the regulation of fibulin 3 expression by p38α MAPK and a potential role for fibulin 3 as a mediator of p38α effects on cell migration and invasion.

3. To investigate whether C3G and p38α MAPK act in a common pathway to regulate cell migration and invasion.

MATHERIALS AND METHODS

1. CELL CULTURE

1.1. Cell lines

The cell lines used for our studies are mouse embryonic fibroblasts (MEFs) derived from wt and p38α knock-out E10.5 embryos, which were immortalized by passages in our laboratory. Then, they were additionally modified to knock-down C3G gene (using a shRNA)or to express a dominant negative Rap-1 (DNRap-1) construct permanently (Gutierrez-Uzquiza *et al.* 2010) (Table 1). p38γ, p38δ and p38γ/δ knock-out MEFs (from E13.5 embryos) (Cerezo-Guisado *et al.* 2011) were obtained from Dr. A. Cuenda's lab (CNB, Madrid).

Table1: Cell lines

Type of cell line	Organism	Genotype	Genetic modification using shRNAs
Mouse embryonic fibroblasts (MEFs) immortalized by passages	*Mus musculus* E10.5	wt	+/- pRS
		shC3G	pRS shC3G
		DNRap-1	pCEP4 DNRap-1
		p38α–/–	+/- pRS
		p38α–/– shC3G	pRS shC3G
		p38α–/– DNRap-1	pCEP4 DNRap-1
	Mus musculus E13.5	p38γ–/–	
		p38δ–/–	
		p38γ/δ–/–	

1.1.1. Gene silencing by shRNA and siRNA

Permanent Fibulin 3 knock-down (in wt and p38α Knock-out MEFs) was performed by infection with mouse Fibulin 3 shRNA Lentiviral Particles (Santa Cruz, sc-44625-V) which are a pool of transduction-ready viral particles containing 3 target-specific constructs encoding 19-25 nt shRNAs. Cells were seeded in a 6-well plate 4-6h 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 virus were added in the presence of 10 μg/ml Polybrene (Santa Cruz; sc-134220). Cells were incubated overnight and the medium was replaced. After 48h, cells were trypsinized and splitted in a puromycin (1 μg/ml)-containing medium to select clones expressing the shRNA. Different clones were picked, expanded and analyzed for Fibulin 3 expression.

Transient ATF-2 knock-down was performed by transfection with a siRNA targeting mouse ATF-2 (Cell signaling, 6433) and a control scrambled siRNA (Ambion) diluted in media without serum at a final concentration of 50 nM using siPORT NeoFX Transfection reagent (Ambion) and following the manufacture instructions. Tripsinized cells were resuspended and overlaid onto the transfection complexes. After 24h, proteins or RNA were isolated or cell viability was quantified.

1.1.2. p38α MAPK rescue

To re-express p38α MAPK in p38α-/- MEFs, a p38α construct containing human p38α c-DNA cloned into the EcoRI site of the pEFmlink expression vector (Porras *et al.* 2004) was transiently transfected using Metafectene-Pro (Biontex, T040), following the protocol supplied by the manufacturer. Cell assays were performed 48h after transfection.

1.1.3. Expression of p70S6K wt and constitutive active

To express wt and constitutive active p70S6K, transient transfections were performed using Metafectene-Pro and the following constructs containing: (i) wtp70 and (ii) p70Δ29-46 ΔCT104 (deletion of aminoacids 29-46 and C-terminal tail, 422-525) active mutant cloned in PMT2 vector containing HA-tag (Addgene plasmid 1892) (Weng *et al.* 1995). The protocol supplied by the manufacturer was followed. Cell assays were performed 48h after transfection.

1.2. Cell culture conditions and cryopreservation

1.2.1. Cell culture conditions

All cell lines were grown in DMEM (4.5 g/l glucose) medium supplemented with 10% fetal bovine serum (FBS) (Gibco), 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). MEFs with permanent Fibulin 3 or C3G knock-down were grown in the same medium, but in the presence of puromycin (1 μg/ml) and DNRap-1 in the presence of hygromycin (2 ug/ml), which was removed 48h before the experiments. Cells were maintained at 37ºC with 5% CO_2 in a humidified atmosphere.

Cells were splitted when they reached 90% confluence by trypsinization with 0.25% Trypsin-0.02% EDTA, stopping trypsin action with 10% FBS supplemented medium.

1.2.2. Freezing, cryopreservation and thawing of cells

Cells were stored in 10% DMSO-FBS in liquid nitrogen (-170ºC). They were progressively frozen: -20ºC (for 20-30 min), -80ºC (for 12-16h) and liquid nitrogen.

Thawing was performed quickly at 37ºC and cells were placed in culture medium.

1.3. Cell treatments

90% confluent cells were treated with the following compounds:

- Stimuli:
    - H_2O_2: 0.1-1 mM H_2O_2 for 20 min for signaling experiments or for 6-24h for cell death analysis.
    - Serum: 10% FBS for 10 min after serum-deprivation (24h) for signaling experiments.

- Inhibitors:
    - SB203580 (Calbiochem, 559389), at 5 μM or 10 μM for p38α and/or p38β.
    - Rapamycin (Sigma, R0395) at 1-10 μM for mTORC1.
    - Actinomycin D (Sigma, A9415) at 5 μg/ml for transcription.
    - MG-132 (Sigma, M7449) at 1 μM for proteasome-dependent protein degradation.
    - 5-Aza-2´-deoxicytidine (Sigma, A3656) at 0.5 and 1 μM for 48h for DNA methylation.
- Anti-oxidants: (1h pre-treatment)
    - N-Acetyl-cysteine (Sigma, A-9165) at 2.5 mM.
    - 50 units/ml of catalase (Sigma, C-1345)

2. PROTEIN ANALYSIS

2.1. Cell extracts preparation

Cells were washed twice with cold PBS and lysed with the following buffer:

Table 2: Lysis buffer

Tris pH 7.5	50 mM
NaCl	150 mM
NP40	1%
EGTA	5 mM
EDTA	50 mM
PMSF	1 mM
Aprotinin	10 μg/ml
Leupeptin	10 μg/ml
Na_3VO_4	1 mM
NaF	20mM

Cells were detached from the plate by scraping, collected in an eppendorf tube, and maintained on ice for 20 min. Every 5 min the tubes were shaken using a vortex. Then, cell lysates were centrifuged at 13000 rpm for 10 min at 4°C and

the supernatant (total protein extracts) was transferred to a new tube and stored at -80°C.

For some experiments, the cell culture medium was also collected into tubes and stored at -80ºC until it was processed (to analyze the levels of Fibulin 3 secreted to the medium).

2.2. Protein quantification

Protein quantification was performed using the method described by Bradford in 1976. 5min after the addition of Bradford reagent, the absorbance was measured at 595 nm. For each experiment, a standard curve with different known concentrations of BSA was prepared using (0 to 5 μg/μl).

2.3. Western-blot analysis

2.3.1. Protein electrophoresis

Protein electrophoresis was performed using SDS-polyacrylamide gels (SDS-PAGE) (Table 3) and non-SDS-polyacrylamide gels (Anderson gels) (Table 4). The last ones allow a better separation of phosphorylated proteins with similar electrophoretic mobility. Acrylamide concentrations were different depending on the proteins size to be studied: for proteins of a relatively small molecular weight (25-80 KDa), we used higher percentages of separating gel (12-15 %) and for proteins of high molecular weight (more than 80 KDa), we used lower percentages of separating gel (7.5-10 %).

SDS-PAGE gels composition:

Table 3: 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	-
Tris 1 M pH 6.8	-	-	-	0.63 ml
10% SDS (w/v)	100 µl	100 µl	100 µl	50 µl
10% Ammonium persulfate (APS)	100 µl	100 µl	100 µl	50 µl
TEMED	4 µl	4 µl	4 µl	5 µl

Anderson gels composition:

Table 4: 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 (APS)	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl

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) was added to protein samples and then, they were heating at 95ºC and loaded into the gel, as well as the molecular weight markers. Electrophoresis was developed at a constant amperage of 20mA 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.

2.3.2. 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).

2.3.3. Proteins immunodetection

Membranes were washed several times with distilled water, and twice with 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 1h at room temperature (RT). After this time, they were rinsed with TTBS and incubated with the primary antibody in 5% non-fat milk-TTBS or BSA-TTBS (overnight at 4ºC for phospho-proteins or 1h at RT for non-phosphorylated proteins) at the dilution indicated in Table 5. Then, membranes were washed 4 times with TTBS (5 min/each) and incubated with the secondary antibody at a dilution of 1:5000 in 1% milk TTBS for 1h at RT. Finally, they were washed 4 times with TTBS and incubated with a chemiluminescent solution (ECL from Amersham Boisciences) to visualize proteins in an X-ray film.

Table 5: Antibodies		
Antibody	**Laboratory**	**Reference**
β-Actin	Sigma-Aldrich	A-5441
Phospho-Ser473-Akt	Cell-Signaling	9271
Akt	Cell-Signaling	9272
Phospho-ATF2	Cell-Signaling	9221
ATF-2	Cell-Signaling	9226
Catalase	Sigma-Aldrich	C-0979
C3G (H-300)	Santa Cruz	sc-15359
DNMT3A	Cell-Signaling	2160
Phospho-ERK1/2	Cell-Signaling	9101
Fibulin-3	Santa Cruz	sc-99177
GAPDH	Cell-Signaling	2118
Phospho-p38	Cell-Signaling	9211
p38α	Santa Cruz	sc-535
Phospho-Thr389-p70S6K	Cell-Signaling	4376
p70S6K	Cell-Signaling	9202
SOD-2	Upstate Biotech	06-984
Tubulin	Sigma-Aldrich	T-5168

3. ANALYSIS OF mRNA EXPRESSION

3.1. Total RNA isolation

Total RNA isolation was performed 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 it was used.

RNA concentration was determined spectrophotometry at 260 nm (1 unit of absorbance at 260 nm corresponds to 40 µg of RNA per ml (cuvette). To

estimate RNA purity, the ratio of absorbance at 260 and 280 nm was calculated. A ratio of A260/A280 of 1.8-2 is accepted for pure RNA.

3.2. c-DNA synthesis

Total RNA (1-3 μg) was reverse-transcribed to generate the cDNA using the SuperScript III RT kit (Invitrogen 18080-040) following instructions from the manufacturer. Essentially, RNA, oligo (dT) (0.5 mM) and random primers (2.5 μM) were incubated at 65 ºC for 5 min to ensure RNA denaturation and then, placed on ice for at least 1min. 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 70ºC for 15 min to inactivate the enzyme. C-DNA was stored at -20 ºC for its use for PCR or qPCR analysis.

3.3. Semi-quantitative PCR

RT-qPCR analysis was used to evaluate SOD-1 and SOD-2 mRNAs levels. PCR reactions were carried out using 0.4 μM specific primers (Table 6), dNTPs at 200 μM each, 2 mM $MgCl_2$ and 1 unit of Taq Polymerase (Biotools, 10-014). The amplified bands were normalized using GAPDH as a control. PCR conditions were: 94°C, 45 sec; 55°C, 45 sec; and 72°C, 1 min for 30 cycles.

Table 6: Primer sequences used in semi-quantitative PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
SOD-1	GATGAAGAGAGGCATGTTGG	CCAATGATGCAATGGTCTCC
SOD-2	TGGGGCTGGCTTGGCTTCAA	GCGTGCTCCCACACGTCAAT
GAPDH	CATCAAGAAGGTGGTGAAGC	CATCGAAGGTGGAAGAGTTGG

The obtained PCR products were analyzed in 1% agarose gels containing Gel Red Nucleic Acid Stain (Biotium, 41003).

3.4. Quantitative PCR

Real Time PCR or quantitative PCR (qPCR) was performed using specific primers (Table 7) and SYBR Green (Roche) to detect DNA in the 7900 Fast Real Time System (Life Technologies). PCR reactions were done in triplicate. GAPDH was used as the normalizing gene, as its expression was constant under the experimental conditions.

Table 7: Primer sequences used in quantitative PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Catalase	GTCACCGGCACATGAATGGCT	TGATGCCCTGGTCGGTCTTGT
Fibulin 3	GAATGTGATGCCAGCAACC	TCACAGTTGAGTCTGTCACTGC
GAPDH	CATCAAGAAGGTGGTGAAGC	CATCGAAGGTGGAAGAGTTGG

During the exponential phase of real-time PCR, a fluorescence signal threshold was determined, so that it was significantly greater than background fluorescence. The fractional number of PCR cycles required to reach this threshold is defined as the cycle threshold, or Ct. Based on this, quantification of RNA levels was performed through calculation of RQ ($2^{-\Delta\Delta Ct}$). First, ΔCt value for each sample and primer is obtained as follows: Ct for a primer minus -Ct for GAPDH=ΔCt under a particular experimental condition and then, this is referred to wt ΔCt control values (sample ΔCt-wt ΔCt= ΔΔCt) to calculate the RQ value. We consider a significant difference when there is a minimum of two-fold change: RQ more than 2 or less than 0.5.

4. CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Protein-DNA interactions were cross-linked with 1% formaldehyde solution (15 min incubation of cultured cells at RT). Cross-linking was stopped by incubation with 125 mM glycine for 5 min at RT. Cells were then washed, harvested in PBS (with protease and phosphatase inhibitors) and centrifuge at 2000 rpm (5 min at 4ºC). Pelleted cells were lysed on ice (10 min) in a buffer

containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1 and protease and phosphatase inhibitors. Lysates were sonicated 10 seconds, 6 times on ice. DNA was fragmented in a range of 200-600 bp. Then, samples were centrifuged and equal amounts of the chromatin solution were diluted in ChIP buffer (0.01% SDS, 1.1% Triton-X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) and incubated o/n at 4ºC with a P-ATF-2 antibody or a rabbit IgG (negative control), followed by 1h incubation with salmon Sperm DNA/protein A Agarose-beads. 10% of the volume was retained as the input. To reduce non-specific background, the chromatin solution was previously pre-cleared by incubation with 50% protein A sepharose containing salmon sperm DNA (20 µg) and BSA (1 mg/ml) in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 30 min at 4ºC. Then, samples were centrifuged and protein A agarose-beads pellets were sequentially washed with the following buffers: a low salt buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl); a high salt buffer (0.1 % SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl); a LiCl wash buffer (0.25 M LiCl, 1% NP40, 1 % deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and TE. Protein-DNA complexes were eluted from the beads by incubation with elution buffer (1% SDS and 0.1 M $NaHCO_3$) at RT by orbital mixing. Protein-DNA cross-linking was reversed by incubation with 200 mM NaCl for 4h at 65ºC and proteins were eliminated by incubation with proteinase K (20 µg) in 40 mM Tris-HCl pH 6.5 and 10 mM EDTA for 1h at 45ºC. DNA was then extracted with phenol/chloroform and precipitated. DNA fragments were amplified by PCR using primers for an AP-1 site (positions 844-853) from mouse SOD-2 promoter: Forward: 5´-GCAAGCAGCAGAACTCGCAGC-3´; and Reverse: 5´-AGCACTCAGGAGGCAGAGGCA-3´. Inputs were also amplified by PCR (Fig. 14).

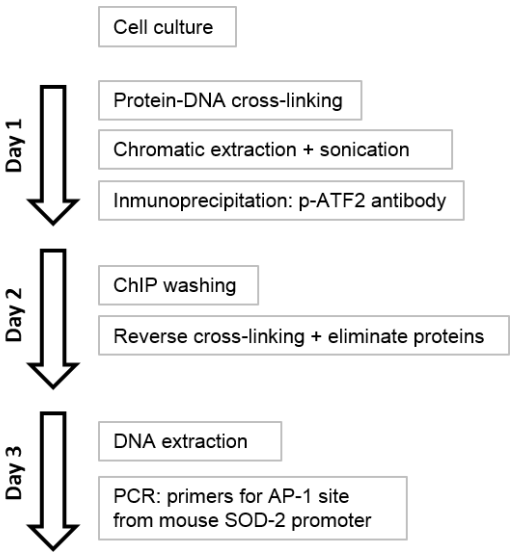

Figure 14: Schematic ChIP protocol

5. ANALYSIS OF DNA METHYLATION THROUGH PYROSEQUENCING

5.1. DNA isolation

Genomic DNA was extracted from 24h serum-deprived wt and p38α-/- MEFs. Once cells were washed twice with cold PBS solution 1 (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.15 M NaCl), proteinase K (10 mg/ml) was added. Then, they were scraped and 20% SDS was added and mixed. The homogenate was digested at 65ºC for 2-3h and Solution 2 (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.65 M NaCl) was added to each tube. Then, DNA was purified by phenol-chloroform method and precipitated with ethanol (two volumes). Finally, samples were resuspended in ultrapure water. DNA was stored at -20ºC until it was processed. DNA concentration was determined spectrophotometrically at 260 nm. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml (A260 = 1 = 50 µg/ml).

5.2. Bisulphite modification

To analyze DNA methylation, the DNA was treated with bisulphite to induce cytosine deamination leading to uracil, while leaving 5-methylcytosine intact. Thus, genomic DNA (1 µg) was modified in this way using the BisulFlash DNA Modification Kit from Epigentek (P-1026) following manufacturer instructions.

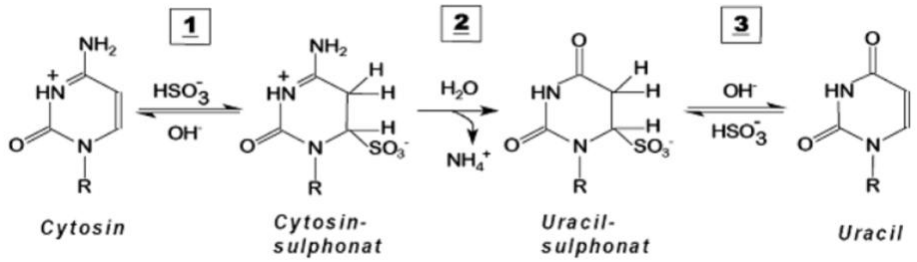

Figure 15: DNA bisulphite modification. Reaction between cytosine and bisulphite (step 1) leads to deamination (step 2) at acid pH. Afterward, desulphonation (step 3) at alkaline pH produces uracil (Adapted from (Darst *et al.* 2010)).

5.3. PCR amplification

The specific region of interest, a fragment of the fibulin 3 (efemp 1) gene promoter with twelve CpG islands were amplified using the bisulfite modified DNA. The PyroMark PCR kit (Qiagen 978703) was used and the following primers: forward: 5´-CCTCCTGTGGCTGCTGCTGCAG-3´; reverse (biotinylated): 5´-CACTTTGACATGTCTCTTCTACCTCCA-3´ (Figure 16: mi_EfempF and mi_EfempR). PCR cycles were as follows: 30 seg at 95 ºC, 30 seg at 52ºC and 30 seg at 72ºC (45 cycles). PCR products were converted into single-stranded DNA (ssDNA) fragments and one strand was isolated using Streptavidine-sepharose beads in the PyroMark workstation for its use as a template in the Pyrosequencing reaction.

5.4. Pyrosequencing

The resulting PCR fragments were sequenced (by synthesis) using two different primers: 5´-GCTGCCCTCCCCTACGCACTCCTT-3´ (mi_Efemp1_S2) for the analysis of the methylation status of five CpG sites and 5´-CCCGCAGGTAGGAGCCCAAAGC-3´ (mi_Efemp1_S3) for the analysis of seven additional CpG sites (Fig. 16). The degree of methylation of each CpG site was estimated by measuring the relative peak height of the cytosine (5-methylcytosine in the original sequence) versus thymine (cytosine in the original sequence) profile. This was performed in the Unitat de Genètica Molecular, Institut de Biomedicina de València – CSIC.

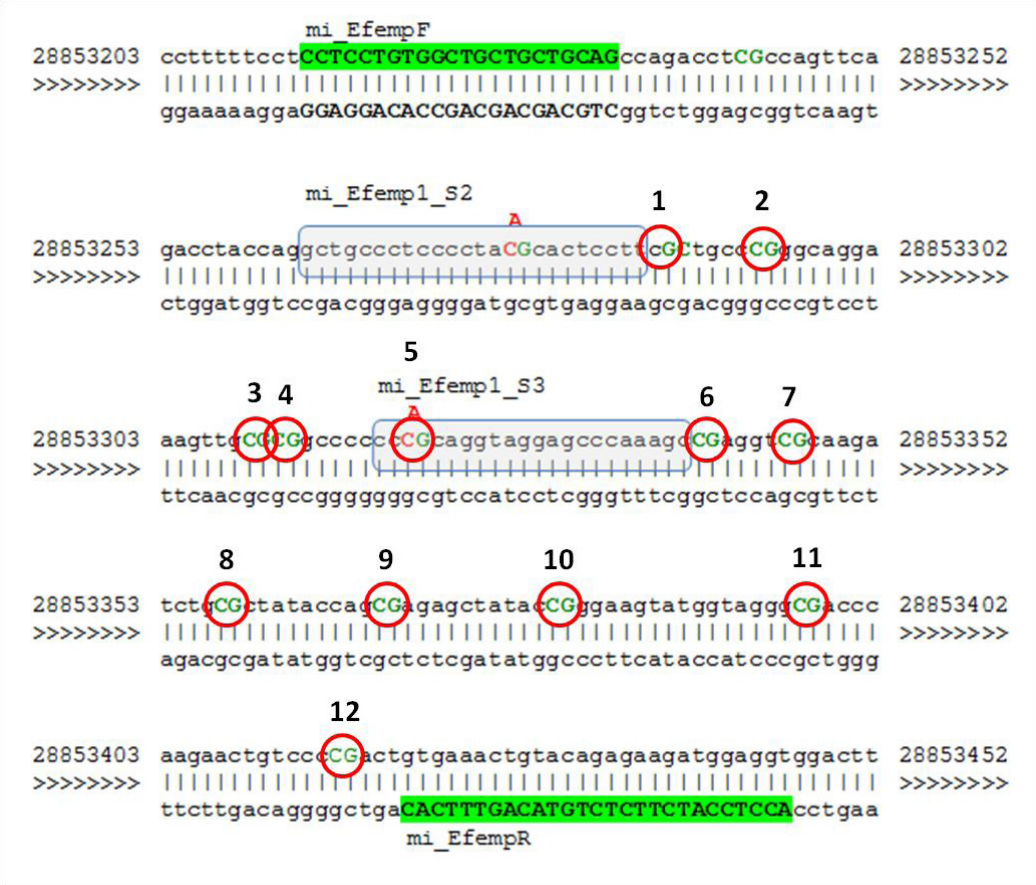

Figure 16: Analyzed region of fibulin 3 gene. mi_EfempF and mi_EfempR (in green) are respectively, forward and reverse primers used for amplifying the region of interest. mi_Efemp1_S2 and mi_Efemp1_S3 are the primers used for sequencing.

6. ANALYSIS OF CELL VIABILITY AND APOPTOSIS

6.1. Quantification of cell viability by Crystal Violet staining

Cells were treated with the different stimuli or antioxidants. Then, the medium was removed, cells were washed twice with PBS and incubated with a Crystal Violet solution at 0.2% (w/v) (in 2% ethanol) for 20 min. Cells were then washed several times with distilled water until the excess staining was eliminated. The plate was air-dried and the stained cells were lysed in 1% SDS and absorbance at 595 nm was measured. Results are expressed as the percentage of viable cells as compared to control cells.

6.2. Analysis of nuclei morphology by fluorescent microscopy

This method is based on the identification of apoptotic (condensed and/or fragmented) nuclei by fluorescence microscopy after fixation and staining with propidium iodide. Cells were washed twice with PBS and fixed by incubation with a methanol-acetic acid (3:1) solution for 30 min at room temperature. Then, cells were washed three times with PBS and stained with a solution containing: 5 μg/ml propidium iodide in PBS, 0.1% Triton X-100, 0.1 M EDTA, supplemented with 5 μg/ml RNAse. After 30 min of incubation at 37ºC in the dark, cells were washed with PBS. Samples were assembled to visualize the nuclei by fluorescent microscopy. Apoptotic indices were calculated after counting 500-1.000 cells per treatment in an inverted fluorescence microscope (Eclipse TE300, Nikon).

7. MEASUREMENT OF INTRACELLULAR ROS

In order to measure the intracellular content of reactive oxygen species (ROS), a fluorimetric method was used. Cells were incubated with 5 μM $DCFH_2$-DA (2',7'-dichlorfluorescein-diacetate; Sigma, 35845) in PBS for 30-60 min at 37ºC in the dark and were either treated with H_2O_2 (0.1-1 mM) for 15 min or maintained untreated (control). Then, cells were collected by trypsinization, centrifuged at 2000 rpm for 5 min and washed twice with warmed PBS. Cells were then resuspended in PBS and propidium iodide was added at 1 μg/ml, to distinguish between dead and alive cells. $DCFH_2$-DA is incorporated into the cells and converted into 2',7'-diclorodihidrofluorescein ($DCFH_2$) by intracellular esterases. In turn, $DCFH_2$ is converted into 2',7'-diclorofluorescein (DCF) when oxidized by hydrogen peroxide, which is the fluorescent compound. The fluorescent intensity of DCF was detected by a FACScan Cytometer. Results were calculated as a percentage of fluorescence positive cells compared to the control.

One extra dish from each condition was also treated with $DCFH_2$-DA and analyzed in an inverted fluorescence microscope (Eclipse TE300, Nikon), taking representative pictures.

8. MIGRATION AND INVASION ASSAYS

8.1. Wound healing

To study cell migration, wound healing assays were carried out. Confluent cells were pre-treated with mitomycin C (25 µg/ml, Sigma M0503) for 30 min to inhibit cell growth. Then, they were washed twice with PBS to eliminate mitomycin C and a straight scratch was performed with a pipette tip. Cells were washed again with PBS and fresh medium without serum was added. Cells were maintained for 24h at 37ºC in a humidified atmosphere and 5% CO_2 to allow migration. Migration was followed by phase-contrast microscopy (Eclipse TE300 Nikon microscope coupled to a digital sight DS-U2 camera) at different time points (0, 4, 8, 12 and 24h) up to wound healing closure. Photographs were taken to quantify (using TScratch program) the percentage of wound healing closure at the different time points.

8.2. Invasion through matrigel

To study cell invasion capacity, cells were allowed to migrate through matrigel using transwells inserts (8 µm filter, BD 353097) coated with matrigel (444 µg/cm^2) (BD Biosciences, 356234).

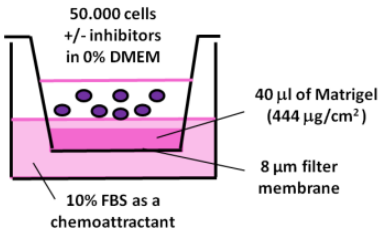

Figure 17: Schematic protocol of invasion assay through matrigel

Cells (50.000) were seeded in the upper chamber in a serum-free medium with or without additional treatments (SB203580 for p38α/β inhibition or marimastat for MMPs inhibition). In the lower chamber, a medium supplemented with 10% FBS was added to act as a chemoattractant and cells were left in the incubator for 24h at 37ºC and 5% CO_2 (Fig. 17). Then, the medium and the matrigel from the upper chamber were removed with cotton-tipped swabs. Cells present in the lower chamber were fixed in 4% paraformaldehyde, washed twice with PBS and stained with crystal violet 0.2% w/v (Sigma Ref C-0775). Finally, inserts were washed with distilled water and

dried at room temperature and cells were counted using a phase-contrast microscope.

9. CUANTIFICATION OF SOD-2 PROMOTER ACTIVITY

To asses SOD-2 promoter activity in wt and p38α-/- MEFs upon H_2O_2 treatment, cells were cotransfected with a construct containing the SOD-2 promoter coupled to a luciferase reporter (kindly provided by Daret St Clair, Kentucky University) and a plasmid-encoding Renilla luciferase (Clontech) (100–500 ng). Then, cells were treated with H_2O_2 (0.5 mM for 4 and 8h) and lysed using the passive lysis buffer from Promega. Luciferase activity was detected by a luminometer (Molecular Devices Spectramax M5E) using the Dual Luciferase Reporter Kit from Promega following the manufacturer's instructions. Luciferase activity was normalized to Renilla luciferase activity.

10. ENZYMATIC ASSAYS

10.1. Catalase activity assay

Catalase activity was measured by quantification of H_2O_2 decomposition in a 50 mM phosphate buffer at pH 7 containing 3 mM H_2O_2. To do it, the sample was added to the cuvette, mixed well and H_2O_2 decomposition was monitored spectrophotometrically at 240 nm for 1min. Catalase specific activity was calculated by the formula: mU CAT/ mg prot = (380 x log Abs t0/Abs t60)/mg prot/ml).

10.2. SOD activity assay

SOD activity from cell extracts was quantified using a Kit (BioVision, K335-100), where WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2H tetrazolium, monosodium salt) is the substrate. WST-1 produces a water-soluble formazan dye upon its reduction with the superoxide anion, which can be monitored spectrophotometrically at 450nm (Fig. 18). This rate of reduction is

linearly related to the xanthine oxidase activity and inhibited by SOD, so the IC50 of SOD is determined as a measure of SOD activity.

Treated cells were lysed in a buffer containing 0.1 M Tris-HCl pH 7.4, 0.5% Triton X-100, 5 mM β-mercaptoethanol and 0.1 mg/ml PMSF on ice. After centrifugation at 14000 rpm (5 min at 4ºC), supernatant fractions (that contains total SOD activity from cytosol and mitochondria) were collected. Then, SOD activity was measured following the manufacture instructions by incubating the WST-1 working solution and the sample at 37ºC for 20min. Finally, the absorbance was read at 450 nm and SOD activity was calculated.

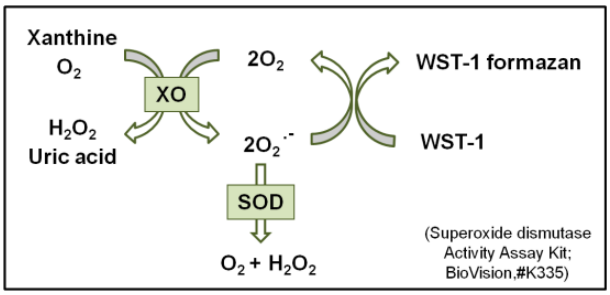

Figure 18: Schematic reaction of superoxide dismutase activity assay

10.3. Quantification of MMP2 and MMP9 activities by zymography

MMP2 (Gelatinase A) and MMP9 (Gelatinase B) activity was determined using conditioned medium from serum-deprived cells. Total protein levels in cell extracts were measured to establish the amount of medium used. Samples were prepared under non-reducing conditions (absence of heating and reducing agent), mixing the medium with 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 loaded in a 8% SDS-PAGE gel polymerized in the presence of 0.1% gelatin. The electrophoresis was carried out at constant voltage of 80 V for 3-4h. Then, SDS was removed from the gel by incubation with 2.5% Triton X-100 for 30min. The gel was then rinsed with the substrate buffer (0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3, 50 mM Tris pH 7.5) and incubated in this substrate buffer at 37ºC overnight to allow protein renaturation and MMP activation. Next day, to visualize gelatin digestion areas (MMP2/9 activity) as clear bands, the gel (zymogram) was stained with Coomassie Brilliant Blue (BioRad, 161-0400).

11. TUMOURIGENESIS STUDIES

11.1. Focus formation assay

To measure anchorage dependent growth, 300 cells (MEFs) were seeded in a 10 cm dish. After 10-13 days, the medium was removed and cells foci were fixed and stained with a 0.2% crystal violet solution (in 2% ethanol). The total number of foci was quantified using Image J program and their size using OpenCFU program.

11.2. Growth in soft-agar

To measure anchorage-independent growth in soft agar, cells were cultured in 24-well dishes containing two agar layers. Cells ($3x10^3$) were resuspended in 0.7 % granulated agar (BD, 214530) diluted in complete medium (2X) and poured onto a 0.5 % layer of agar (diluted in complete medium). Fresh medium was added to the top layer every 3 days to prevent gel drying. After 2 weeks, colonies were stained with MTT and counted.

11.3. Xenografts assays

For xenograft experiments, MEFs cells ($1.5x10^6$ cells/100 μl DMEM) were resuspended in DMEM and injected subcutaneously into the flank of eight-week old male nude mice (Harlam Laboratories). Tumour growth was monitored twice a week for 6 weeks. All animal experiments were carried out in compliance with the institutions guidelines.

12. STATISTICAL ANALYSIS

Data have been represented as the mean value of 3-10 independent experiments and ±SEM. The comparisons were made between two experimental groups. An unpaired Student's t-test was used and alternatively, ANOVA test was carried out for comparisons of more than two experimental groups.

RESULTS

1. p38α MAPK MEDIATES CELL SURVIVAL IN RESPONSE TO OXIDATIVE STRESS VIA INDUCTION OF ANTIOXIDANT GENES IN MEFs

p38 MAPKs play an important role in the coordination of cellular stress responses to signals such as ROS, mediating cell death or survival. Interestingly, previous results from our group indicated that p38α MAPK induced cell survival in response to relatively low levels of H_2O_2 (Gutierrez-Uzquiza *et al.* 2010). However, the precise mechanisms by which p38α MAPK signaling achieves cell survival were poorly understood. In addition, in this context of moderate oxidative stress, the Akt/mTORC1/p70S6K pathway was differentially regulated by p38α MAPK. Although Akt phosphorylation was enhanced in p38α-/- MEFs treated with H_2O_2, p70S6K activation was very low and the potential relevance of this pathway in the control of this process of cell death was unclear. Therefore, we decided to fully characterize this pro-survival role of p38α MAPK in response to these moderate levels of oxidative stress and the potential implication of the Akt/mTORC1/p70S6K pathway.

1.1. Loss of p38α sensitizes cells to H_2O_2-induced cell death

As explained in the group background section, wt MEFs exhibited a higher resistance to a level of oxidative stress, able to generate damage but not massive toxicity (0.1-0.5 mM H_2O_2), than p38α-deficient MEFs (Fig 11 background). This suggests that p38α allows cells to survive under mild levels of oxidative stress. This was confirmed by p38α reconstitution in p38α-/- cells (Fig 11 background).

We have now analyzed nuclei morphology and found a significantly higher number of condensed and/or fragmented nuclei in p38α-deficient MEFs than in wt cells upon H_2O_2 treatment (Fig. 19), which suggests that cells would be dying by a process of apoptosis. All these data demonstrate that p38α protects from low levels of H_2O_2-induced cell death.

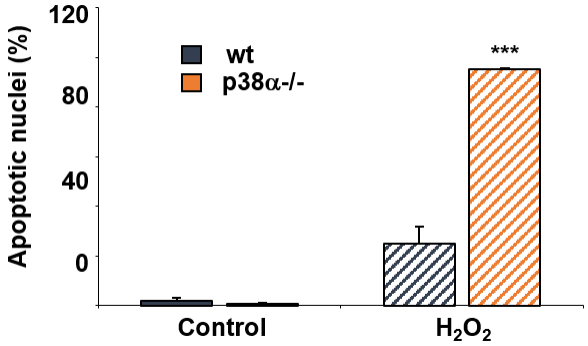

Figure 19: Loss of p38α increases the number of apoptotic nuclei in MEFs treated with H_2O_2. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 0.5 mM H_2O_2 for 3h. Apoptotic (condensed and/or fragmented) nuclei were counted in an inverted fluorescence microscope after fixation and staining with propidium iodide. The histogram shows the mean values ± S.E.M (n=3). ***p<0.001, p38α-/- versus wt MEFs upon treatment with H_2O_2.

1.2. Loss of p38α MAPK impairs mTORC1/p70S6K activation in response to H_2O_2 through Akt independent mechanisms

Our previous results indicated that Akt activity is negatively regulated by p38α, so that Akt is hyperactivated in p38α-/- cells, leading to increased survival in response to serum-deprivation (Zuluaga *et al.* 2007a). However, whether this had any effect on ROS sensitivity remained unknown. Results from Nogueira et al., showed that Akt activation sensitized cells to oxidative stress through down-regulation of ROS scavengers, which increased intracellular ROS (Nogueira *et al.* 2008). This led us to hypothesize that p38α, through inhibition of Akt, might allow a proper expression of anti-oxidant genes.

As previously mentioned (group background section), under mild levels of oxidative stress, Akt activation was uncoupled from mTORC1-mediated p70S6K phosphorylation in p38α-deficient cells. (Fig. 12 background). Thus, we next confirmed this result by p38α reconstitution in p38α-/- cells, which rescued the levels of p70S6K activation (Fig. 20), reaching a level comparable to that of wt cells treated with H_2O_2. In the absence of p38α, although Akt phosphorylation was enhanced in response to H_2O_2 treatment, p70S6K activation was very low.

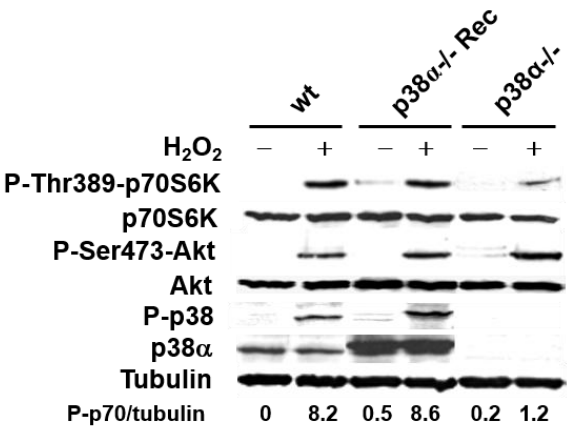

Figure 20: p38α MAPK positively regulates p70S6K through an Akt independent mechanism. MEFs (wt, p38α-/- and p38α-/- with reconstitution of p38α (Rec)) maintained in the presence of serum were treated with H_2O_2 (1 mM) for 20 min. Western-blot analysis of the levels of P-Thr389-p70S6K, P-Ser473-Akt, P-p38, as well as total levels of p70S6K, Akt, and p38α normalized with tubulin. P-p70/tubulin represents the relative value resulting from the densitometric analysis of P-Thr389-p70S6K versus tubulin levels multiplied by 10.

We next considered the possibility that the higher mTORC1/p70S6K activation in cells expressing p38α could be responsible for the increased survival in response to H_2O_2. Thus, we inhibited this pathway with rapamycin to evaluate it. However, rapamycin did not sensitize wt cells to ROS-induced cell death (Fig. 21A). Therefore, the higher activation of mTORC1/p70S6K appears not to be responsible for the p38α-mediated increased survival. According to this, transfection of either an active p70S6K (p70Δ29-46 ΔCT104 mutant) or a wt p70S6K construct did not increase cell viability of p38α-deficient MEFs treated with H_2O_2 (Fig. 21B). In contrast, basal viability was slightly reduced upon expression of these p70S6K constructs.

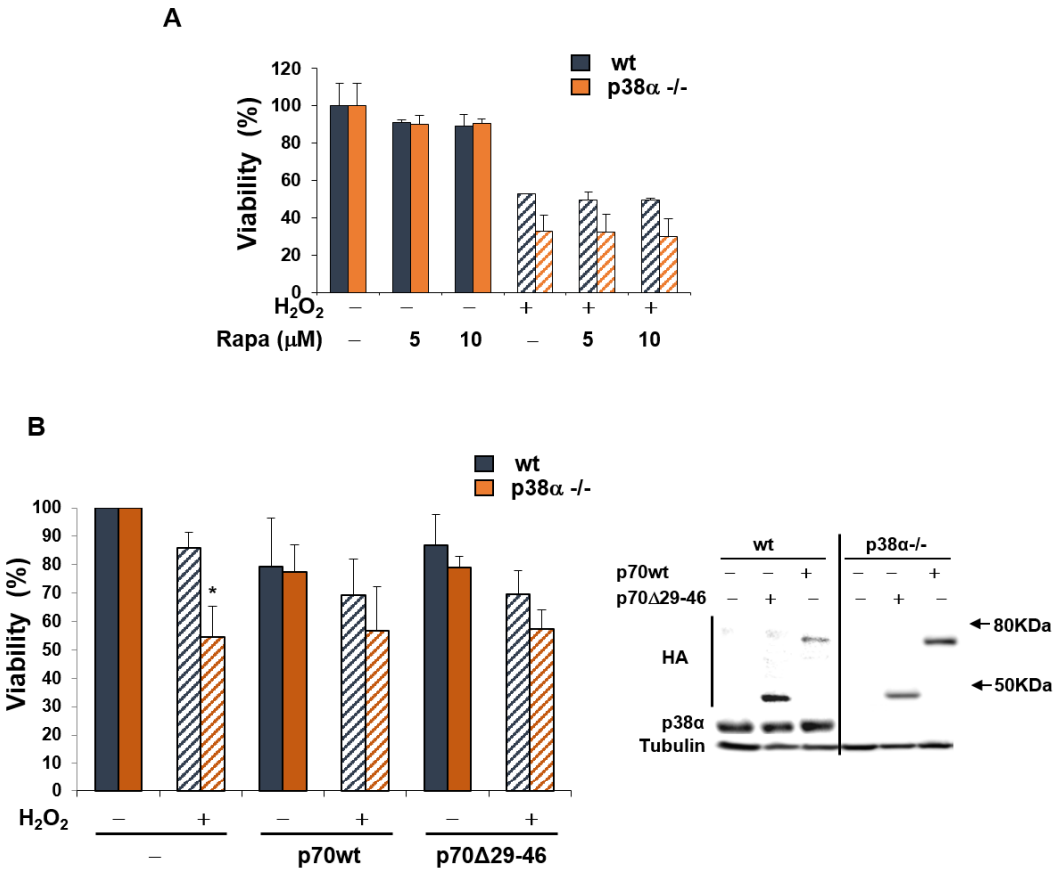

Figure 21: mTORC1/p70S6K does not mediate p38α-dependent survival in response to H_2O_2. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with H_2O_2 (0.1, 0.5 or 1 mM). A) Effect of rapamycin on H_2O_2-induced cell death. Cells were pretreated for 1h with rapamycin (5 and 10 μM). Results show the mean values ± S.E.M (n=3) of the percentage of viable cells determined by crystal violet staining. *p<0.05, p38α-/- versus wt MEFs treated with H_2O_2. B) Left panel, effect of the expression of transfected p70S6Kwt (p70wt) and p70Δ29-46ΔCT104 active mutant on cell viability. *p<0.05, p38α-/- versus wt MEFs treated with H_2O_2. Right panel, western-blot analysis showing HA expression in cells transfected with p70S6Kwt and p70Δ29-46ΔCT104 active mutant. Arrows indicate the migration of 80 and 50 KDa molecular weight markers.

On the other side, Akt inhibition with the selective chemical inhibitor, A443354, had no effect on H_2O_2-induced cell death, either in wt or p38α-/- MEFs (Fig. 22), which suggests that the Akt pathway does not play a major role in p38α-mediated survival.

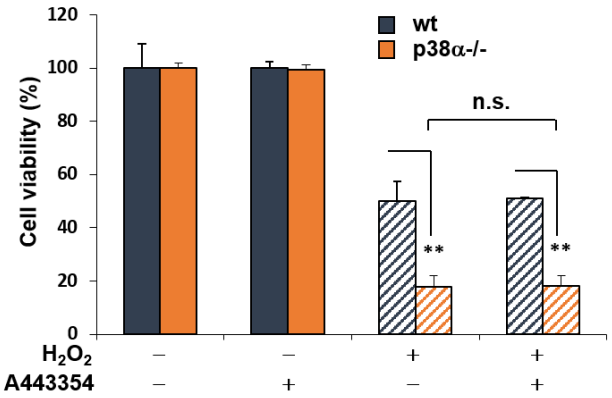

Figure 22: Inhibition of Akt activity has no effect on H_2O_2-induced cell death. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 1 mM H_2O_2 for 3h. When indicated, cells were pretreated for 1h with A443354 (0.5 µM). Results show the percentage of viable cells determined by crystal violet staining and are the mean values ±S.E.M (n=3). **p<0.01, p38α-/- versus wt MEFs treated with H_2O_2.

All these data indicate that either the higher activation of mTORC1/p70S6K or the lower Akt activation is responsible for the p38α-mediated increased survival. Therefore, we decided to explore alternative mechanisms by which p38α might protect cells from ROS damage, allowing cell survival in response to oxidative stress.

1.3. Activation of p38α prevents the accumulation of ROS

Although p38α MAPK is known to mediate cell death in response to oxidative stress (Dolado *et al.* 2007;Tobiume *et al.* 2001;Wada and Penninger 2004), p38 has also been shown to induce the expression of antioxidant enzymes (Aggeli *et al.* 2006;Sen *et al.* 2005). This led us to hypothesize that p38α through the regulation of the anti-oxidant response might maintain low ROS intracellular levels, leading to cell survival.

As shown in figure 23, the percentage of cells with detectable levels of ROS (positive for DCFH) was slightly higher in untreated p38α-deficient cells compared with wt cells, and was highly increased upon treatment with H_2O_2 in a dose dependent manner. This suggests that p38α-/- cells are unable to efficiently

scavange ROS in response to H_2O_2, leading to a high and progressive accumulation of ROS (Fig. 23).

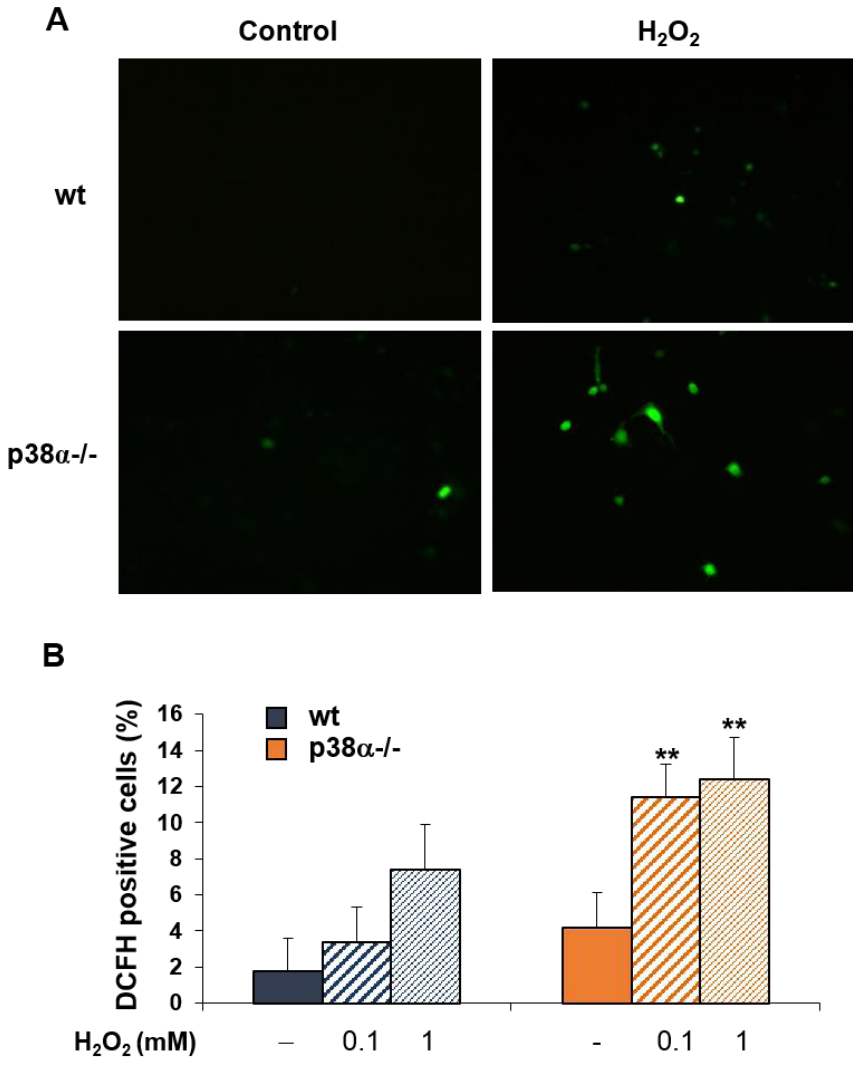

Figure 23: ROS accumulation upon H_2O_2 treatment is higher in cells lacking p38α. MEFs (wt and p38α-/-) were incubated with DCFH and treated with H_2O_2 (1 mM for A and 0.1 and 1 mM for B) for 15 min. Analysis of DCFH-positive cells using an inverted fluorescence microscope. A) Representative images of DCFH-positive cells in green. B) Percentage of DCFH-positive cells. Results are the mean values ±S.E.M (n=3). ** $p<0.01$, p38α-/- versus wt MEFs treated with the same dose of H_2O_2.

1.4. The presence of p38α increases basal and H_2O_2-induced expression of the antioxidant enzymes

This high ROS accumulation in p38α-deficient cells could be a consequence of a deficiency in the activation of the anti-oxidant mechanisms. In agreement with this potential role of p38α as a positive regulator of antioxidant enzymes expression, it was previously reported that p38 MAPK up-regulated catalase (Sen *et al.* 2005) and heme oxygenase-1 mRNAs (Aggeli *et al.* 2006) in response to H_2O_2. For this reason, we decided to analyze the effect of p38α in the expression of relevant antioxidant enzymes such as superoxide-dismutases (SOD-1 and SOD-2) and catalase.

We first analyzed the protein levels of SOD-2 and catalase under basal conditions and after H_2O_2 treatment. As shown in figure 24A, p38α-/- cells expressed lower protein levels of SOD-2 and catalase than wt cells under basal conditions. Moreover, upon treatment with H_2O_2 these cells were either unable to efficiently induce the expression of these enzymes, as observed for SOD-2, or had a delay and a reduced induction, as happens for catalase (Fig. 24A). These results suggest that impaired or delayed induction of antioxidant enzymes in p38α-/- MEFs could be responsible for its higher sensitivity to H_2O_2-induced cell death.

To demonstrate that the absence of p38α was responsible for the lower catalase protein levels, we re-introduced p38α in p38α-deficient cells. Results presented in Fig.24B indicate that p38α reconstitution in p38α-/- cells led to an increase in catalase protein levels after 2h of treatment with H_2O_2.

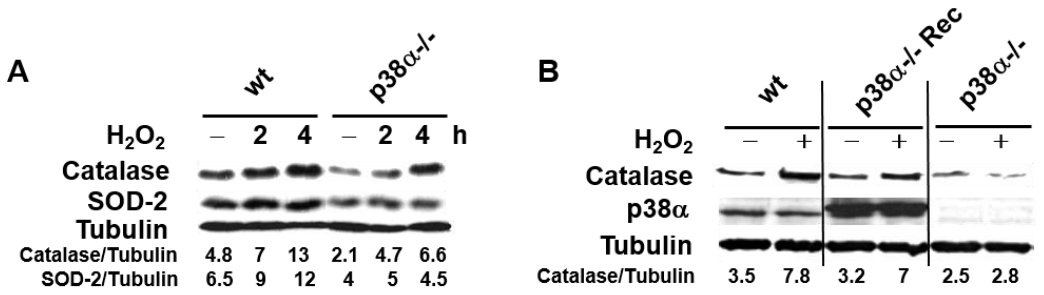

Figure 24: Loss of p38α reduces de expression of antioxidant enzymes. MEFs (wt, p38α-/- reconstituted with p38α (Rec) and p38α-/-) maintained in the presence of serum were treated with 0.5 mM H_2O_2 for 2 (A and B) and 4h (A). A) Catalase and SOD-2 protein levels determined by western-blot analysis and normalized with tubulin. B) Rescue of catalase protein expression by p38α reconstitution in p38α-/- cells (Rec) treated with H_2O_2. p38α MAPK was transiently re-expressed in p38α-/- MEFs. Western-blot analysis of catalase and p38α normalized with tubulin.

In addition, basal and H_2O_2-induced catalase and SOD activities were significantly higher in wt than in p38α-/- MEFs (Fig. 25A and R25B, respectively). Therefore, all these data indicate that the presence of p38α highly increases the anti-oxidant activity of cells.

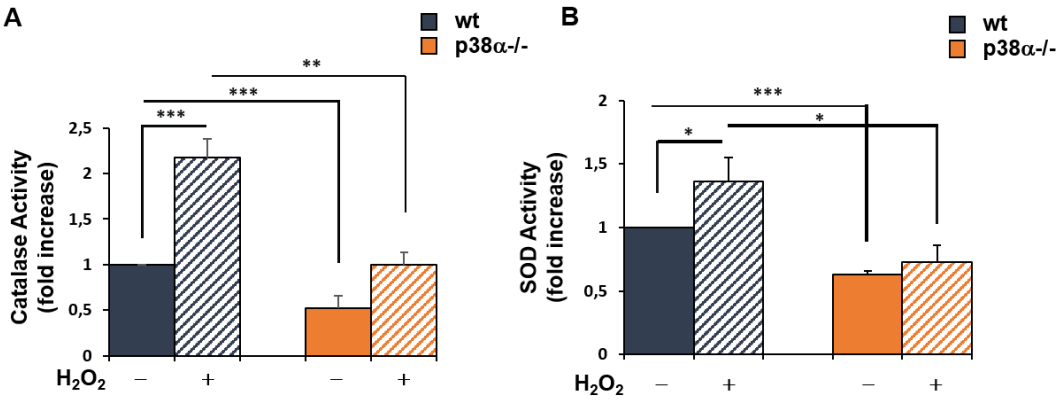

Figure 25: Loss of p38α reduces the activity of catalase and SOD-2. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 0.5 mM H_2O_2 for 20 min. Catalase and SOD-2 activity are shown as a fold increase of that of wt untreated cells (9.35 mU/mg protein for catalase and 7.32 mU/mg protein for SOD-2) and are the mean values ±S.E.M (n=3) *p<0.05, **p<0.01 or ***p<0.001, compared as indicated.

These results led us to conclude that p38α was increasing the antioxidant capacity in this moderate oxidative stress context.

To get further insight into the role of p38α as a regulator of antioxidant enzymes expression, we measured SOD-1 and SOD-2 mRNA levels by RT-PCR. They were significantly lower in p38α-/- cells under basal conditions and H_2O_2 only induced an increase in SOD-1 mRNA after 20-60 min, while SOD-2 mRNA remained unchanged (Fig. 26).

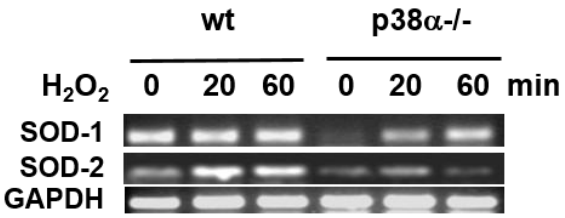

Figure 26: Loss of p38α reduces the expression of SOD-1 and SOD-2. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 0.5 mM H_2O_2 for 20 and 60 min. RT-PCR analysis of the expression of SOD-1 and SOD-2 mRNAs normalized with GAPDH.

1.5. p38α through ATF-2 regulation induces SOD-2 expression and resistance to H_2O_2 treatment

To better understand the regulation of SOD-2 expression by p38α in response to H_2O_2, we started analyzing SOD-2 promoter activity using luciferase as a reporter (colaboration with Dr. P. Bragado and Dr. J. A. Aguirre-Ghiso (Mount Sinai Hospital, New York, USA)).

As shown in figure 27, H_2O_2 treatment induced a significant increase in SOD-2 reporter activity in wt cells after 8h, but not in p38α-/- MEFs.

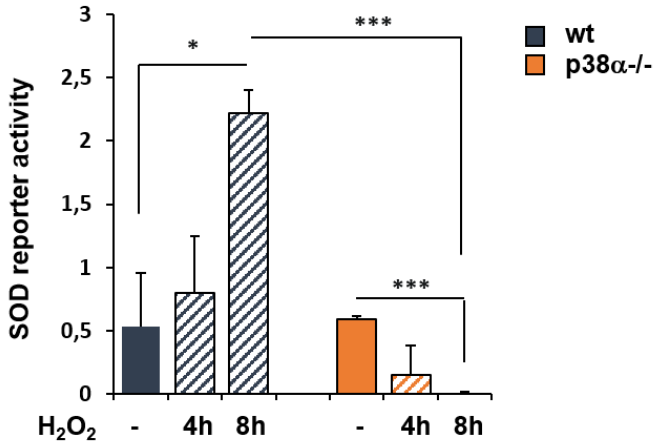

Figure 27: **H_2O_2 treatment induced an increase in SOD-2 reporter activity in wt cells, but not in p38α-/- MEFs**. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with 0.5 mM H_2O_2 for 4 and 8h. SOD-2 reporter activity using luciferase as reporter. Results are expressed as arbitrary units and are the mean values ±S.E.M (n=3). *p<0.05, ***p<0.001 compared as indicated.

In an effort to define possible substrates of p38α that helped us to understand the link between p38α and SOD-2, we found that ATF-2, a p38 MAPK substrate, was more phosphorylated in wt than in p38α-deficient cells in response to H_2O_2 (Fig. 28). Therefore, the lack of activation of SOD-2 promoter in the absence of p38α could be a consequence of the lower activation of the transcription factor ATF-2.

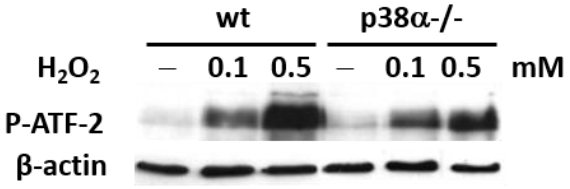

Figure 28: H_2O_2 induced a higher ATF-2 phosphorylation in wt cells than in p38α-/- MEFs. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with H_2O_2 (0.1 and 0.5 mM) for 20 min. Western-blot analysis of P-ATF-2 levels normalized with β-actin.

To address this issue, ATF-2 knock-down experiments were performed using an ATF-2 siRNA, which markedly reduced ATF-2 protein levels in wt MEFs (Fig. 29A). A high decrease in SOD-2 mRNA levels was observed in wt cells upon

ATF-2 knock-down, either when they are maintained untreated or treated with H_2O_2 (Fig. 29). This suggests that ATF-2 is a mediator of p38α-induced SOD-2 expression and it would bind SOD-2 promoter to exert its action.

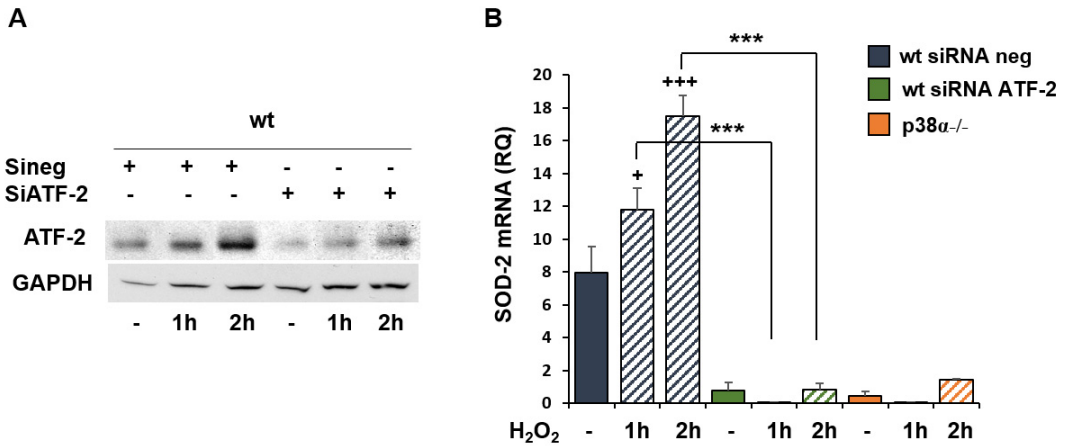

Figure 29: **ATF-2 is an important mediator of p38α in the induction of SOD-2 mRNA expression upon H_2O_2 treatment.** Effect of ATF-2 siRNA on SOD-2 mRNA levels. A negative control (siRNA neg) was also used. A) Western-blot analysis of ATF-2 levels normalized with GAPDH. B) Histogram shows the mean values ±S.E.M. (n=3) of SOD-2 mRNA levels of wt, siATF-2-wt and p38α-/- MEFs untreated or treated with 0.5 mM H_2O_2 for 1 or 2h. (*p<0.05), (***p<0.001), compared as indicated.

To demonstrate that ATF-2 was able to bind to SOD-2 promoter under these conditions, we next performed chromatin immunoprecipitation (CHiP) assays. As shown in figure 30, CHiP assays revealed a significant binding of P-ATF-2 to SOD-2 promoter in wt MEFs treated with H_2O_2, while in p38α-/- cells it was no detectable binding.

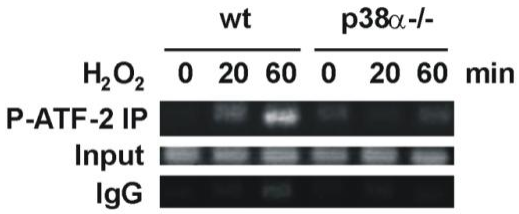

Figure 30: **P-ATF-2 binds to SOD-2 promoter in wt MEFs, but not in p38α-/- MEFs.** CHIP analysis of P-ATF-2 binding to SOD-2 promoter. PCR analysis of DNA inmunoprecipitated by a P-ATF-2 antibody and of input DNA.

In parallel to these studies, and to know whether p38α was mediating cell survival through the up-regulation of ATF-2 and consequently SOD-2, viability assays were performed using wt, ATF-2 knock-down and p38α knock-out cells. Results presented in figure 31 show that ATF-2 siRNA significantly decreased cell viability of wt MEFs treated with H_2O_2. Therefore, these results indicate that p38α through ATF-2 regulation induces SOD-2 expression and resistance to H_2O_2 treatment.

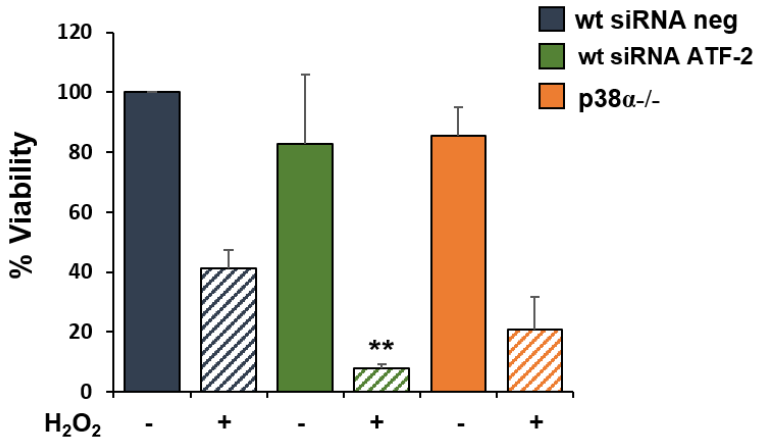

Figure 31: ATF-2 activation is required for p38α-mediated cell survival upon H_2O_2. Effect of ATF-2 siRNA on cell viability upon treatment of MEFs (wt, ATF-2 knock-down and p38α knock-out) with 0.5 mM H_2O_2 for 24h. A negative control (siRNAneg) was also used. Results are expressed as the percentage of viable cells, showing the mean values ±S.E.M. (n=3). **p<0.01, ATF-2 knock-down (wt siRNA ATF-2) as compared with wt cells treated with H_2O_2.

1.6. p38α positively regulates catalase through mechanisms involving protein stabilization and mRNA expression and/or stabilization

We have demonstrated that catalase levels and activity were significantly lower in p38α-/- than in wt MEFs. Next, we wanted to explore the mechanisms involved in the regulation of catalase expression by p38α.

As shown in figure 32A, catalase mRNA levels were increased by H_2O_2 progressively at 2h and 4h in both, wt and p38α-deficient cells, but to a higher extent in wt cells at 4h. However, after 8h of treatment, catalase mRNA highly decreased to the level of control in p38α-/- cells, while in wt cells these levels

remained above control values. Inhibition of transcription by actinomycin D abolished the increase in catalase mRNA levels observed at 2h and 8h, regardless of the presence of p38α. However, at 4h it was just a partial decrease in catalase mRNA upon actinomycin D treatment, specially, in wt cells. This would suggest that increases in catalase mRNA levels are only partially dependent on transcription and p38α might stabilize catalase mRNA at 4-8h of treatment. In addition, the evaluation of the effect of the proteasome inhibitor MG-132 revealed that p38α could also regulate catalase protein stability. As shown in figure 32B, MG-132 increased catalase protein levels only in p38α-deficient cells, which strongly suggests that catalase protein would be also stabilized by p38α.

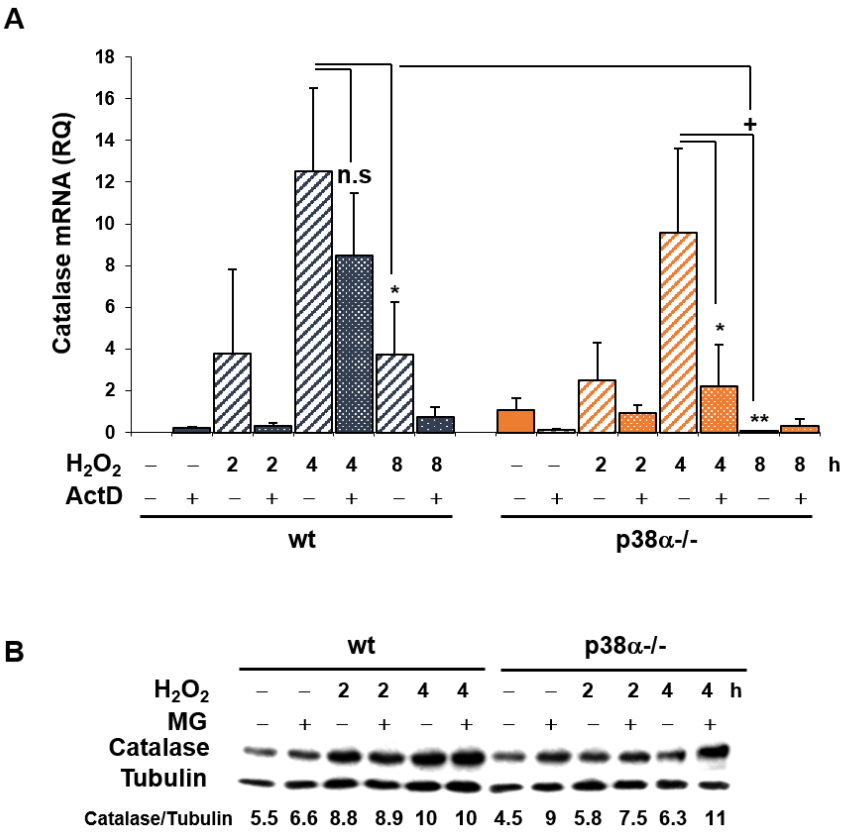

Figure 32: p38α positively regulate catalase through mRNA and protein stability. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with H_2O_2 (0.5 mM) in the presence or absence of actinomycin D or MG-132 for the indicated time periods. A) Analysis of catalase mRNA levels by RT-qPCR in the presence or absence of actinomycin D. Catalase mRNA expression was normalized using GAPDH. Histograms show mean values ±S.E.M (n=3). *p<0.05, +p<0.05, **p<0.01, compared as indicated. B) Effect of the proteasome inhibitor, MG-132, on catalase protein expression. Western-blot analysis of catalase protein levels normalized with tubulin. Catalase/tubulin represents the relative value resulting from the densitometric analysis of catalase versus tubulin levels multiplied by 10.

Hence, based on these results, p38α might be a positive regulator of catalase through protein stabilization and mRNA expression and/or stabilization.

1.7. Antioxidants protect p38α-deficient MEFs from ROS-induced cell death

Our data indicate that p38α activation in MEFs can protect from H_2O_2-induced cell death through a mechanism that reduces ROS accumulation via induction of antioxidant enzymes. Thus, we next studied the effect of the antioxidant N-acetyl cysteine (NAC) on H_2O_2-induced cell death.

As shown in figure 33A, NAC significantly decreased the number of apoptotic nuclei induced by H_2O_2 in p38α-/- cells. Moreover, NAC not only protected from cell death, but also induced an increase in phosphoThr389-p70S6K levels, which was particularly strong in p38α-deficient cells, reaching a similar level to that found in wt cells (Fig. 33B). In contrast, Akt and p38α phosphorylation decreased in NAC treated cells.

All these results suggest that H_2O_2-induced cell death would be a consequence of the damage induced by ROS accumulation, which is higher in p38α-/-MEFs. Hence, because catalase levels were lower in these cells, we added exogenous catalase to mimic wt cells situation. As shown in figure 33A, exogenous catalase highly decreased the number of apoptotic nuclei in p38α-deficient MEFs treated with H_2O_2. In addition, a parallel increase in P-Thr389-p70S6K levels was observed (Fig. 33C).

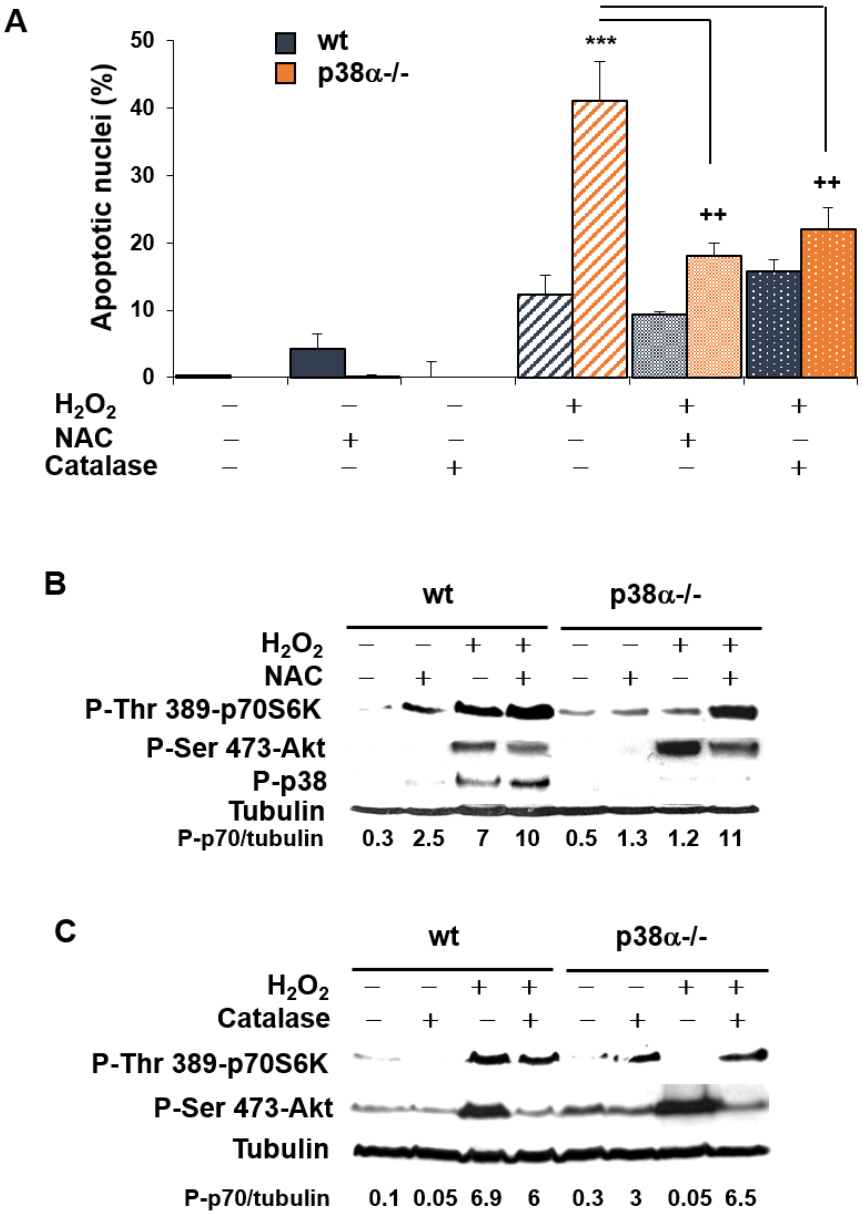

Figure 33: N-acetyl cysteine or catalase protects from ROS-induced apoptosis and allows H_2O_2-induced activation of p70S6K by mTOR in p38α-/- MEFs. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with H_2O_2 (1 mM) for 20 min (B and C) or 3h (A). When indicated, cells were pretreated for 1h with N-acetyl cysteine (NAC) (2.5 mM) or catalase (50 units/ml). A) Results show the percentage of apoptotic nuclei. ***p<0.001, p38α-/- versus wt MEFs upon treatment with H_2O_2 and $^{++}$p<0.01, compared to p38α-/- MEFs treated with H_2O_2 plus NAC or catalase, as indicated. B) and C) Western-blot analysis of the levels of P-Thr389-p70S6K and P-Ser473-Akt normalized with tubulin. P-p70/tubulin represents the relative value resulting from the densitometric analysis of P-Thr389-p70S6K versus tubulin levels multiplied by 10.

Moreover, in agreement with the idea that a high accumulation of ROS impairs p70S6K activation, we observed that treatment of wt MEFs with a very high dose of H_2O_2 (5 mM) impaired p70S6K activation in wt cells (Fig. 34), which indicates that a very high accumulation of ROS, either prevents activation or inactivates mTORC1/p70S6K pathway.

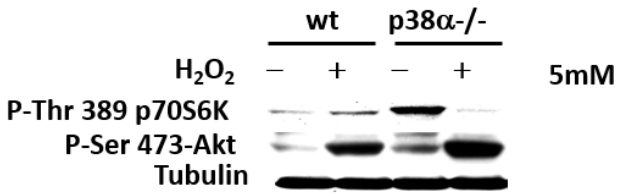

Figure 34: A high dose of H_2O_2 impairs p70S6K activation, even in MEFs expressing p38α. MEFs (wt and p38α-/-) maintained in the presence of serum were treated with H_2O_2 (5 mM) for 20 min. Western-blot analysis of P-Thr389-p70S6K and P-Ser473-Akt normalized with tubulin.

2. p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH HYPERMETHYLATION OF GENE REGULATORY SEQUENCES, PROMOTING MIGRATION AND INVASION

As described in the group background section, affymetrix microarrays analyses revealed that fibulin 3 mRNA was highly up-regulated in p38α-/- MEFs. Fibulin 3 is an extracellular matrix protein, which is involved in the regulation of migration and invasion in some tumour cell lines (Chen *et al.* 2014;Hu *et al.* 2009;Kim *et al.* 2012a). p38α also regulates these cellular functions (del, I and Nebreda 2012;Wagner and Nebreda 2009). This prompted us to deeply characterize the role played by p38α in the regulation of Fibulin 3 expression, as well as to investigate if p38α could act through Fibulin 3 to regulate migration and invasion. Additionally, we wanted to test the involvement of the others p38 MAPK isoforms in the regulation of Fibulin 3.

2.1. p38 MAPKs down-regulate fibulin-3 expression

Based on our data derived from Affymetrix microarrays showing that fibulin 3 was down-regulated by p38α and taking into account that there were so far no studies linking fibulin 3 expression and p38 MAPKs, we decided to characterize the regulation of fibulin 3 expression by p38 MAPKs. First, we validated data from microarrays assays and quantified fibulin 3 expression by RT-qPCR using wt and p38α knock-out MEFs. As shown in figure 35A, fibulin 3 mRNA levels were significantly higher in p38α knock-out than in wt MEFs. Moreover, western-blot analysis using total cell extracts or mediums (to analyze Fibulin 3 secretion) showed an increase in the levels of the secreted and intracellular Fibulin 3 protein in p38α deficient MEFs (Fig. 35C). 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 35B, treatment of wt MEFs with SB203580 led to an up-regulation of fibulin 3 mRNA, while it had no effect on p38α-/- cells, even when using a higher SB203580 concentration (data not shown). According to mRNA data, treatment of wt MEFs with SB203580 led also to the up-regulation of secreted Fibulin 3 protein

levels (Fig. 35D).These results confirmed that p38α is a negative regulator of Fibulin 3 expression. As SB203580 had no effect on p38α-/- MEFs, it is very likely that p38β is not a relevant regulator of fibulin3 expression as compared to p38α.

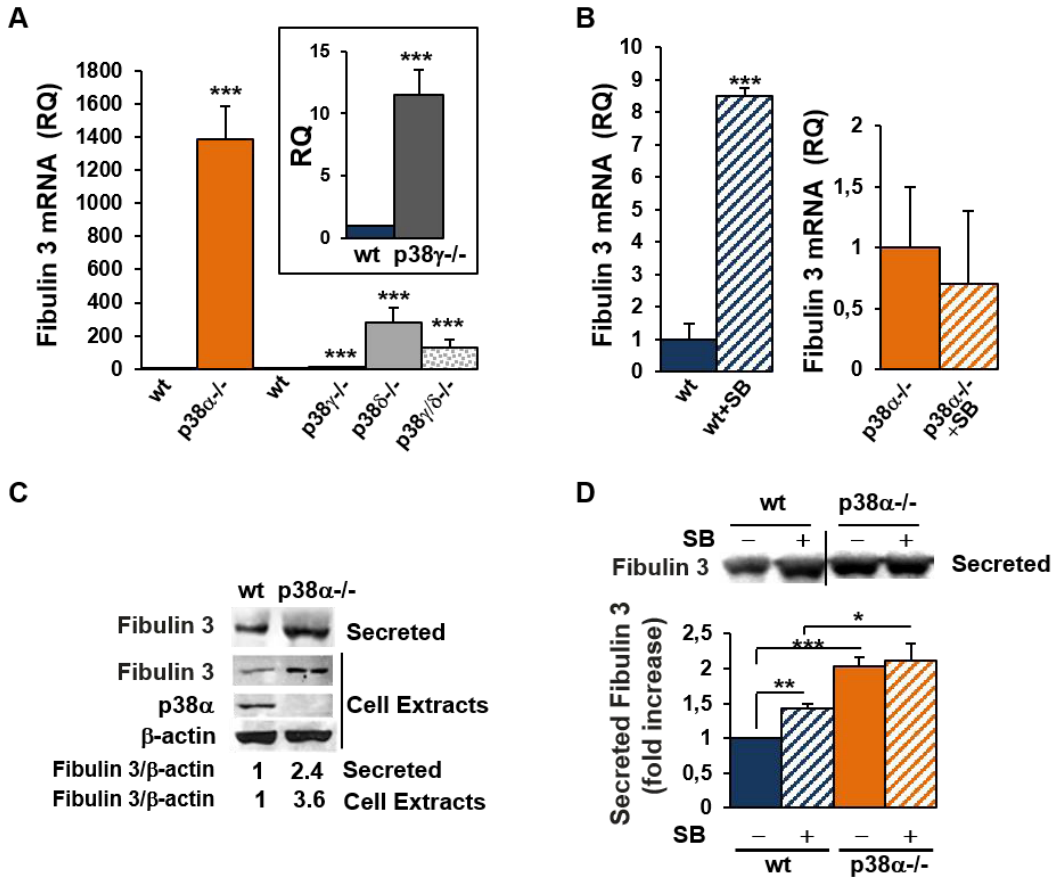

Figure 35: p38α, p38γ and p38δ are negative regulators of fibulin 3 mRNA expression. MEFs (wt, p38α-/-, p38γ-/-, p38δ-/- and p38γ/δ-/-) were maintained in the absence of serum and in the presence or absence of SB203580 (5 μM) for 24h and then, total RNA or proteins were isolated. A) and B) Analysis by RT-qPCR of fibulin 3 mRNA levels. Results represent the means ± S.E.M. of RQ values (n=3-8). ***p<0.001, as compared with wt cells. B) Effect of SB203580 (5 μM) in wt and p38α-/- MEFs. C) and D) 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. D) Effect of SB203580 (5 μM) in wt and p38α-/- MEFs. Histograms show the mean values ± S.E.M. of the densitometric analyses of the blots normalized with β-actin. *p<0.05, **p<0.01, ***p<0.001 compared as indicated.

As explained in the introduction section, four p38 MAPK isoforms (p38α, p38β, p38γ and p38δ) have been identified so far, which have either overlapping

or specific functions according to the cellular context (Nebreda and Porras 2000). Thus, we analyzed whether p38γ and p38δ isoforms could also regulate fibulin 3 expression. In order to perform this study, we used p38γ-/-, p38δ-/- and p38δ/γ-/- MEFs and analyzed fibulin 3 mRNA levels by RT-qPCR. Results from figure 35A show increased levels of fibulin 3 mRNA in MEFs lacking p38γ, p38δ or both, as compared to wt cells, although at a lower extent than in p38α-/- MEFs.

All these data indicate that p38α, p38γ and p38δ are all capable to down-regulate fibulin 3 expression, although p38α elicits the strongest effect. Therefore, we decided to focus our studies on the analysis of the function of p38α in the regulation of Fibulin 3 expression.

2.2. p38α MAPK induces methylation of regulatory sequences of fibulin 3 gene

Data from the literature indicated that Fibulin 3 expression was mainly regulated at the transcriptional level. According to this, in 2003, it was described in mammalian retina the relevance of different regulatory sequences present at the 5´end of the fibulin 3 gene controlling its transcription (Blackburn *et al.* 2003). Moreover, in some tumours, Fibulin 3 expression has been found to be down-regulated as a consequence of promoter methylation (Kim *et al.* 2011;Tong *et al.* 2011).

Based on all this, we wondered whether p38α might down-regulate Fibulin 3 expression through promoter methylation and consequently, might be responsible for its low expression in wt MEFs.

To examine the potential involvement of DNA methylation in the down-regulation of Fibulin 3 in wt MEFs, we first determined whether the expression of Fibulin 3 could be restored in wt cells upon treatment with the DNA methylation inhibitor, 5-aza-2´-deoxicytidine (5A2dC). As shown in figure 36A, fibulin 3 mRNA levels highly increased in wt MEFs treated with 5A2dC, both at 0.5 and 1 μM, while they remained unchanged in p38α-/- MEFs. Accordingly, 5A2dC treatment induced an increase in the levels of the secreted Fibulin 3 protein in wt MEFs, while it had no effect in p38α knock-out MEFs (Fig. 36B).

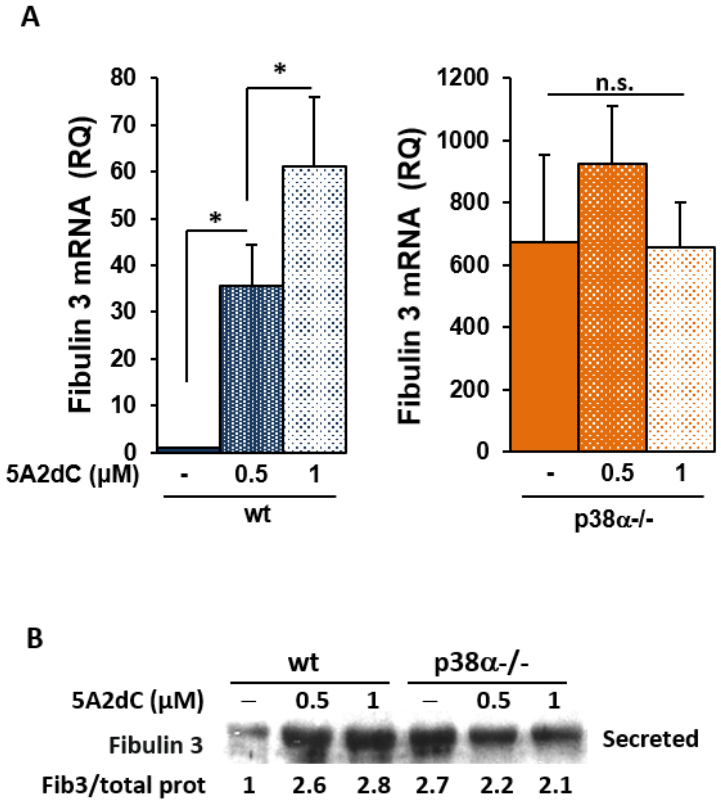

Figure 36: 5-Aza-2´-deoxicytidine treatment leads to an up-regulation of Fibulin 3 expression in wt MEFs. wt and p38α-/- MEFs were maintained with 5-Aza-2´-Deoxicytidine (5A2dC) (0.5 μM or 1 μM) for 48h (24h pretreatment in the presence of serum plus 24h treatment in the absence of serum) and then, total RNA or proteins were isolated. A) RT-qPCR analysis of fibulin 3 mRNA levels. Results represent the mean ± S.E.M. of RQ values. *p<0.05, compared as indicated. B) Western-blot analysis of Fibulin 3 protein levels secreted to the culture medium. Densitometric analysis of the blots normalized with total intracellular protein levels are shown.

These results suggest that p38α might down-regulate fibulin 3 expression through a mechanism dependent on DNA hypermethylation. Nevertheless, 5A2dC is a general inhibitor of DNA methylation that helps us to know qualitatively if the regulatory sequences of a gene are methylated or not. However, it does not give us an accurate information about the methylation status of the different CpG islands.

To demonstrate and quantitatively evaluate the effect of p38α on the methylation levels of fibulin 3 regulatory sequences, we analyzed methylation status of fibulin 3 gene regulatory sequences in wt and p38α knock-out MEFs. In

particular, methylation levels of twelve CpG islands sites present in the 5´untranslated region (UTR) of fibulin 3 gene (1210bp upstream of the ATG translation start site) were determined by pyrosequencing in wt and p38α-/- cells (Fig. 37).

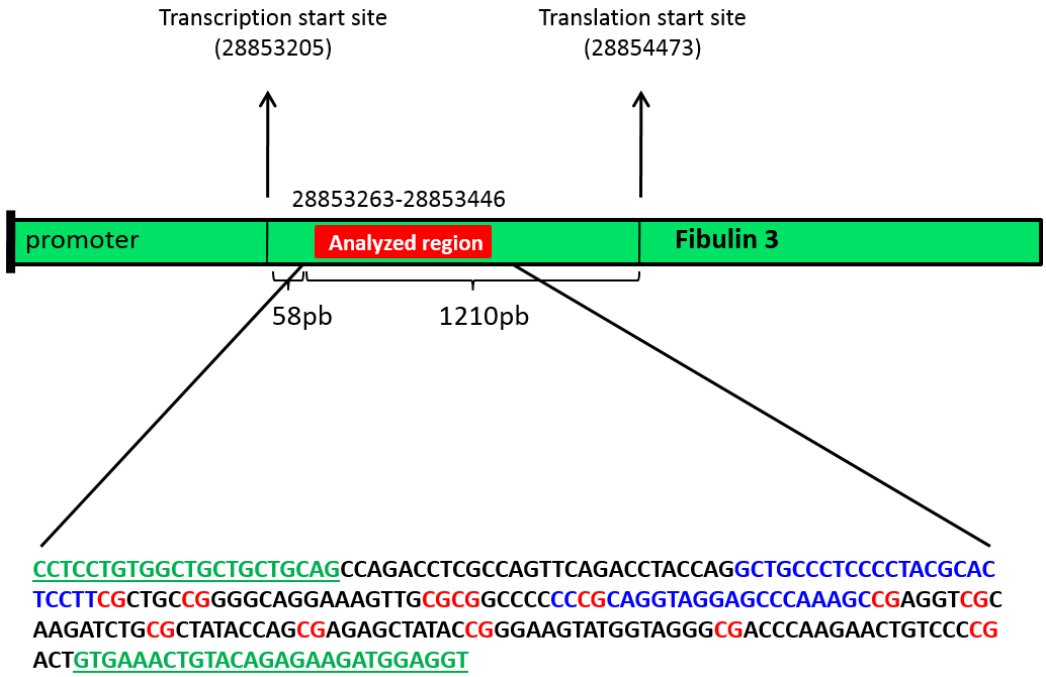

Figure 37: Fibulin 3 regulatory sequences analyzed by pyrosequencing (red box). The sequences labeled in green correspond to the primers used to amplify the region; those labeled in blue correspond to the primers used for the PCRs in the pyrosequencing process; the twelve analyzed CpG islands are in red.

Figure 38A shows the pyrosequencing diagram of the twelve CpG islands analyzed in the DNA from wt and p38α-deficient MEFs. The average methylation percentage of 10 out of 12 CpG sites was higher in wt as compared to p38α-/- MEFs (Fig. 38B).

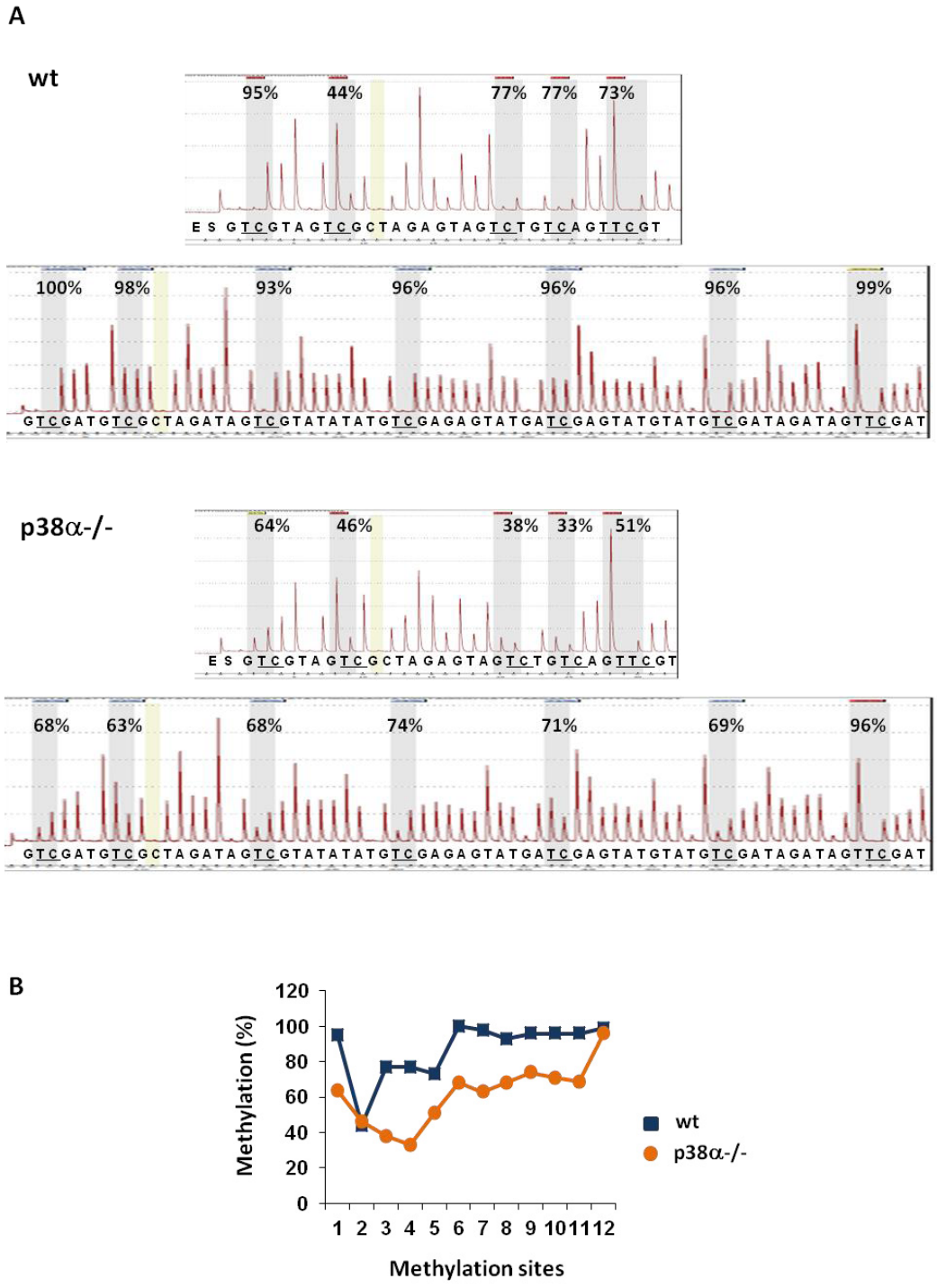

Figure 38: p38α leads to hypermethylation of fibulin 3 gene regulatory sequences. Percentages of methylation of CpG sites from fibulin 3 gene regulatory sequences (5´UTR region) in wt and p38α-/- MEFs. A) Representative pyrosequencing diagrams showing the methylation status of the 12 CpG sites. B) Graphic showing the percentages of methylation of the 12 analyzed CpG sites.

These results strongly indicate that p38α represses fibulin 3 expression through hypermethylation of 5´UTR regulatory sequences.

2.3. DNMT3A protein levels are down-regulated in p38α MAPK knock-out MEFs

DNA methylation results from the activity of DNA methyltransferases (DNMTs), leading to gene regulation (Denis *et al.* 2011). Among the different DNMTs present in mammals, DNMT3A/3B are thought to function as de novo DNA methyltransferases (Miremadi *et al.* 2007). Their levels can be regulated, being of relevance its post-transcriptional regulation (Denis *et al.* 2011). In particular, binding of HuR protein (an RNA-binding protein that stabilizes and/or modulates the translation of target mRNAs) to the 3´UTR of DNMT3B mRNA enhances its stability and increases its protein levels in colorectal RKO cells (Lopez, I *et al.* 2009). p38α MAPK can phosphorylate HuR, which enhances its binding to certain mRNAs such as p21mRNA, increasing its protein levels (Lafarga *et al.* 2009). This raised the possibility that p38α MAPK could be regulating DNMT3A/3B protein levels through a HuR-dependent mechanism. Thus, we initially analyzed DNMT3A protein levels in wt and p38α-/- MEFs and we found a significant decrease in DNMT3A protein levels in p38α-/- MEFs as compared to wt cells (Fig. 39).

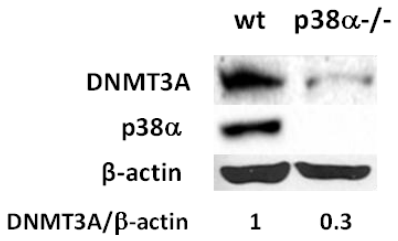

Figure 39: p38α positively regulates DNMT3A protein levels. wt and p38α-/- MEFs were maintained in the absence of serum for 24h. DNMT3A protein levels were determined in cell extracts by western-blot analysis using β-actin to normalize and p38α as a control of its expression in wt cells.

These results indicate that p38α MAPK positively regulates DNMT3A protein levels. Thus, DNMT3A could lead to the p38α-induced hypermethylation of fibulin 3 gene regulatory sequences detected by pyrosequencing. Therefore, it

is likely that DNMT3A might be a mediator of the p38α-induced hypermethylation of fibulin 3 regulatory sequences, although we cannot exclude the possibility of the participation of other DNA methyltransferases.

2.4. Fibulin 3 knock-down promotes migration and invasion of MEFs

p38α is a mediator of cell migration and invasion (Nebreda and Porras 2000;Wagner and Nebreda 2009). Fibulin 3 is an extracellular matrix (ECM) protein that can regulate migration and invasion properties of distinct tumour cells, either promoting or inhibiting cell invasiveness depending on the tumour type (Hu *et al.* 2009;Kim *et al.* 2012a). Therefore, we next wondered whether p38α, through down-regulation of Fibulin 3, could favour cell migration and invasion.

To perform these studies, Fibulin 3 was permanently knocked-down in wt and p38α-/- MEFs using a mouse Fibulin 3 shRNA. Different Fibulin 3 knock-down clones were generated, which showed a similar behaviour. As shown in figure 40, secreted Fibulin 3 protein levels were decreased in both, wt and p38α-/- MEFs by this shRNA, but not by a control shRNA (data not shown).

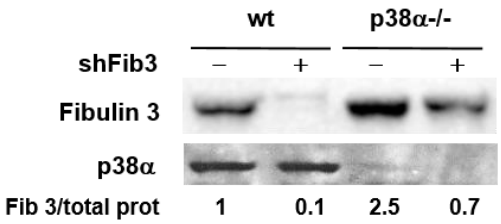

Figure 40: Fibulin 3 knock-down in wt and p38α-/- MEFs. Western-blot analysis of Fibulin 3 protein levels secreted to the culture medium by MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) maintained in the absence of serum for the last 24h. p38α was used as a control. Fibulin 3 quantification referred to total protein levels is shown.

To study the migratory capacity of these cells, wound healing assays were carried out. As expected, a faster migration of wt MEFs as compared with p38α-/- cells was observed. Fibulin 3 knock-down highly increased migration of p38α-/-

MEFs up to the levels of wt cells, while no significant changes were observed in wt MEFs (Fig. 41).

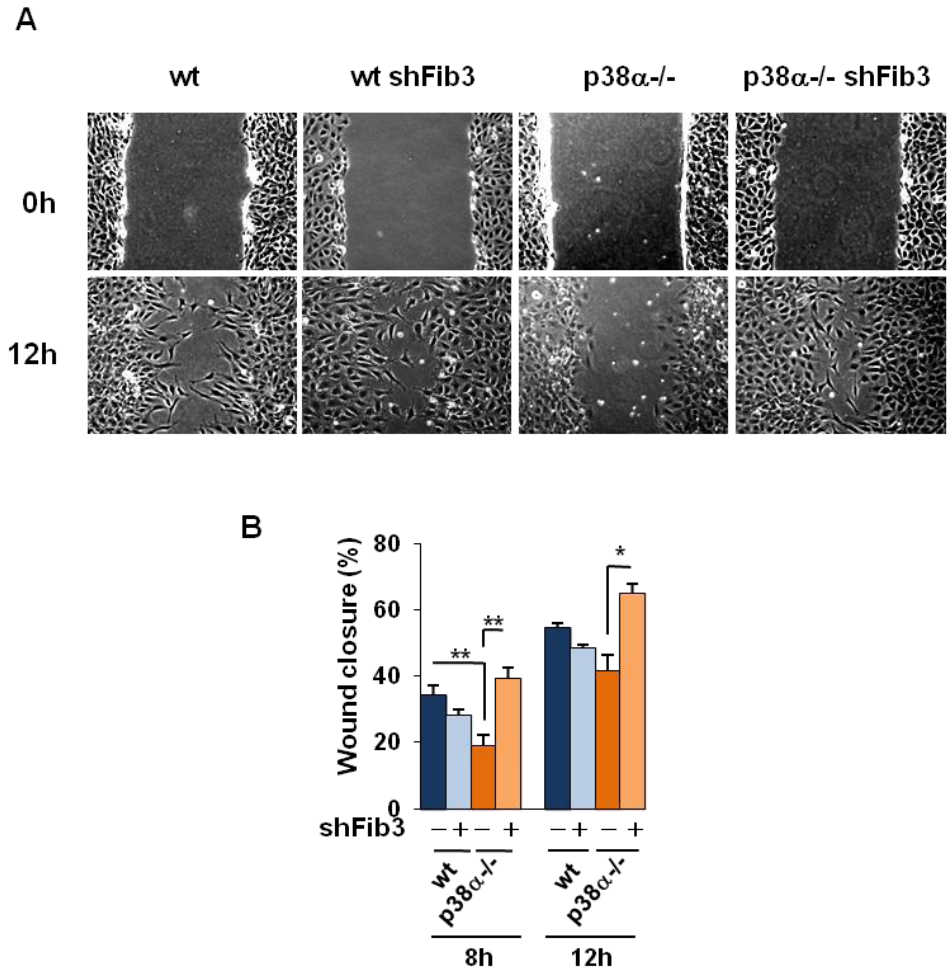

Figure 41: Fibulin 3 knock-down enhances migration of MEFs. Wound healing assay. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were pre-treated with mitomycin C for 30 min in serum free medium and, then, a straight scratch was performed. Cells were maintained in the absence of serum and allowed to migrate. A) Representative images from phase contrast microscope at 0 and 12h. B) Histograms show the mean value ± S.E.M. of the percentage of wound closure at 8 and 12h. *p<0.05, **p<0.01, compared as indicated.

Based on these results, the effect of Fibulin 3 knock-down in the invasive capacity of MEFs was also evaluated. Fibulin 3 knock-down promoted invasion through matrigel of p38α knock-out MEFs, so the number of invading cells was similar to the one found in wt MEFs (Fig. 42). Although wound healing assays did

not reveal any significant change in cell migration in wt MEFs upon fibulin 3 knock-down, invasion through matrigel was increased (Fig. 42).

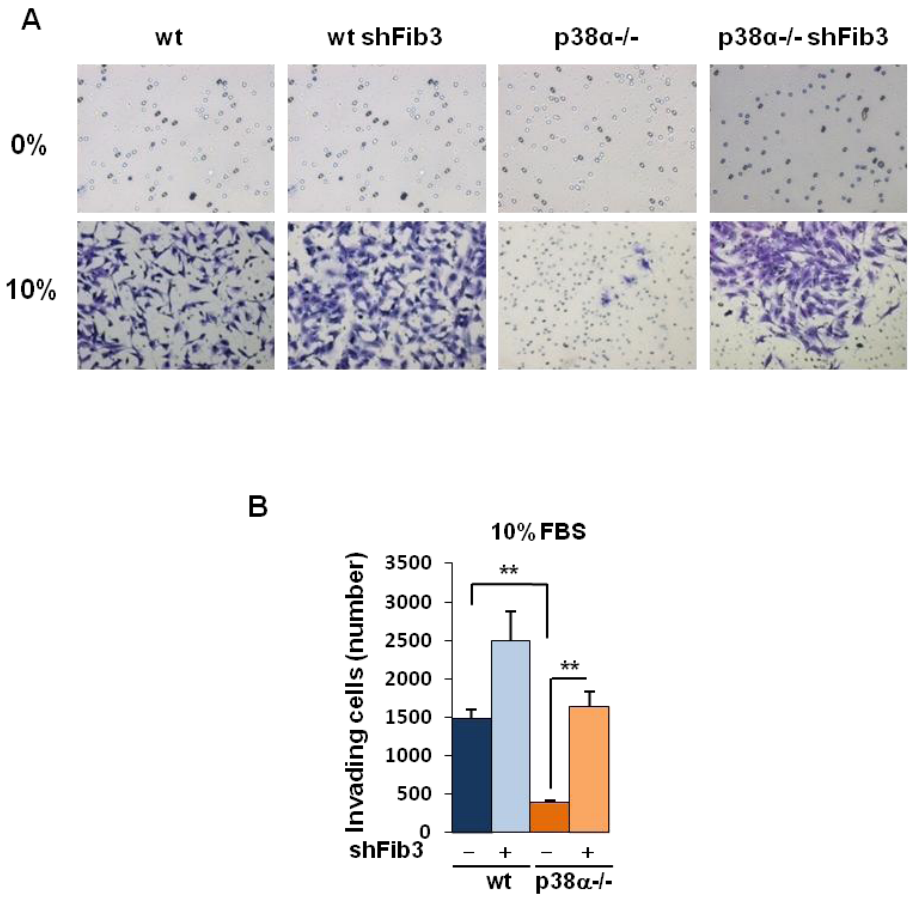

Figure 42: Fibulin 3 knock-down enhances invasion of MEFs. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were seeded in the upper transwell chamber covered with matrigel in serum-free medium. Medium with FBS (10%), to act as a chemoattractant, or without FBS (0%) was placed in the lower chamber. Cells were allowed to pass through the matrigel for 24h. A) Representative images of invading cells after staining with crystal violet (phase contrast microscope). B) Histograms show the mean values ± S.E.M. of the number of invading cells (n=3). *p<0.05,**p<0.01, compared as indicated.

Accordingly with other data from the literature, these results support a role for p38α as a mediator of cell migration and invasion. Moreover, Fibulin 3 acts as an inhibitor of cell migration and invasion in MEFs as it happens in certain tumours, such as non-small lung carcinoma (Kim *et al.* 2012a). Additionally, we

have found that the p38α pro-migratory/invasive effect might be, at least in part, mediated by fibulin 3 down-regulation in MEFs.

2.5. Mechanisms involved in the regulation of cell migration and invasion by Fibulin 3 in MEFs. Role of p38α/β.

As MMPs are relevant for extracellular matrix degradation during cell migration/invasion (Bourboulia and Stetler-Stevenson 2010;Kessenbrock *et al.* 2011), we evaluated whether the increased invasion observed in fibulin 3 knock-down MEFs was due to changes in MMPs levels and/or in their activities. We did not find significant changes in the levels of MMP2, 7, 9, 10, 11 and 13 mRNAs that could explain invasion results (data not shown). Thus, we next evaluated MMP2 and MMP9 activities. As observed in figure 43A and XB, MMP2 and 9 activities were lower in p38α-/- than in wt MEFs and MMP9 activity increased in p38α Knock-out MEFs upon fibulin 3 knock-down, which could be responsible for their enhanced invasion. However, MMP2/9 activities were decreased in fibulin 3 knock-down wt MEFs (Fig. 43A and 43B), which did not correlate with its enhanced invasive capacity. Therefore, we determined the effect on cell invasion of a broad spectrum MMP inhibitor, marimastat. We found that it impaired invasion in all cell lines (Fig. 43C and 43D). This suggests that other MMPs, different from MMP2 and 9, could be responsible for the increased invasion of fibulin 3 knock-down wt MEFs. Overall, these results indicate that MMPs are involved in the fibulin 3 knock-down-induced cell invasion in MEFs.

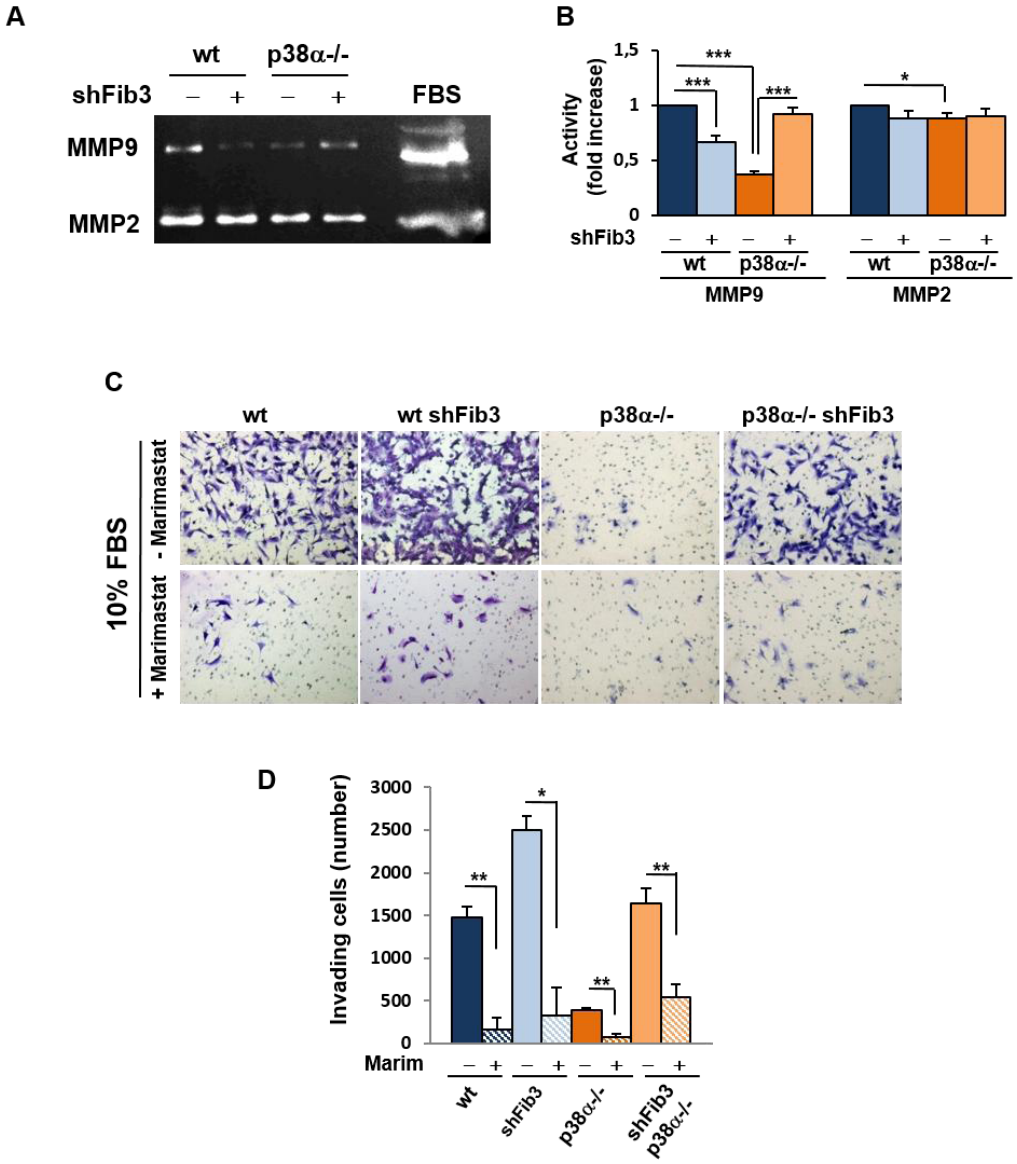

Figure 43: Role of MMPs in Fibulin 3 knock-down induced invasion: MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were maintained in absence of serum for 24h. A) and B) MMP2/9 activities analysis by zymography. FBS was used as a positive control. A) Representative zymogram. B) Histograms show the mean values ± S.E.M. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value (n=6). *p<0.05, ***p<0.001, compared as indicated. C) and D) Effect of MMPs inhibition on invasion. The MMPs inhibitor, marimastat was added to the cells 24h prior to the invasion assay through matrigel. FBS (10%) was used as a chemoattractant. C) Representative images of invading cells after staining with crystal violet (phase contrast microscope). D) Histograms show the mean ± S.E.M. of the number of invading cells (n=3). *p<0.05,**p<0.01, compared as indicated.

We also evaluated the impact of fibulin 3 knock-down in some of the signaling pathways regulating cell migration and invasion such as p38 MAPKs, PI3K/Akt and ERKs. As shown in figure 44, an increase in P-ERKs and P-p38 MAPKs levels was induced by fibulin 3 knock-down, mainly in cells stimulated with serum. Moreover, upon fibulin 3 knock-down p38α MAPK phosphorylation was enhanced in wt MEFs, while in p38α-/- cells there was a strong increase in the phosphorylation of another p38 MAPK isoform (potentially, p38β) with a lower mobility (Fig. 44).

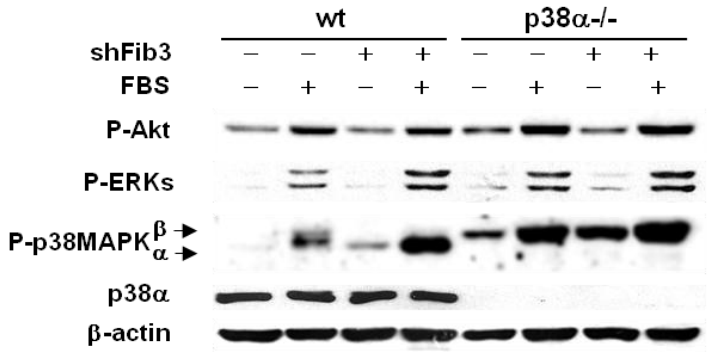

Figure 44: Fibulin 3 knock-down up-regulates the levels of p38 phosphorylation. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were serum deprived for 24h and then, treated with 10% FBS for 10 min. Western-blot analysis of P-Akt, P-ERKs and P-p38 levels normalized with β-actin. p38α was used as a control. P-p38/β-actin ratio derived from the densitometric analysis is shown (arbitrary units).

To determine the relevance of the hyper-activation of these p38 MAPKs, we evaluated the effect of the treatment with SB203580 on migration and invasion. As shown in figure 45, inhibition of p38α/β impaired migration of fibulin 3 knock-down cells and wt MEFs.

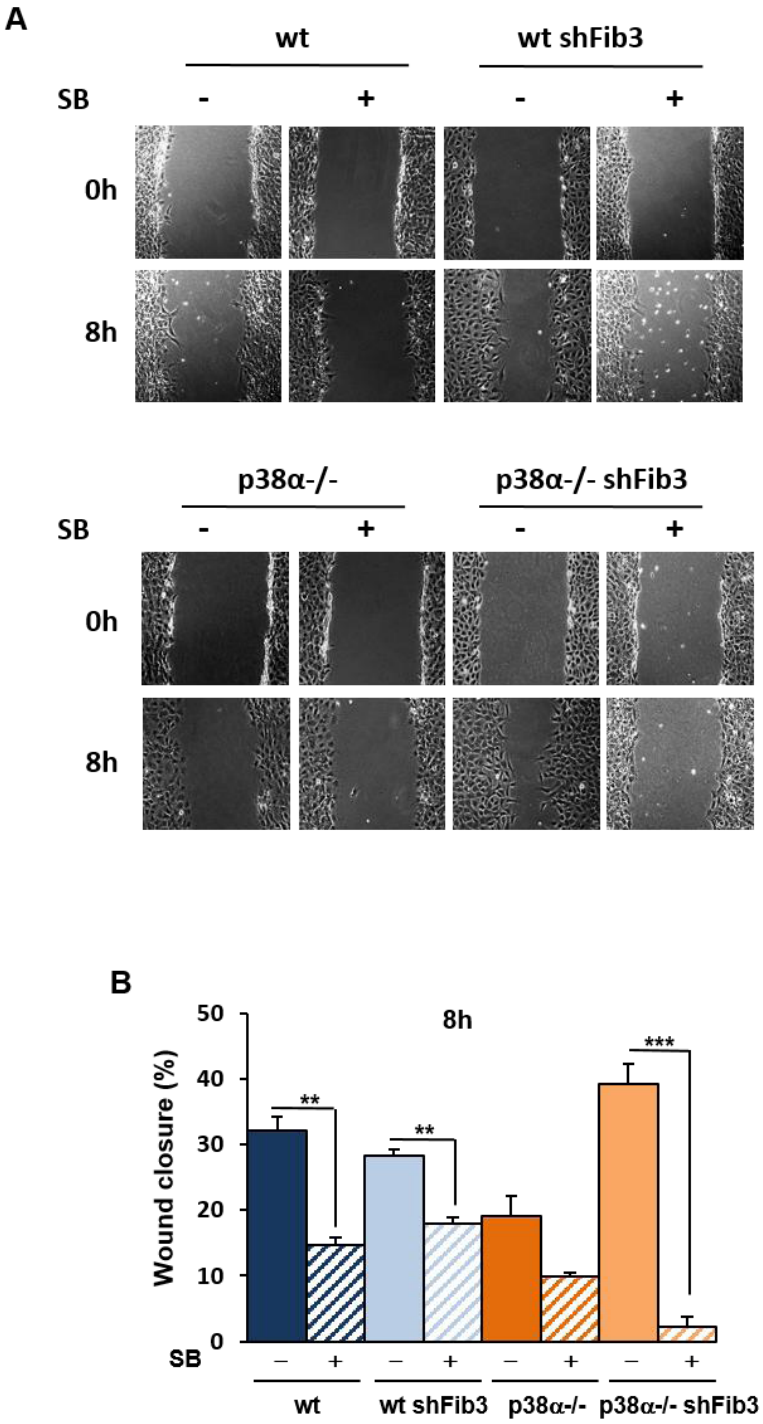

Figure 45: Analysis of the function of p38α/β in migration of fibulin 3 knock-down MEFs. Wound healing assay. Cells (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were pre-treated for 24h with 10 μM SB203580 before performing the scratch. Then, cells were allowed to migrate in the absence of serum (with or without SB203580). A) Representative images from phase contrast microscope after 0 and 8h of migration. B) Histograms show the mean value ± S.E.M. of the percentage of wound closure at 8h. **p<0.01, *** p<0.001, compared as indicated.

According to these results, p38α/β inhibitor also impaired cell invasion through matrigel of fibulin 3 knock-down cells and wt MEFs (Fig. 46). All these data indicate that the enhanced activation of p38α in wt and of p38β in p38α-/- cells induced by fibulin 3 knock-down is necessary for migration and invasion of these cells.

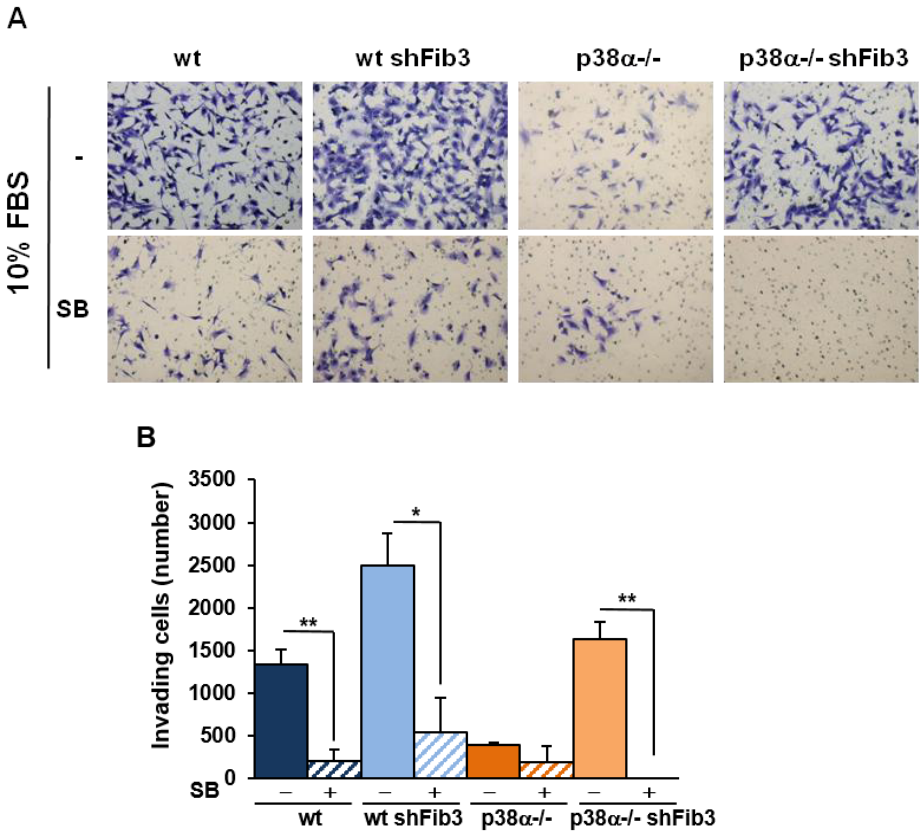

Figure 46: Inhibition of p38α/β impairs cell invasion. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were pretreated with 10 μM SB203580 for 24h prior to the invasion assay through matrigel. FBS (10%) was used as a chemoattractant. A) Representative images of invading cells after staining with crystal violet (phase contrast microscope). B) Histograms show the mean values ± S.E.M. of the number of invading cells (n=3). *p<0.05,**p<0.01, compared as indicated.

The observed effect of p38α/β inhibition with SB203580 on cell migration and invasion partially correlates with the decrease in MMP9 activity upon treatment with SB203580 (Fig. 47). Therefore, MMP9 would contribute to

mediate p38α/β actions on migration/migration in MEFs, including those where fibulin 3 was knocked-down.

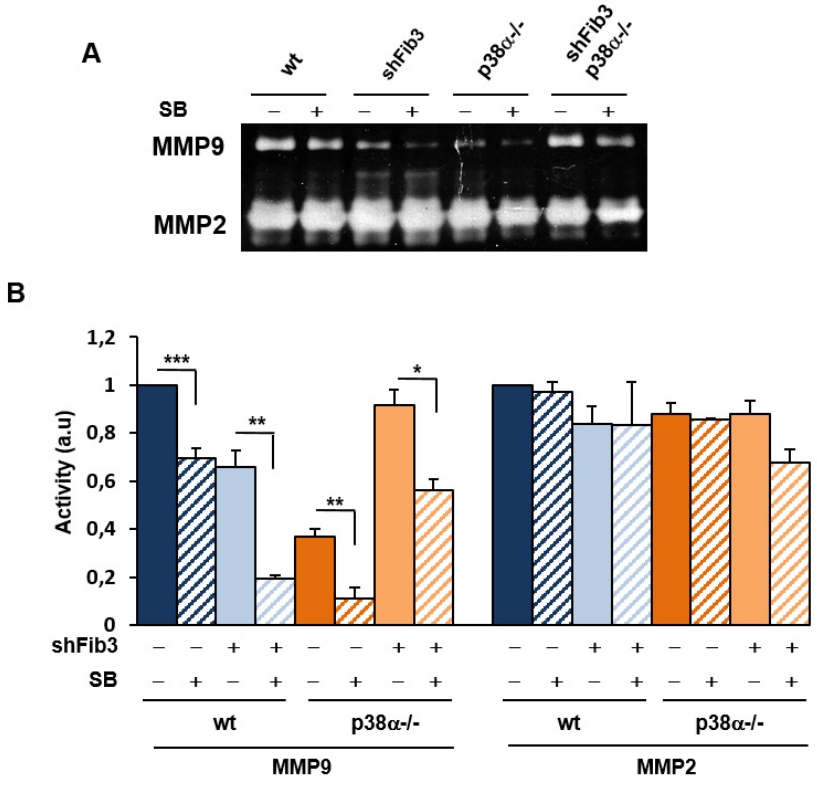

Figure 47: Effect of p38α/β inhibition onMMP9 activity. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) were maintained in absence of serum and in the absence or presence of 10 μM SB203580 for 24h. FBS was used as a positive control. A) Representative zymogram. B) Histograms show the mean values ± S.E.M. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value (n=6). *p<0.05, ** p<0.01, ***p<0.001, compared as indicated.

2.6. Role of Fibulin 3 in cell transformation

As fibulin 3 knock-down had an impact on the invasive capacity of MEFs, mainly in p38α-/- cells, we further analyzed the behavior of fibulin 3 knock-down MEFs. They grew faster than wt and p38α-/- MEFs (data not shown). In addition, anchorage-dependent growth assays revealed an enhanced foci formation upon fibulin 3 knock-down (in wt and p38α-/- MEFs) (Fig. 48A and 48B). In contrast,

foci size was only increased in p38α knock-out cells, mainly in those where fibulin 3 has not been knocked-down (Fig. 48C).

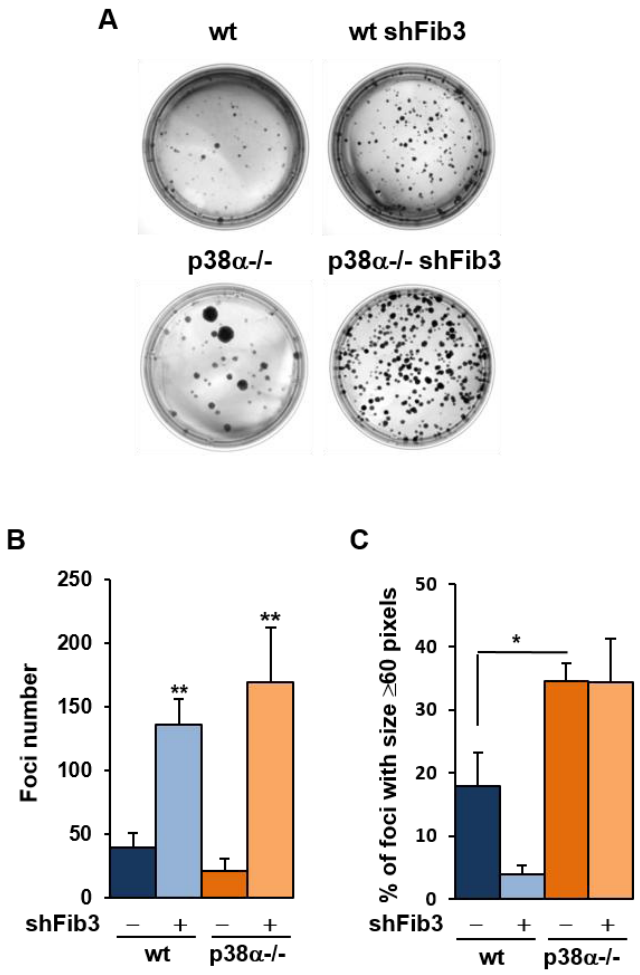

Figure 48: Fibulin 3 knock-down enhances focus formation. A) Representative images of anchorage dependent growth of MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) after 13 days. B) and C) Number and size of foci, respectively. Histograms show the mean values ± S.E.M. of foci number (B) and the percentage of those with a size ≥60 pixels, respectively. **p<0.01, shFib3 MEFs as compared with non-silenced cells; *p<0.05, compared as indicated.

All this indicates that contact inhibition is lost in fibulin 3 knock-down cells. This suggests that these cells could have suffered a process of transformation as impaired contact inhibition is considered a hallmark of cell transformation (Hanahan and Weinberg 2000). To further characterize the potential role of fibulin 3 in cell transformation, we also determined the ability of

these cells to grow in soft-agar, which is *in vitro* assay to determine *in vivo* tumourigenesis. However, fibulin 3 knock-down MEFs were unable to grow in soft agar (data not shown). Accordingly, *in vivo* xenograft assays revealed that fibulin 3 knock-down MEFs were unable to induce tumours in nude mice (Fig. 49).

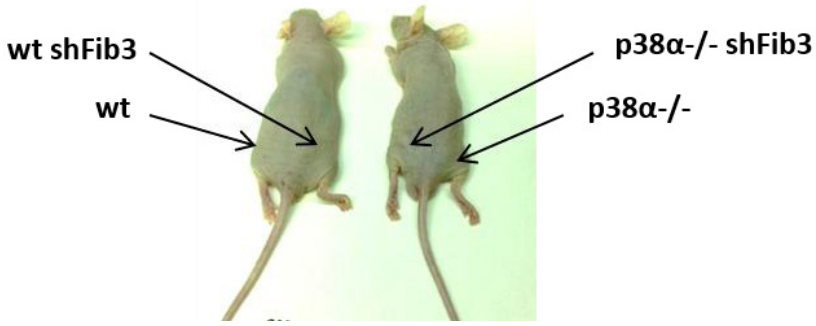

Figure 49: Fibulin 3 knock-down MEFs does not induce tumours *in vivo*. Xenograft assay. MEFs (wt and p38α-/-, with (shFib3) or without fibulin 3 knock-down) ($1.5x10^6$ cells) were injected in both flanks of nude mice. Representative image of two mice 6 weeks after injection of the cells.

Results shown here suggest that fibulin 3 knock-down is not sufficient to induce transformation, but it may collaborate with other genes to induce it.

3. C3G INHIBITS CELL MIGRATION AND INVASION THROUGH DOWN-REGULATION OF p38α MAPK ACTIVITY

As explained in the group background section, we have previously described a functional relationship between C3G and p38α MAPK in the regulation of apoptosis in CML and in MEFs (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009) as well as in the regulation of cell adhesion in CML (Maia *et al.* 2013). Moreover, both p38α MAPK and C3G, play important roles in the regulation of cell adhesion (Guo and Yang 2006;Ohba *et al.* 2001;Pannekoek *et al.* 2009;Voss *et al.* 2003;Zuluaga *et al.* 2007b) and migration (Cuenda and Rousseau 2007;Dayma and Radha 2011;Ohba *et al.* 2001;Rufanova *et al.* 2009;Wagner and Nebreda 2009). However, the function of C3G in cell migration and invasion has not been fully characterized. Based on all this, we hypothesized that C3G could act though p38α to control cell migration and invasion. We aim to study whether C3G and p38α MAPK can act in a common regulatory pathway to modulate cell migration and invasion in MEFs and if Rap-1 is a mediator of C3G.

3.1. C3G knock-down promotes cell migration through p38α by a mechanism not mediated by Rap-1

To study the cross-talk between C3G and p38α, MEFs (wt and p38α-/-) with permanent C3G knock-down (Fig. 50) (Gutierrez-Uzquiza *et al.* 2010) were used.

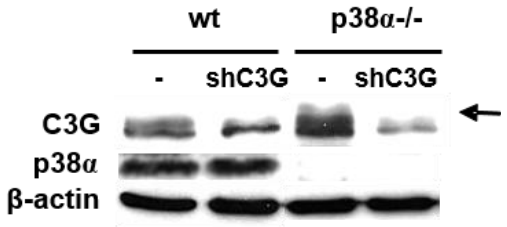

Figure 50: Decrease in C3G expression upon C3G silencing. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down) were maintained in the presence of serum and proteins were isolated. Western blot analysis of C3G and p38α levels normalized with β-actin.

To determine whether C3G regulates cell migration through a mechanism dependent on p38α, wound healing assays were carried out. As shown in results section 2 (Fig. 41), wt cells migrate faster than p38α-/- cells. C3G knock-down highly enhanced cell migration in wt cells, but not in p38α-/- MEFs, which presented even a lower migration than p38α-/- cells (Fig. 51).

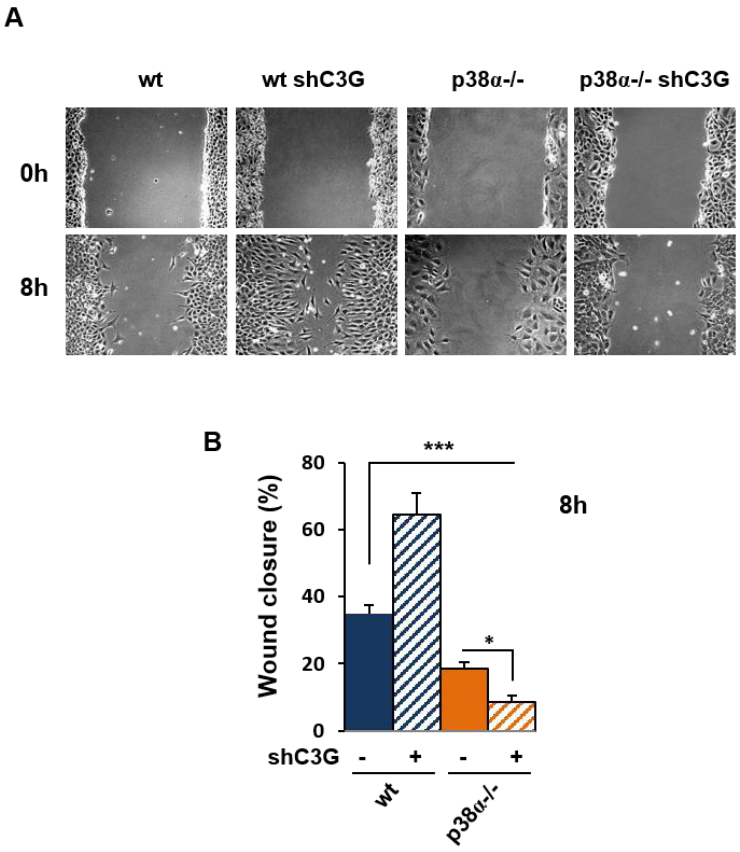

Figure 51: C3G knock-down enhances migration of MEFs expressing p38α. Wound healing assay. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down were pre-treated with mitomycin C for 30 min in serum free medium. Then, a straight scratch was performed and cells were allowed to migrate in a serum free medium. A) Representative images from phase contrast microscope at 0 and 8h of migration. B) Histograms show the mean value ± S.E.M. of the percentage of wound closure at 8h. *p<0.05, ***p<0.001, compared as indicated.

According to other data from the literature (Ohba *et al.* 2001), C3G inhibits cell migration. Additionally, our data suggest that C3G regulates migration through a p38α dependent mechanism. To confirm this hypothesis, we evaluated the effect of the selective p38α/β inhibitor, SB203580. As shown in figure 52, treatment with SB203580 decreased migration of wt cells, with or

without C3G knock-down, while it had not effect in p38α-/- cells. These results confirm that C3G knock-down increases cell migration through the up-regulation of p38α activity in MEFs.

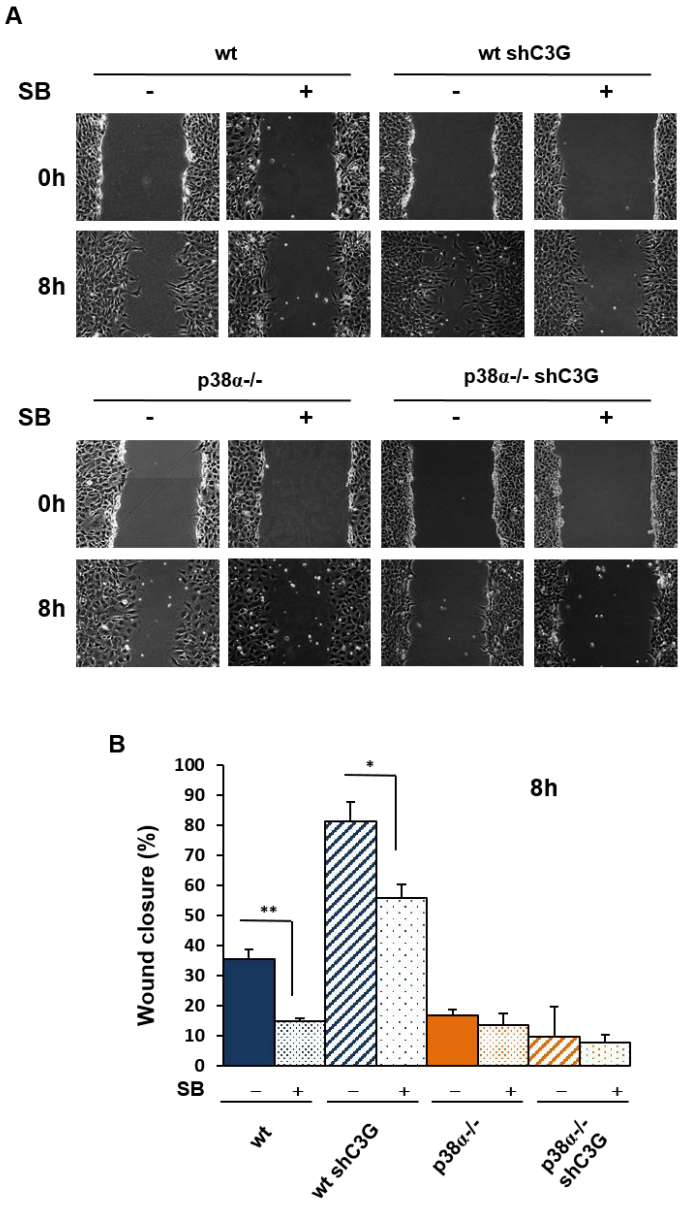

Figure 52: C3G knock-down enhances cell migration through p38α. Wound healing assay. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down) were pre-treated for 12h with 5μM SB203580 before performing the scratch. Then, cells were pre-treated with mitomycin C for 30 min in serum free medium. Then, a straight scratch was performed and cells maintained in the absence of serum (with or without SB203580) were allowed to migrate. A) Representative images from phase contrast microscope after 0 and 8h of migration. B) Histograms show the mean value ± S.E.M. of the percentage of wound closure at 8h. *p<0.05, **p<0.01, compared as indicated.

Next, we wanted to know if C3G was acting through its main target, Rap-1. Thus, we used MEFs with permanent expression of a dominant negative Rap-1 (DNRap-1) construct (Gutierrez-Uzquiza *et al.* 2010) to perform migration assays. As shown in figure 53, the effect of DNRap-1 expression and C3G knock-down on cell migration was opposite. While C3G knock-down increased migration of wt MEFs, the expression of the DNRap-1 impaired this process. Moreover, in p38α-/- cells, C3G knock-down slightly decreased cell migration, whereas the expression of a Rap-1 dominant negative induced a small increase in cell migration.

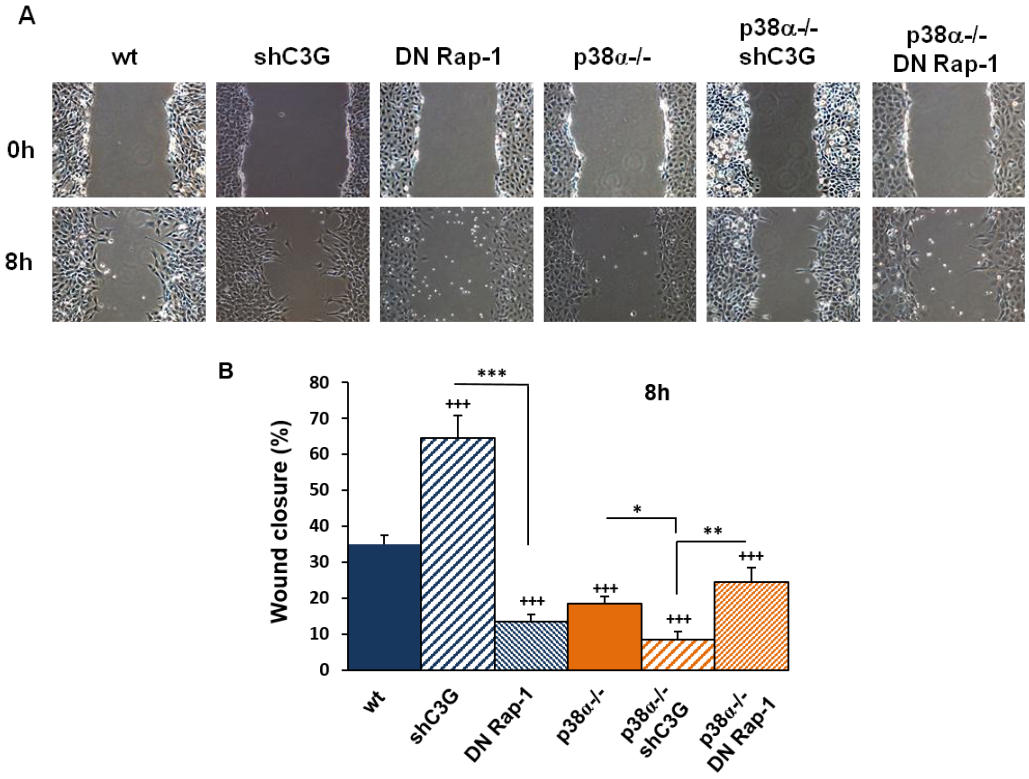

Figure 53: Rap-1 does not mediate C3G effects on cell migration. Wound healing assay. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down, or expressing a dominant negative Rap-1 mutant (DNRap-1)) were pre-treated with mitomycin C for 30 min in serum free medium. Then, a straight scratch was performed and cells were allowed to migrate in serum free medium. A) Representative images from phase contrast microscope at 0 and 8h of migration. B) Histograms show the mean value ± S.E.M. of the percentage of wound closure at 8h. +++ $p<0.001$ versus wt, **$p<0.01$, ***$p<0.001$, compared as indicated.

Therefore, all these data are in favour of a model where C3G would inhibit cell migration through the negative regulation of p38α activation, while Rap-1 would play an opposite role.

3.2. C3G knock-down increases cell invasion through p38α by a mechanism not mediated by Rap-1

We next examined the invasiveness of these cells. According to the results derived from the migration assays, the effect of C3G knock-down and DNRap-1 expression on cell invasion was opposite in wt MEFs. While C3G knock-down enhanced invasion through matrigel, the expression of the DNRap-1 impaired this process. In p38α-/- MEFs invasion through matrigel was very low as compared to wt cells, and there was no additional effect of C3G knock-down, or even a reduction upon DNRap-1 expression (Fig. 54).

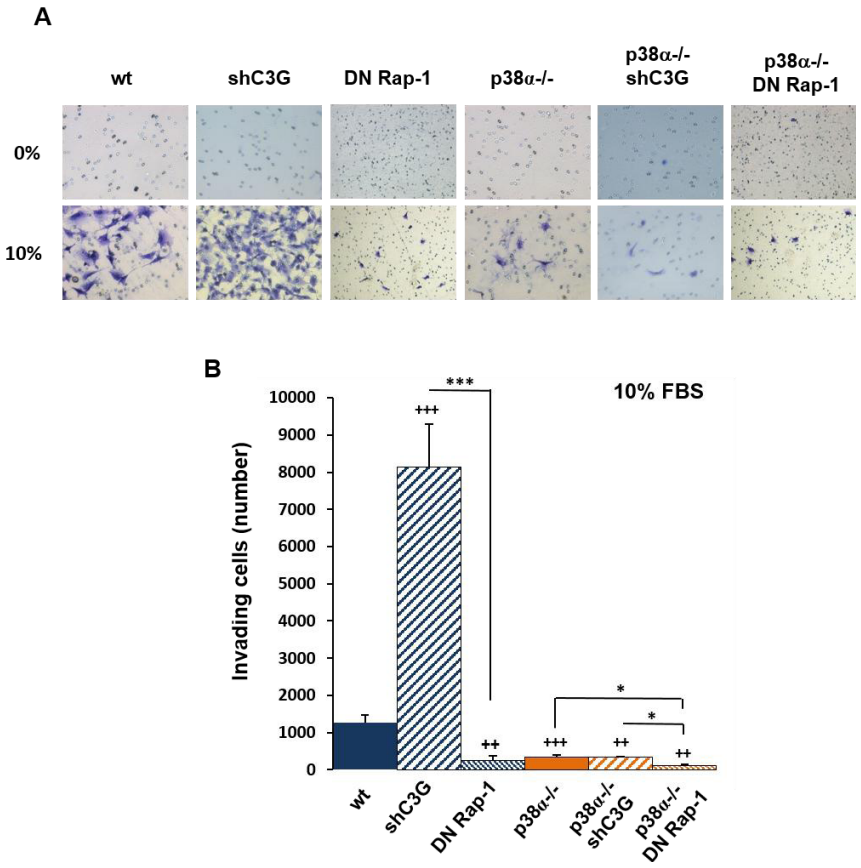

Figure 54: C3G knock-down enhances invasion of MEFs expressing p38α, while the expression of a DNRap-1 impaired cell invasion. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down, or expressing a dominant negative Rap-1 mutant (DNRap-1)) were seeded in the upper transwell chamber covered with matrigel in serum-free medium. Medium with 10% FBS (10%), to act as a chemoattractant, or without FBS (0%), was placed in the lower chamber. Cells were allowed to pass through matrigel for 24h. A) Representative images of invading cells after staining with crystal violet (phase contrast microscope). B) Histograms show the mean value ± S.E.M. of the number of invading cells (n=3). +++ $p<0.001$, ++ $p<0.01$ versus wt; ***$p<0.001$, *$p<0.05$, compared as indicated.

These results suggest that C3G would inhibit cell invasion through the negative regulation of p38α activation, while Rap-1 would promote this process through a mechanism independent of C3G GEF-activity.

3.3. Role of MMP2 and MMP9 as mediators of C3G/p38α effects on cell invasion

In order to further understand the role of C3G/p38α pathway in cell invasion and the potential implication of MMPs, the effect of C3G knock-down on MMP2 and 9 activities was determined in wt and p38α-/- MEFs (Fig. 55). As previously shown, MMP2/9 activities were lower in p38α-/- than in wt MEFs (Fig. 43A and 43B). Consistent with the enhanced invasive capacity, in C3G knock-down MEFs, MMP2 and MMP9 activities increased as compared to wt cells, while they decreased in p38α-/- MEFs. The expression of the DNRap-1 significantly reduced MMP2/9 activities in wt MEFs, which is in agreement with the lower invasion observed in these cells. However, the expression of the DNRap-1 in p38α-/- cells increased MMP9 activity.

Therefore, all these results indicate that MMP2/9 activities are up-regulated by p38α, mainly that of MMP9. In contrast, C3G through down-regulation of p38α activity would decrease MMP2/9 activities, which correlates with its effect on cell invasion in wt MEFs. Curiously, C3G, in the absence of p38α, would be a positive regulator of MMP2 activity. In contrast, Rap-1 would be a negative regulator of MMP9 in p38α-/- MEFs. Thus, although the role of Rap-1 in the control of MMP2/9 activities is more complex, it is different from that of C3G, which indicate that Rap-1 is not the mediator of C3G actions on cell invasion.

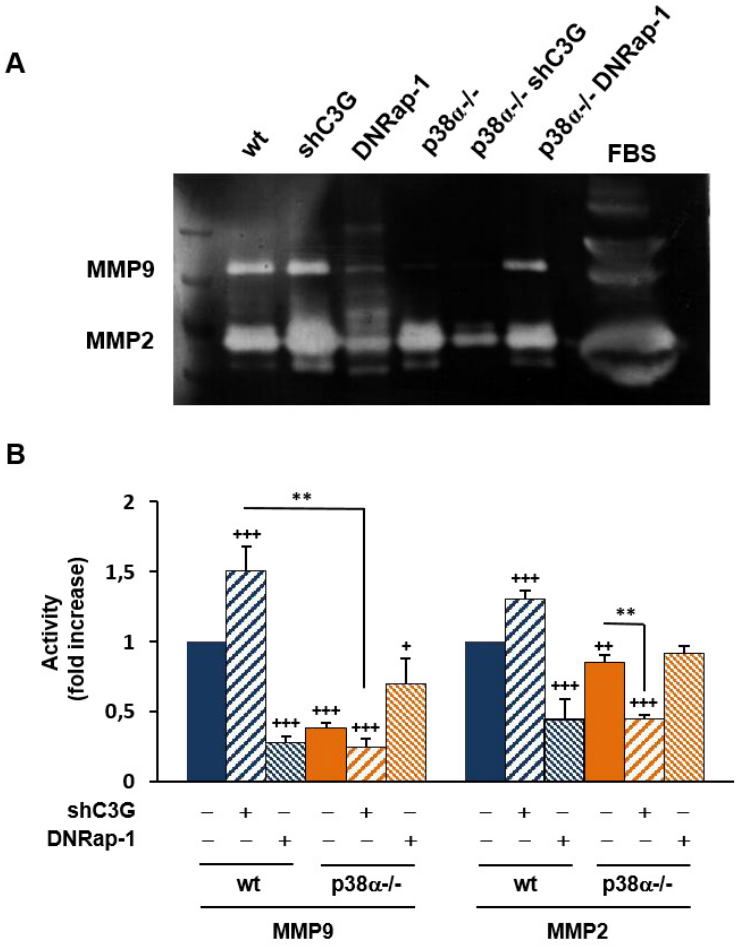

Figure 55: C3G knock-down increases MMP2/9 activities through a mechanism dependent on p38α MAPK. MEFs (wt and p38α-/-, with (shC3G) or without C3G knock-down, or expressing a dominant negative Rap-1 mutant (DNRap-1)) were maintained in absence of serum for 24h. Then, the conditioned media from the cells was used to perform zymographic analyses of MMP2/9 activities. FBS was used as a positive control. A) Representative zymogram. B) Histograms show the mean values ± S.E.M. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value (n=4). +++ $p<0.001$, ++ $p<0.01$, +$p<0.05$, versus wt; **$p<0.01$, compared as indicated.

More studies will be necessary to fully characterize the precise mechanism by which C3G/p38α cascade is regulating cell migration and invasion.

DISCUSSION

1. p38α MAPK MEDIATES CELL SURVIVAL IN RESPONSE TO OXIDATIVE STRESS VIA INDUCTION OF ANTIOXIDANT GENES IN MEFs

It is well established that ROS play important biological roles in cell homeostasis, but several studies have also reported that high intracellular ROS levels are usually associated with apoptosis (Dolado *et al.* 2007; Wang and Yi 2008).

Concerning the function of p38α in response to oxidative stress, there are data in favour of both, a pro-survival and a pro-apoptotic function that might depend on the stimuli, dose, cell type and other factors. Sustained activation of p38α by ROS has been implicated in apoptosis induction (Dolado *et al.* 2007; Tobiume *et al.* 2001; Wada and Penninger 2004). According to this, it has been demonstrated that oncogenic H-Ras-induced ROS activate p38α, leading to apoptosis, which contributes to inhibition of tumour initiation (Dolado *et al.* 2007). In contrast, we have recently found a pro-survival effect of p38α in response to H_2O_2 in MEFs (Gutierrez-Uzquiza *et al.* 2010), which agrees with some data from other groups (Cai *et al.* 2011; Kurata 2000; Zhang *et al.* 2005). As the precise mechanism by which p38α MAPK protected cells from oxidative stress was poorly understood, we have further evaluated it.

Here, we reveal a pro-survival function of p38α, which is dependent on the regulation of the antioxidant response. In addition, we have found that although this response is associated with reduced Akt activity and enhanced mTOR/p70S6K signaling, these cascades are not immediately responsible for the p38α-mediated survival effect, but instead, their modulation is a consequence of the ROS content in the cells.

In agreement with the proposed role for p38α as a positive regulator of antioxidant enzymes expression, it was previously reported that p38 MAPK increased the basal expression and activation of SOD-2 (Qadri *et al.* 2004) and up-regulated catalase (Aggeli *et al.* 2006; Sen *et al.* 2005) and heme oxygenase-1 (Aggeli *et al.* 2006) mRNAs in response to H_2O_2. We have now characterized the precise role of p38α in the regulation of the expression and activity of different antioxidant enzymes, as well as the mechanisms involved. Our data reveal a novel

function for p38α controlling (i) H_2O_2-induced SOD-2 expression through direct regulation of transcription via ATF-2 activation and (ii) basal and H_2O_2-induced catalase expression through regulation of mRNA expression and/or stability and protein stability. Thus, p38α may negatively regulate ROS accumulation at different levels. In agreement with these results, we have more recently found that HGF acts through p38α MAPK to up-regulate SOD-2 and catalase expression and activity in cardiomyocytes (Arechederra *et al.* 2013). Other reports also indicates that p38 MAPK signaling negatively regulates ROS accumulation in cancer cells, but through the up-regulation of *GPX5* and *TXNDC2* genes, which encode a glutathione peroxidase and a thioredoxin, respectively (Pereira *et al.* 2013). However, they found no evidence for the regulation of SOD-2 and catalase by p38 in their cells. This suggests that p38α signaling can probably regulate ROS accumulation by different mechanisms in different cell types.

It is worth highlighting that we describe, for the first time, a p38α-ATF-2 dependent transcriptional regulation of SOD-2 in response to H_2O_2. Previously, it had been shown that low levels of H_2O_2 induced ATF-2 expression (Kurata 2000) or activation through p38α MAPK (Frippiat *et al.* 2002), leading to growth arrest. A role for JNKs/ATF-2 and p38 MAPK in the regulation of heme oxygenase-1 in response to oxidative stress was also demonstrated (Aggeli *et al.* 2006). However, we now show that the p38α MAPK/ATF-2 cascade also mediates SOD-2 up-regulation and cell survival in response to low levels of oxidative stress.

Regarding the regulation of mTORC1/p70S6K by H_2O_2, there are also conflicting data in the literature. In contrast to our results, demonstrating that mTORC1/p70S6K cascade is not responsible for the p38α-mediated survival effect in response to H_2O_2, there are a number of data in the literature proposing that p38 MAPK activation and/or mTORC1 inhibition are required for H_2O_2-induced cell death in different cell types (Cao *et al.* 2008; Chen *et al.* 2010; Matsuzawa and Ichijo 2008; Tobiume *et al.* 2001). For example, some data indicate that mTORC1 can be inhibited by H_2O_2 treatment through different mechanisms such as up-regulation of REDD1 (Jin *et al.* 2009a) or inhibition of PDK1 and Akt, accompanied by AMPK activation (Chen *et al.* 2010). In addition, AMPK through p38-dependent and -independent mechanisms can decrease mTORC1 activation by H_2O_2 (Cao *et al.* 2008), mediating cell death (Cao *et al.* 2008; Chen *et al.* 2010).

However, our results indicate that p38α do not act as a negative regulator of mTORC1/p70S6K. This would be in agreement with previous data demonstrating a pro-survival role for p38α and a p38α-dependent activation of mTORC1/p70S6K signaling in response to different types of stresses (Cully *et al.* 2010; Schewe and Aguirre-Ghiso 2008). Hence, consistent with Cully´s results (Cully *et al.* 2010), we also found that in cells treated with H_2O_2 at a low dose, mTORC1 activation is dependent on p38α. We additionally found that p38α regulates mTORC1/p70S6K in an indirect way, which is dependent on ROS accumulation. So, in cells lacking p38α, a high accumulation of ROS is produced, which leads to cell death and inactivation of the mTORC1/p70S6K pathway. However, in the presence of either catalase or N-acetyl-cysteine, which protects from a high ROS accumulation, mTORC1/p70S6K can be activated in p38α deficient cells treated with H_2O_2. Moreover, although we did not measure this process, autophagic cell death could contribute to the loss of cells observed in p38α-deficient cells treated with H_2O_2, since autophagy is inhibited by mTOR (Noda and Ohsumi 1998;Ravikumar *et al.* 2004). Nevertheless, the enhanced mTORC1/p70S6K signaling due to p38 activation appears to favour cell size maintenance during stress (data not shown), a function recently identified in D. melanogaster cells (Cully *et al.* 2010).

Based on our results, it is unclear whether Akt has any potential role in the response to H_2O_2 mediated by p38α in MEFs. The expression of SOD and catalase is higher in wt than in p38α-/- cells under basal conditions, when Akt activation is quite similar in both cell lines. Upon H_2O_2 treatment, Akt activation is higher in p38α-/- cells and antioxidant enzymes are down-regulated, however, the Akt inhibitor does not affect survival. Hence, these findings do not support that Akt antagonizes the anti-oxidant response in these cells, as found in other systems (Nogueira *et al.* 2008).

We conclude that p38α has a pro-survival function due to its ability to up-regulate antioxidant genes expression, preventing from a high accumulation of ROS upon exposure to low or moderate doses of H_2O_2 (Fig. 56). In this way, in the presence of p38α, cell damage can be overcome, allowing cell survival and mTORC1/p70S6K pathway activation. In contrast, in the absence of p38α, the antioxidant defense is not properly activated, leading to ROS accumulation and high cell damage. As a consequence, the mTORC1/p70S6K pathway is inactivated,

which might avoid protein synthesis and cell growth of damaged cells. In agreement with other studies (Jin *et al.* 2009b), we also found that mTORC1/p70S6K activation is dependent on p38α activation in an oxidative microenvironment. We believe this could be critical for other cellular responses such as autophagy that can also control cell size and to maintain cellular homeostasis and function.

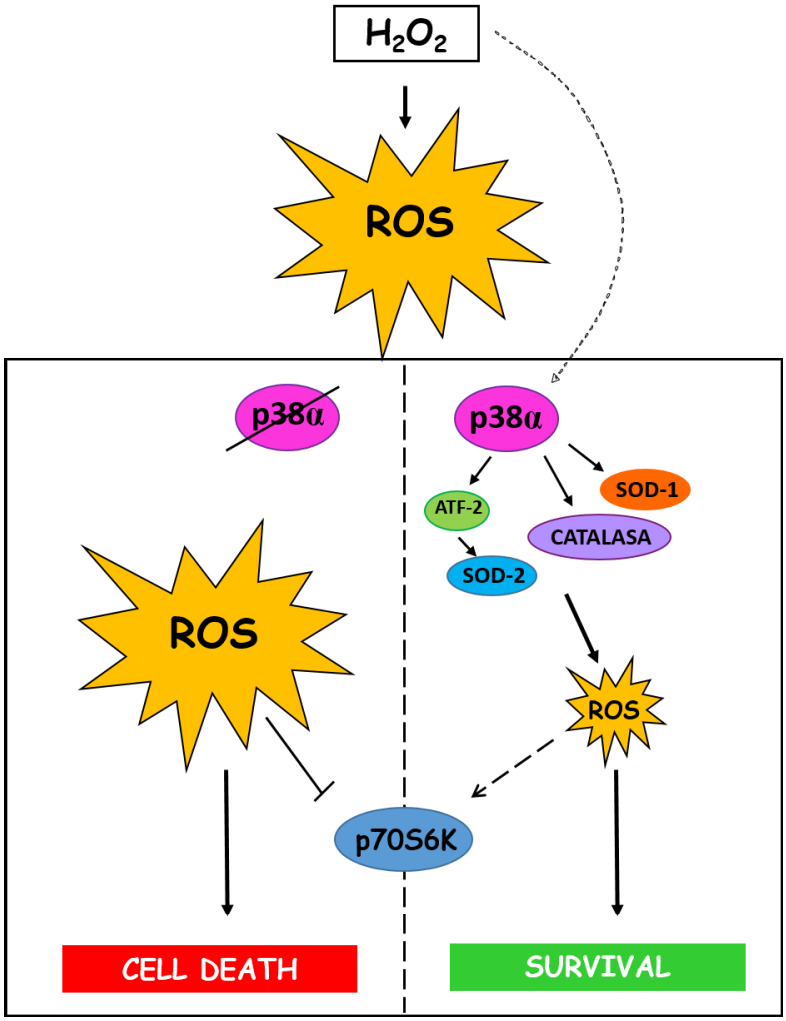

Figure 56: Proposed model showing the effect of p38α MAPK in response to oxidative stress. The up-regulation of antioxidant enzymes, SOD-1, SOD-2 and catalase, by p38α MAPK in response to H_2O_2 through different mechanisms, leads to the removal of ROS. As a consequence, ROS levels decrease, which allows cell survival and mTORC1/p70S6K activation.

2. p38α MAPK DOWN-REGULATES FIBULIN 3 EXPRESSION THROUGH HYPERMETHYLATION OF GENE REGULATORY SEQUENCES PROMOTING MIGRATION AND INVASION

p38α can play a dual role in cancer acting either as a tumour suppressor or promoter depending on the type of cancer and the tumour stage (Wagner and Nebreda 2009). In some tumours, p38α inhibits tumour initiation (Dolado *et al.* 2007), but at later stages, it can promote survival (Aguirre-Ghiso 2007; Schewe and Aguirre-Ghiso 2008), migration and invasion leading to metastasis (del Barco Barrantes and Nebreda 2012; Wagner and Nebreda 2009). Moreover, p38α also promotes migration in non-tumour cells (Rousseau *et al.* 2006), which might be relevant for different physio-pathological process such the migration processes occurring during embryonic development or tissue regeneration.

The function of p38γ and p38δ in cancer has not been well characterized, although it has been recently shown that they can play a role. Using MEFs lacking p38δ or p38γ, it was demonstrated that both isoforms inhibit cell migration and MMP2 secretion. In addition, in K-Ras-transformed MEFs, p38γ suppresses tumour growth *in vitro* and *in vivo* (Cerezo-Guisado *et al.* 2011). In contrast, recent data also suggest that p38γ and p38δ might promote tumour development. p38δ promotes the malignant phenotype of squamous cell carcinoma (Junttila *et al.* 2007) and p38γ is involved in Ras-increased invasion of breast cancer cells (Qi *et al.* 2006).

Our data uncover Fibulin 3 as a new target of p38 MAPK, which participates in the regulation of migration and invasion in MEFs. p38α, p38γ and p38δ regulate fibulin 3 expression. However, the effect of p38α is more significant, so, we have characterized it. We described for the first time that p38α down-regulates fibulin 3 expression through hyper-methylation of fibulin 3 gene regulatory sequences. Moreover, p38α might do so through modulation of DNMT3A protein levels, as they are highly decreased when p38α is not present. This might be a consequence of a p38α-mediated post-transcriptional regulation of DNMT3A. As previously mentioned, it is known that DNMT3B mRNA, highly homologous to DNMT3A mRNA, is stabilized by the binding of HuR protein to its 3´UTR (Lopez de Silanes *et al.* 2009). Therefore, it can be hypothesized that

DNMT3A mRNA could be regulated in a similar way. Moreover, it is likely that p38α might stabilize it through HuR phosphorylation, as it happens with p21 mRNA (Lafarga *et al.* 2009). In that case, p38α MAPK phosphorylates HuR, leading to cytoplasmic accumulation of HuR and enhancement of its binding to the 3´UTR of the mRNA. In agreement with this hypothesis, p38 MAPK mediated cytoplasmic accumulation of HuR stabilizes survival motor neuron mRNA (Farooq *et al.* 2009). Similarly, but involving the participation of additional proteins, p38 MAPK via MK2 regulates the stability of other mRNAs such as TNF mRNA through regulation of HuR and tristetraprolin (TTP) (Tiedje *et al.* 2012). MK2 phosphorylates 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 the hypermethylation of its regulatory sequences in cancer (Kim *et al.* 2012a; Tong *et al.* 2011). This down-regulation of fibulin 3 is associated with poor prognosis in some tumours, such as non-small cell lung cancinoma (Kim *et al.* 2012a; Kim *et al.* 2014). However, the mechanisms controlling fibulin 3 gene hypermethylation remain unknown. Therefore, the finding of p38α MAPK as a novel regulator of this process opens new possibilities to fully characterize how fibulin 3 expression is controlled under physiological conditions and in cancer.

Our findings also show that Fibulin 3 inhibits cell migration and invasion in MEFs. Data from the literature describe opposite effects of Fibulin 3 in the control of these processes in cancer models, which might depend on the type of tumour and/or its context (Obaya *et al.* 2012). For example, in glioma and pancreatic adenocarcinoma, Fibulin 3 is overexpressed, promoting migration and invasion (Camaj *et al.* 2009; Hu *et al.* 2009). In contrast, Fibulin 3 is a negative regulator of invasiveness in non-small cell lung cancer (Kim *et al.* 2012a) or in nasopharyngeal carcinomas (Hwang *et al.* 2010).

It is interesting the finding of Fibulin 3 as a target of p38α in MEFs and its role inhibiting cell migration and invasion. As they are embryonic mesenchymal cells, it is likely that p38α-mediated regulation of Fibulin 3 might play a role during development. Moreover, it would be of great interest to know whether

this p38α/Fibulin 3 cascade might play a similar role in cancer cells with mesenchymal characteristics, acquired after an EMT process.

During cell migration and invasion, MMPs are relevant for extracellular matrix degradation (Kessenbrock *et al.* 2011). In agreement with data from the literature showing that p38 MAPK is a positive regulator of MMPs (Hou *et al.* 2009; Kessenbrock *et al.* 2011; Loesch *et al.* 2010; Ren *et al.* 2013; Xu *et al.* 2006), we have found that MMP2/9 activities were lower in p38α-/- cells than in wt MEFs. Furthermore, the enhanced invasion induced by fibulin 3 knock-down in p38α-/-cells correlates with an increase in MMP2/9 activities. However, in the presence of p38α, MMP2/9 activities do not explain the higher invasiveness. The fact that inhibition of MMPs with a broad spectrum MMP inhibitor (marimastat) blocks invasion, suggests that other MMPs might be also involved, although other proteases might also play a role.

Curiously, Fibulin 3 also regulates p38α and/or p38β activity. Fibulin 3 down-regulates p38α activation and most likely that of p38β, which would limit migration and invasion. So, fibulin 3 knock-down enhances p38α activation in wt cells and potentially, p38β in p38α-/- MEFs and this activation of p38α/β is required for migration and invasion as demonstrated by the effects of SB203580 (10 μM) abolishing these processes.

Finally, fibulin 3 knock-down not only increases the migratory and invasive capacity of MEFs, but it also contributes to deregulation of contact inhibition and foci formation in anchorage-dependent growth assays. However, results from soft agar (data not shown) and xenograft assays showed that these cells are not tumourigenic. This suggests that fibulin 3 knock-down is not sufficient to induce cell transformation. However, fibulin 3 might collaborate with other genes to induce cell transformation as it happens in lung carcinoma (Kim *et al.* 2012a;Kim *et al.* 2014).

In summary (Fig. 57), working with MEFs, we have described for the first time that p38α down-regulates fibulin 3 expression through hypermethylation of regulatory sequences of the gene. p38α might do so through the up-regulation of DNMT3A. Moreover, Fibulin 3 acts as a negative regulator of cell migration and invasion, through mechanisms involving inhibition of MMPs and p38α/β MAPKs.

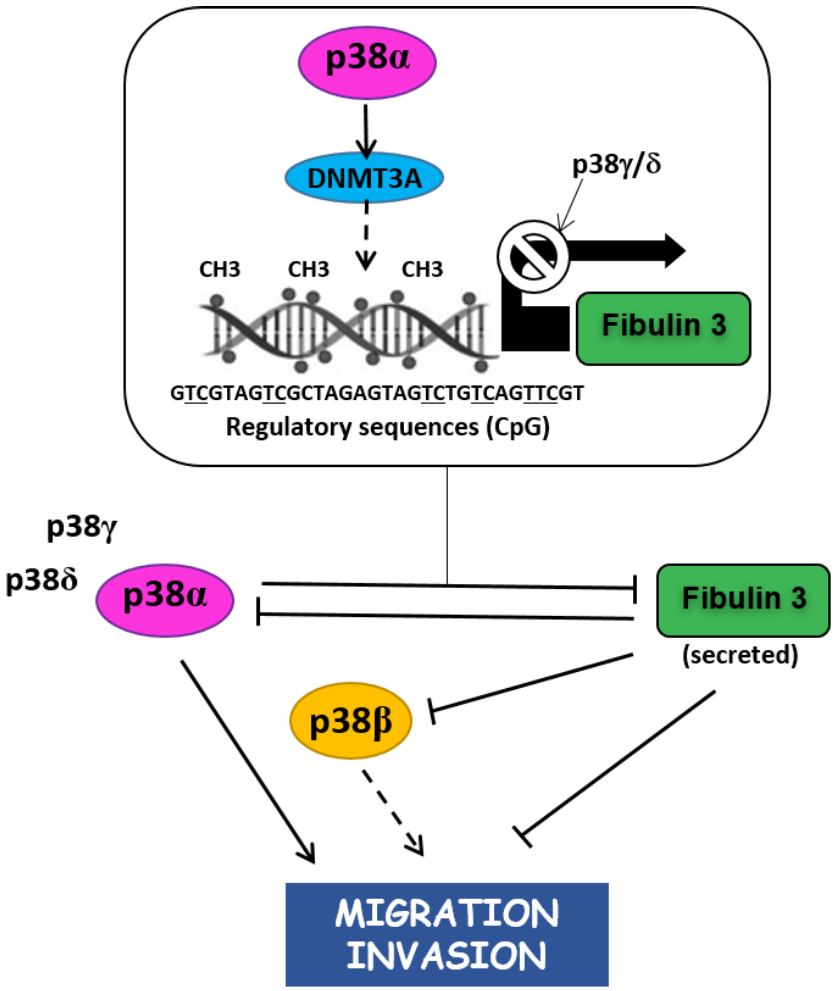

Figure 57: p38 MAPK down-regulates fibulin 3 expression regulating cell migration and invasion in MEFs. Model showing that p38α, γ and δ decrease fibulin 3 transcription, leading to low levels of secreted fibulin 3. p38α-mediated DNMT3A up-regulation might be responsible for hypermethylation of regulatory sequences of fibulin 3 gene and its silencing. Fibulin 3 negatively regulates migration and invasion through p38α/β inhibition.

3. C3G INHIBITS CELL MIGRATION AND INVASION THROUGH DOWN-REGULATION OF p38α MAPK ACTIVITY

Our group has previously described, using different cell models deficient in p38α and/or with C3G knock-down, a new functional relationship between C3G and p38α MAPK in the regulation of apoptosis, where C3G down-regulates p38α MAPK activity (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009). However, depending on the cell type, Rap-1 is a mediator of C3G (Maia *et al.* 2009) or plays an opposite role (Gutiérrez-Uzquiza et al., 2010). Moreover, both proteins participate in the same signaling pathway regulating cell adhesion in CML (Maia *et al.* 2013). In the present work, we have explored whether C3G and p38α could also act in a common pathway regulating cell migration and invasion.

We have found, in agreement with previous data from the literature, that p38α MAPK plays a role in different aspects of cell migration and invasion (del Barco Barrantes and Nebreda 2012; Rousseau *et al.* 2006; Wagner and Nebreda 2009). However, although it is clear that C3G plays a relevant role in cell migration, its precise function has not been yet well characterized. Thus, whereas C3G deficiency in MEFs enhances migration (Ohba *et al.* 2001), its overexpression results in opposite outcomes in different cell types: increased migration in glomerulonephritis (Rufanova *et al.* 2009) and decreased migration in highly invasive breast carcinoma cells (Dayma and Radha 2011).

The present work shows that C3G inhibits cell migration and invasion in MEFs, so that C3G silencing promotes these processes. This agrees with the increased migration observed in C3G-/- MEFs (Ohba *et al.* 2001) and with the reduced migration of highly invasive breast carcinoma cells upon C3G overexpression (Dayma and Radha 2011). However, there are other studies showing an opposite role for C3G in the regulation of cell migration. C3G is required for a correct migration of cortical (Voss *et al.* 2008) and sympathetic preganglionic neurons (Voss *et al.* 2008;Yip *et al.* 2012). Similarly, C3G-Rap1 activation also promotes migration in MCF-7 cells in response to IGF-1 (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.

Our data show that C3G controls migration and invasion through the regulation of p38α MAPK activity. Thus, C3G knock-down was only capable to enhance migration/invasion in cells expressing p38α. Therefore, the previously identified C3G/p38α cascade not only regulates cell death (Gutierrez-Uzquiza *et al.* 2010; Maia *et al.* 2009), but also cell migration and invasion.

On the other hand, we have found that C3G and Rap-1 exert opposite effects on cell migration/invasion in MEFs. Thus, contrary to C3G knock-down, the expression of a Rap-1 dominant negative impaired cell migration in wt MEFs, while a slightly faster wound closure was observed in p38α-/- cells. Therefore, Rap-1 would not be the mediator of C3G actions on these processes. This is in agreement with our previous results on cell death showing that C3G acting through p38α, but through a Rap-1 independent mechanism regulated cell death in MEFs (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).

There are different potential reasons that can explain why Rap-1 does not always mediate the effect of C3G. On one side, C3G effects are not always dependent on its GEF activity (Guerrero *et al.* 1998; Guerrero *et al.* 2004; Gutierrez-Uzquiza *et al.* 2010; Martin-Encabo *et al.* 2007; Shivakrupa *et al.* 2003), but on protein-protein interactions. In addition, there are several Rap-1 GEFs, different from C3G, able to activate Rap-1 (Hattori and Minato 2003; Pannekoek *et al.* 2009; Schultess *et al.* 2005; Stork and Dillon 2005). Hence, depending on the cell type and the stimulus, specific GEFs are activated leading to Rap-1 activation. This might explain the different roles played by these two proteins in some contexts.

Our findings suggest that Rap-1 in wt MEFs promotes cell migration and invasion through p38α activation. Although these results should be further confirmed by additional experiments, Rap-1 is known to activate p38α in MEFs in response to different stresses (Gutierrez-Uzquiza *et al.* 2010), so it is likely that it could do so in the context of migration. There are data in the literature supporting our hypothesis. On one hand, Rap-1 contributes to cell migration and invasion in a number of cells. For example, Rap-1 regulates the migration, invasiveness and *in vivo* dissemination of B-cell lymphomas (Lin *et al.* 2010), invasion of renal carcinoma cells (Kim *et al.* 2012b), migration of breast cancer

cells (McSherry *et al.* 2011) and promotes cell movement of MCF-7 cells in response to IGF-1 (Guvakova *et al.* 2014). According to this, optimal cell migration has been associated with cycles of Rap-1 activation (Takahashi *et al.* 2013). On the other hand, although Rap-1 can regulate p38 MAPK, there are contradictory results about it. While Rap-1 has an inhibitory effect on p38 MAPK activation upon IL-1 stimulation (Palsson *et al.* 2000; McDermott and O'Neill 2002), it is a positive regulator of p38α activity in response to oxidative and osmotic stress in MEFs (Gutierrez-Uzquiza *et al.* 2010) or to FGF-2 in endothelial cells (Yan *et al.* 2008). However, it acts in parallel to p38 MAPK to induce chemotaxis in monocytes in response to LPS (lipopolysaccharide) (Yi *et al.* 2012).

Several studies show a direct correlation between MMP2 and/or MMP9 level and/or activity and an increase in cell motility and invasiveness (Kessenbrock *et al.* 2011). We have shown in results section 2 that p38α is a positive regulator of MMP2/9 activities, which agrees with several studies (Hou *et al.* 2009; Kessenbrock *et al.* 2011; Loesch *et al.* 2010; Ren *et al.* 2013; Xu *et al.* 2006). Our results also suggest that C3G acting through p38α regulates MMP2/9 activities, which correlates with its effect on cell migration and invasion in wt MEFs. In contrast, the role of Rap-1 in the control of MMP2/9 activities is different from that of C3G, which indicate that Rap-1 is not the mediator of C3G actions on cell invasion. Moreover, our results suggest that Rap-1 in the presence of p38α increases MMP2/9 activation, which agrees with other data showing that Rap-1 promotes invasion via induction of MMP9 secretion in head and neck squamous cell carcinoma (Mitra *et al.* 2008). In contrast, in p38α-/- MEFs, Rap-1 inhibits MMP9 activation, suggesting that the role of Rap-1 in the regulation of MMP2/9 activities is more complex.

In summary (Fig. 58), results presented here indicate that C3G would be a suppressor of migration and invasion through its inhibitory effects on p38α MAPK activity in a Rap-1-independent pathway.

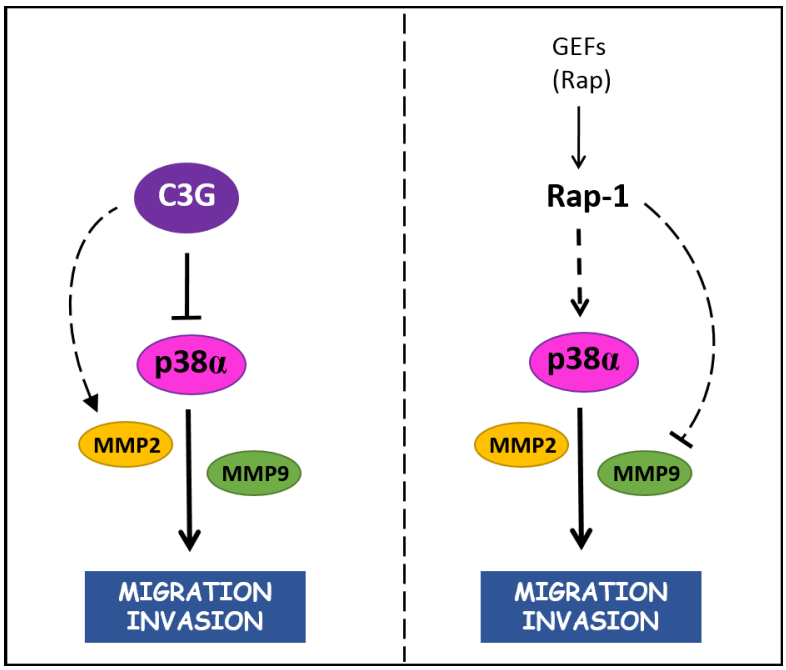

Figure 58: Model showing the regulation of p38α by C3G and Rap-1 in MEFs and their effects on cell migration and invasion. C3G through the negative regulation of p38α MAPK inhibits cell migration, invasion and MMP2/9 activation in a Rap-1 independent mechanism. In contrast, activation of Rap-1 by other GEFs would lead to p38α activation promoting these processes.

GENERAL DISCUSSION

In the present study we have revealed a pro-survival role for p38α MAPK, in response to a level of oxidative stress able to generate damage, but not massive toxicity, which is dependent on the enhancement of the antioxidant response. This result is in agreement with other data from the literature, which proposed a role for p38α as a positive regulator of SOD-2 (Qadri *et al.* 2004), catalase (Sen *et al.* 2005) and heme-oxigenase-1 (Aggeli *et al.* 2006). However, we describe here, for the first time, a p38α-ATF-2 dependent transcriptional regulation of SOD-2 in response to H_2O_2. In addition, although this p38α mediated response is associated with an enhanced mTORC1/p70S6K signaling, this cascade is not immediately responsible for the p38α-mediated survival effect, but instead, their modulation is a consequence of the cellular ROS content.

In the second part of our study, a new target of p38α, Fibulin 3, has been identified. It is described for the first time that p38α down-regulates fibulin 3 expression through hyper-methylation of fibulin 3 gene regulatory sequences in MEFs, which favours migration and invasion. Fibulin 3 acts as a negative regulator of cell migration and invasion, through mechanisms involving a p38α/β negative feedback loop. In some tumours, fibulin 3 is also down-regulated through promoter methylation (Kim *et al.* 2012a; Tong *et al.* 2011), although it can play a dual role in the control of migration/invasion and tumour growth (Obaya *et al.* 2012). Therefore, our findings open new possibilities in order to understand how fibulin 3 expression can be controlled in cancer.

Finally, the present work shows that C3G inhibits cell migration and invasion in MEFs. This agrees with the increased migration observed in C3G-/- MEFs (Ohba *et al.* 2001) and with the reduced migration of breast carcinoma cells upon C3G overexpression (Dayma and Radha 2011). However, we described here that C3G controls migration and invasion through the regulation of p38α MAPK activity. Therefore, the previously identified C3G/p38α cascade not only regulates cell death (Gutierrez-Uzquiza *et al.* 2010; Maia *et al.* 2009), but also cell migration and invasion. Moreover, we have found that C3G and Rap-1 exert opposite

effects on cell migration/invasion in MEFs, suggesting that C3G would be a suppressor of migration and invasion through its inhibitory effects on p38α MAPK activity via a Rap-1-independent pathway.

Therefore, we have identified new mediators and regulators of p38α MAPK actions on cell survival, migration and invasion in MEFs. Moreover, our studies uncover a new function of p38α as a regulator of DNA methylation, which might have a wide impact, as other genes might be controlled in this way (Fig.59). Therefore, all these results open new perspectives for future studies in tumour cells in order to establish the potential relevance of these pathways during tumour progression and to find new targets for novel cancer therapies.

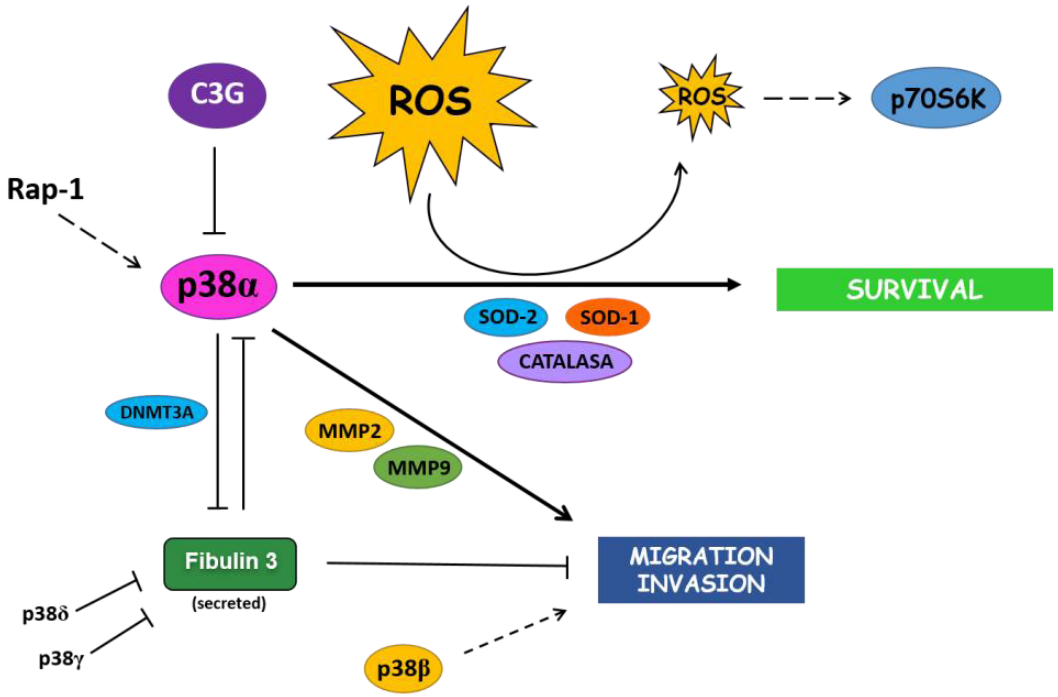

Figure 59: Model showing new mediators and regulators of p38α actions on cell survival, migration and invasión in MEFs: (i) p38α through the up-regulation of antioxidant enzymes (SOD-1, SOD-2 and catalase) promotes cell survival in response to oxidative stress (ii) p38 MAPK down-regulates fibulin 3 expression regulating cell migration and invasion in MEFs and (iii) C3G through the negative regulation of p38α MAPK inhibits cell migration, invasion and MMP2/9 activation in a Rap-1 independent mechanism

CONCLUSIONS

1. p38α MAPK plays a pro-survival role in MEFs exposed to low or moderate doses of H_2O_2 , preventing from a high accumulation of ROS .
2. p38α mediates H_2O_2-induced SOD-2 expression through a direct regulation of transcription mediated by ATF-2.
3. p38α positively regulates H_2O_2-induced catalase expression through regulation of protein stability and mRNA expression and/or stabilization.
4. p38α-dependent antioxidant response allows wt cells to maintain an efficient activation of the mTORC1/p70S6K pathway. However, this activation is not responsible for survival in this context.
5. Fibulin 3 is a new target of p38 MAPKs. p38α, p38γ and p38δ are all capable to down-regulate fibulin 3 expression, although p38α elicits the strongest effect.
6. p38α down-regulates fibulin 3 expression through hypermethylation of regulatory sequences of the gene.
7. p38α up-regulates DNMT3A protein levels.
8. p38α-induced Fibulin 3 down-regulation contributes to p38α pro-migratory/invasive effect.
9. Fibulin 3 knock-down is not sufficient to induce cell transformation, but it might do it in collaboration with other genes.
10. C3G acts through p38α MAPK in a common regulatory pathway to regulate cell migration and invasion of MEFs. Thus, C3G through down-regulation of p38α activity would inhibit these processes.
11. Rap-1 is not a mediator of C3G inhibitory effects on MEFs migration/invasion. In contrast, it plays an opposite role.
12. p38α up-regulates MMP2/9 activities, while C3G down-regulates them through its effect on p38α.

CONCLUDING REMARKS

p38α MAPK plays a pro-survival role in response to a level of oxidative stress able to generate damage, but not massive toxicity. This involves the up-regulation of the antioxidant defenses. Additionally, p38α promotes cell migration and invasion through mechanisms involving fibulin 3 down-regulation through hypermethylation of regulatory sequences. On the other hand, C3G limits cell migration/invasion through the negative regulation of p38α activity.

Moving to a tumour model, these pathways would contribute to the control of tumour progression and they might represent potential targets for novel cancer therapies.

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ABSTRACT

NEW MEDIATORS OF p38ALPHA MAPK ACTIONS ON CELL SURVIVAL AND MIGRATION/INVASION. CROSS-TALK WITH C3G

INTRODUCTION

p38 MAPKs (p38α, p38β, p38δ and p38γ) are activated by several stimuli, leading to the regulation of different cellular functions (Nebreda and Porras 2000;Wagner and Nebreda 2009). p38α MAPK is ubiquitously expressed and is the most abundant isoform (Cuenda and Rousseau 2007). p38 MAPKs play an important role in the coordination of cellular responses to ROS (reactive oxygen species) (Dolado *et al.* 2007;Tobiume *et al.* 2001). Hence, although p38α MAPK can induce apoptosis in response to oxidative stress (Bragado *et al.* 2007;Dolado *et al.* 2007), it has been recently revealed that it can also promote cell survival (Gutierrez-Uzquiza *et al.* 2010;Thornton and Rincon 2009). Moreover, p38 MAPKs regulate cell migration, invasion and metastasis at different levels (del Barco Barrantes and Nebreda 2012).

C3G is a Crk-binding protein with guanine nucleotide exchange (GEF) activity against two proteins of the Ras family: Rap1 and R-Ras. It also regulates different cellular functions such as apoptosis, adhesion and migration (Radha *et al.* 2011) . Recently, our team in collaboration with Dr. Guerrero´s group has identified a new functional interaction between C3G and p38α MAPK involved in the regulation of cell death (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009) and cell adhesion (Maia *et al.* 2013).

Fibulins family is a newly recognized family of extracellular matrix proteins with at least seven members (de Vega *et al*. 2009). Fibulins expression can be deregulated in some human cancers, which correlates with tumour progression (Obaya et al. 2012). In particular, Fibulin 3 is known to regulate cell migration and invasion, playing both (i) antitumour, in lung cancer (Kim *et al.* 2012a) or nasopharyngeal carcinomas (Hwang *et al.* 2010), and (ii) oncogenic activities, in glioma (Hu *et al.* 2009) or pancreatic adenocarcinomas (Camaj *et al.* 2009).

OBJECTIVES/AIMS

1. To characterize the pro-survival function of p38α MAPK in response to oxidative stress and the potential implication of the Akt/mTORC1/p70S6K pathway.

2. To identify the mechanisms involved in the regulation of fibulin 3 expression by p38α MAPK and a potential role for fibulin 3 as a mediator of p38α effects on cell migration and invasion.

3. To investigate whether C3G and p38α MAPK act in a common pathway to regulate cell migration and invasion.

RESULTS

1. p38α MAPK mediates cell survival in response to oxidative stress via induction of antioxidant genes in MEFs

Loss of p38α sensitizes cells to H_2O_2-induced cell death and impaired mTORC1/p70S6K activation

Our group had previously reported that wt MEFs exhibited a higher resistance to moderate oxidative stress than p38α-/- cells (Gutierrez-Uzquiza *et al.* 2010). We validated this result, demonstrating that p38α protects from low levels of H_2O_2-induced cell death.

In addition, we found that p38α MAPK positively regulated mTORC1/p70S6K activation in response to H_2O_2 through an AKt independent mechanism. However, this pathway did not mediate p38α-dependent survival.

p38α increases basal and H_2O_2-induced expression of antioxidant enzymes: SOD-1, SOD-2 and Catalase

Based on these results, we decided to explore alternative mechanisms by which p38α might protect cells from ROS damage, allowing cell survival in response to oxidative stress. Our data indicated that upon treatment with H_2O_2, p38α-/- cells were unable to efficiently scavange ROS, which led to an accumulation of ROS.

The presence of p38α increased basal and H_2O_2-induced expression of the antioxidant enzymes: SOD-1, SOD-2 and catalase through different mechanisms, which protected from ROS accumulation and prevented cell death. p38α was found to regulate (i) H_2O_2-induced SOD-2 expression through a direct regulation of transcription mediated by ATF-2 (activating transcription factor-2) and (ii) H_2O_2-induced catalase expression through regulation of protein stability and mRNA expression and/or stabilization. As a consequence, SOD and catalase activities were higher in wt MEFs.

2. p38α MAPK down-regulates fibulin 3 expression through hypermethylation of gene regulatory sequences, promoting migration and invasion

p38 MAPK down-regulates fibulin 3 expression

Based on our data derived from Affymetrix microarrays showing that fibulin 3 was down-regulated by p38α, we have characterized the role played by p38α in the regulation of Fibulin 3. We have also analyzed if other p38 MAPKs regulated Fibulin 3 expression. Hence, fibulin 3 mRNA levels have been quantified by RT-qPRC in MEFs wt, p38α-/-, p38γ-/-, p38δ-/- and p38γ/δ-/-. In addition, the effect of the selective p38α/β inhibitor, SB203580, has been also evaluated to confirm p38α effect on Fibulin 3 expression.

Our data indicate that p38α, p38γ and p38δ are all capable to down-regulate fibulin 3 expression, although p38α elicits the strongest effect.

p38α MAPK induces the hyper-methylation of regulatory sequences of fibulin 3 gene

Data from the literature indicated that fibulin 3 expression was mainly regulated at the transcriptional level (Blackburn *et al.* 2003;Kim *et al.* 2012a). Thus, we next explored whether p38α might down-regulate Fibulin 3 expression through promoter methylation. Treatment with 5-aza-2´-deoxicytidine (5A2dC) and pyrosequencing analyses strongly indicate that p38α represses fibulin 3 expression through hypermethylation of 5'UTR regulatory sequences. Moreover, this might be mediated by the DNA methylase, DNMT3A, which is up-regulated by p38α MAPK.

Fibulin 3 knock-down promotes migration and invasion of MEFs

To study the function of the p38α/Fibulin 3 circuit in the regulation of cell migration and invasion, we permanently knocked-down Fibulin 3 in wt and p38α-/- MEFs.

According to other data from the literature, our results support a role for p38α as a mediator of cell migration and invasion. Moreover, we have found that Fibulin 3 inhibits migration and invasion in MEFs through mechanisms dependent on p38α/β inhibition. Hence, the p38α pro-migratory/invasive effect might be, at least in part, mediated by fibulin 3 down-regulation in MEFs.

Our data also suggest that MMPs might be involved in the regulation of cell invasion by Fibulin 3.

3. C3G inhibits cell migration and invasion through down-regulation of p38α MAPK

We have previously described that C3G through down-regulation of p38α MAPK activity was able to control apoptosis. Here, we show that the C3G/p38α pathway also modulates cell migration and invasion. Our data

are in favour of a model where C3G would inhibit cell migration and invasion through the negative regulation of p38α activation by a Rap-1 independent mechanism.

We have also seen by zymography that C3G decreases MMP2/9 activities through down-regulation of p38α activity. In contrast, in the absence of p38α, C3G would be a positive regulator of MMP2 activity and Rap-1 would be a negative regulator of MMP9.

DISCUSSION

In the present study we have revealed a pro-survival function of p38α, which is dependent on the enhancement of the antioxidant response. This result is in agreement with other data from the literature, which proposed a role for p38α as a positive regulator of SOD-2 (Qadri *et al.* 2004), catalase (Sen *et al.* 2005) and heme-oxigenase-1 (Aggeli *et al.* 2006). However, we describe here, for the first time, a p38α-ATF-2 dependent transcriptional regulation of SOD-2 in response to H_2O_2. In addition, we have found that although this response is associated with a reduced Akt activity and enhanced mTORC1/p70S6K signaling, these cascades are not immediately responsible for the p38α-mediated survival effect, but instead, their modulation is a consequence of the ROS content in the cells.

The second part of our study describes for the first time that p38α down-regulates fibulin 3 expression through hyper-methylation of fibulin 3 gene regulatory sequences in MEFs. This would favour migration and invasion. Therefore, Fibulin 3 is a target of p38α in MEFs that acts as a negative regulator of cell migration and invasion, through mechanisms involving a p38α/β negative feedback loop. In several tumours, fibulin 3 is also down-regulated through promoter methylation (Kim *et al.* 2012a;Tong *et al.* 2011). However, it can play a dual role in the control of migration and invasion of cancer cells (Obaya *et al.* 2012). Thus, depending on the type of tumour, Fibulin 3 can either promote or inhibit migration, invasion and/or tumour growth.

Finally, the present work shows that C3G inhibits cell migration and invasion in MEFs. This agrees with the increased migration observed in C3G-/- MEFs (Ohba *et al.* 2001) and with the reduced migration of breast carcinoma cells upon C3G overexpression (Dayma and Radha 2011). However, we described here, that C3G controls migration and invasion through the regulation of p38α MAPK activity. Therefore, the previously identified C3G/p38α cascade not only regulates cell death (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009), but also cell migration and invasion. Moreover, we have found that C3G and Rap-1 exert opposite effects on cell migration/invasion in MEFs. Rap-1 promotes cell migration and invasion in wt MEFs, according with other publications (Kim *et al.* 2012b;Lin *et al.* 2010; McSherry *et al.* 2011). Additionally, we have found that it does so through p38α activation.

MAIN CONCLUSION

p38α MAPK plays a pro-survival role in response to a level of oxidative stress able to generate damage, but not massive toxicity. This involves the up-regulation of the antioxidant defenses. Additionally, p38α promotes cell migration and invasion through mechanisms involving fibulin 3 down-regulation through hypermethylation of regulatory sequences. On the other hand, C3G limits cell migration/invasion through the negative regulation of p38α activity.

Moving to a tumour model, these pathways would contribute to the control of tumour progression and they might represent potential targets for novel cancer therapies.

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RESUMEN

NUEVOS MEDIADORES DE LAS ACCIONES DE p38ALFA EN LA SUPERVIVENCIA Y MIGRACIÓN/INVASIÓN CELULAR. INTERACCIÓN CON C3G.

INTRODUCCIÓN

Las p38 MAPKs (p38α, p38β, p38δ y p38γ) son activadas en respuesta a una gran variedad de estímulos, regulando numerosas funciones celulares (Nebreda and Porras 2000;Wagner and Nebreda 2009). p38α es la isoforma más ubicua y de mayor expresión (Cuenda and Rousseau 2007). Las p38 MAPKs desempeñan un papel importante en la coordinación de la respuesta frente a las especies reactivas de oxígeno (ROS) (Dolado *et al.* 2007;Tobiume *et al.* 2001). A pesar de que p38α MAPK media apoptosis en respuesta al estrés oxidativo (Bragado *et al.* 2007;Dolado *et al.* 2007), recientemente se ha descrito que también puede promover la supervivencia celular (Gutierrez-Uzquiza *et al.* 2010;Thornton and Rincon 2009). Además, las p38 MAPKs regulan los procesos de migración e invasión celular, así como la metástasis (del Barco Barrantes and Nebreda 2012).

C3G es una proteína activadora del intercambio de nucleótidos de guanina (GEF) de dos miembros de la familia de proteínas Ras: Rap-1 y R-Ras. Regula diferentes funciones celulares entre las que se encuentran la apoptosis, la adhesión y la migración celular (Radha *et al.* 2011). Recientemente, nuestro grupo, en colaboración con el de la Dra. Guerrero, ha identificado una nueva interacción funcional entre C3G y p38α MAPK en la regulación de la apoptosis (Gutierrez-Uzquiza *et al.* 2010;Maia *et al.* 2009) y la adhesión celular (Maia *et al.* 2013).

Las fibulinas son una familia de proteínas de la matrix extracelular recientemente descubiertas (de Vega *et al.* 2009). Datos en la bibliografía indican que la expresión de las fibulinas se encuentra alterada en varios tipos de cáncer, relacionándose con la progresión tumoral (Obaya et al. 2012). En concreto, se sabe que Fibulina 3 desempeña un papel relevante en la migración e invasión celular. No obstante, su papel en cáncer es dual y dependiente del tipo de tumour: (i) anti-tumoral en cáncer de pulmón (Kim *et al.* 2012a) o carcinoma

nasofaríngeo (Hwang *et al.* 2010), y (ii) pro-tumoral en glioma (Hu *et al.* 2009) o adenocarcinoma pancreático (Camaj *et al.* 2009).

OBJETIVOS

1. Caracterización del papel de p38α MAPK en la regulación de la supervivencia celular en respuesta al estrés oxidativo y la posible implicación de la ruta Akt/mTORC1/p70S6K.

2. Identificación de los mecanismos implicados en la regulación de la expresión de fibulina 3 por p38α y de su papel como mediador de las acciones de p38α MAPK en la migración e invasión celular.

3. Estudio del papel que juega la ruta C3G/p38α en la regulación de la migración e invasión celular.

RESULTADOS

1. p38α MAPK media supervivencia celular en respuesta al estrés oxidativo a través de la inducción de enzimas antioxidantes en fibroblastos embrionarios de ratón (MEFs).

La ausencia de p38α sensibiliza a las células a la muerte inducida por H_2O_2 e impide la activación de la ruta mTORC1/p70S6K.

Previamente nuestro grupo había demostrado que los fibroblastos que expresaban p38α presentaban una mayor resistencia a la muerte inducida por niveles moderados de estrés oxidativo (Gutierrez-Uzquiza *et al.* 2010). Validamos dichos resultados, demostrando que p38α protege frente a la muerte inducida por H_2O_2.

Además, nuestros resultados demuestran que p38α MAPK regula positivamente la activación de la ruta mTORC1/p70S6K en respuesta a H_2O_2 por

un mecanismo independiente de Akt. Sin embargo, dicha ruta no es la responsable de la mayor supervivencia observada en los fibroblastos p38α+/+.

p38α protege frente a la muerte inducida por H_2O_2 mediante la expresión de enzimas antioxidantes: SOD-1, SOD-2 y catalasa

Quisimos analizar otros posibles mecanismos que pudieran ser responsables del efecto protector de p38α frente al daño causado por el exceso de ROS, y, por lo tanto, de la supervivencia de las células. Nuestros datos indicaban que tras el tratamiento con H_2O_2, las células deficientes en p38α no eran capaces de eliminar eficazmente los ROS, acumulándose en su interior y causando la muerte celular. Por tanto, podía haber un déficit en los sistemas antioxidantes.

Nuestros resultados demuestran que p38α incrementa la expresión de enzimas antioxidantes (SOD-1, SOD-2 y catalasa), tanto a nivel basal como tras el tratamiento con H_2O_2 a través de diversos mecanismos, lo que evita la acumulación de ROS y la muerte celular. Por tanto, p38α induce: (i) la expresión de SOD-2 tras la estimulación con H_2O_2, facilitando la unión del factor de transcripción ATF-2 al promotor y (ii) la expresión de catalasa inducida por el H_2O_2 a través de un incremento en la estabilidad y/o expresión de su RNAm y en la estabilidad de la proteína.

2. p38α MAPK inhibe la expressión de fibulina 3 a través de la hipermetilación de secuencias reguladoras, promoviendo la migración e invasión celular.

p38 MAPK inhibe la expressión de fibulin 3

A partir de los datos obtenidos mediante análisis de *microarrays* que indicaban que p38α regulaba negativamente la expresión de fibulina 3, quisimos caracterizar el mecanismo implicado en la regulación de la expresión de fibuina 3 por p38α y si otras isoformas de la subfamilia de las p38 MAPKs podrían también regularla. Por ello, validamos y cuantificamos por RT-PCR cuantitativa los niveles

del RNAm de fibulina 3 en fibroblastos wt y deficientes en las isoformas p38α, p38γ, p38δ y p38γ/δ. Además, evaluamos el efecto del SB203580 (inhibidor de las isoformas α y β). Nuestros datos indican que tanto p38α como p38γ y p38δ regulan negativamente la expresión de fibulina 3, pero p38α presenta un efecto mucho mayor.

p38α MAPK favorece la metilación de secuencias reguladoras en el gen de fibulina 3

Datos de la literatura indicaban que la expresión de fibulina 3 se regulaba principalmente a nivel transcripcional a través de un mecanismo dependiente de la metilación de su promotor (Blackburn *et al.* 2003;Kim *et al.* 2012a). Por ello, analizamos si p38α podría estar inhibiendo a fibulina 3 a través de un incremento en la metilación de secuencias reguladoras del gen. Los resultados obtenidos tras el tratamiento con la 5-aza-2-deoxicitidina y el análisis del grado de metilación de secuencias reguladoras confirman que p38α inhibe la expresión de fibulina 3 a través de la metilación de dichas secuencias reguladoras. Además, dicha metilación se correlaciona con un aumento en los niveles de la metilasa DNMT3A, que es regulada positivamente por p38α.

El silenciamiento de Fibulin 3 promueve la migración e invasión celular en MEFs

Para estudiar el papel de la ruta p38α/Fibulin 3 en la regulación de la migración e invasión celular, se hizo un silenciamiento estable de Fibulina 3 en MEFs wt y deficientes en p38α.

De acuerdo con los datos de la literatura, nuestros resultados demuestran que p38α es un mediador de dichos procesos. Además, la Fibulina 3 inhibe ambos procesos a través de un mecanismo dependiente de la inhibición de p38α/β. Por lo tanto, el efecto positivo de p38α sobre los procesos de migración e invasión, podría estar mediado, en parte, por su inhibición de fibulina 3.

Nuestros datos también sugieren que las MMPs podrían estar desempeñado un papel relevante en la regulación que Fibulina 3 ejerce sobre la invasión celular.

3. C3G regula negativamente la migración e invasión celular a través de la inhibición de la actividad de p38α MAPK

Previamente habíamos descrito que C3G, a través de la regulación negativa de p38α, mediaba procesos de muerte celular/supervivencia (Gutierrez-Uzquiza et al. 2010; Maia et al. 2009). Por ello, quisimos analizar si ambas proteínas participaban también en una ruta común regulando los procesos de migración e invasión celular. Nuestros resultados indican que, de nuevo, C3G estaría inhibiendo la migración e invasión celular a través de la regulación negativa de la actividad de p38α, por un mecanismo independiente de Rap-1.

Analizamos también si C3G modulaba la actividad de las MMPs durante estos procesos. C3G disminuye la actividad de MMP2 y MMP9 a través de la inhibición de p38α. Sin embargo, en ausencia de p38α, C3G parece ser un regulador positivo de la actividad de MMP2, mientras que Rap-1 sería un regulador negativo de MMP9.

DISCUSIÓN

Nuestros resultados demuestran que p38α desempeña una función importante en la regulación de la supervivencia celular en respuesta a niveles moderados de estrés oxidativo, que es dependiente del aumento en la respuesta antioxidante. Estos resultados están de acuerdo con datos de la literatura que proponen a p38α como un regulador positivo de la expresión de SOD-2 (Qadri et al. 2004), catalasa (Sen et al. 2005) y hemo-oxigenasa-1 (Aggeli et al. 2006). Sin embargo, en este trabajo se describe, por primera vez, que p38α, por un mecanismo dependiente de la activación de ATF-2, regula positivamente la transcripción de SOD-2 tras un tratamiento con H_2O_2. Además, aunque en esta

situación de moderado nivel de estrés oxidativo, encontramos disminuida la activación de Akt y aumentada la p70S6K, dichas rutas no son responsables de la mayor supervivencia que presentan las células con p38α. La diferente activación de la ruta mTORC1/p70S6K es consecuencia del contenido intracelular de ROS, de forma que unos elevados niveles de ROS bloquean la activación de dicha ruta.

En la segunda parte de esta Tesis hemos descrito por primera vez que p38α regula negativamente la expresión de fibulina 3 a través del aumento en la metilación de secuencias reguladores del gen de fibulina 3 en MEFs. Además, la menor expresión de fibulina 3 estaría promoviendo los procesos de migración e invasión celular. Por tanto, la Fibulina 3, sería un nuevo mediador de p38α, actuando como un regulador negativo de la migración e invasión celular, a través de un mecanismo que implica la regulación negativa de p38α/β. Ha sido descrito que la expresión de fibulina 3 puede estar desregulada en distintos tipos de tumores (Obaya *et al.* 2012), desempeñando un papel dual. Dependiendo del tipo de tumor, Fibulina 3 favorece o inhibe la migración, invasión y el crecimiento tumoral. Además, de acuerdo con nuestros resultados, en ciertos tumores el silenciamiento de fibulina 3 se correlaciona con un aumento en la metilación de su promotor (Kim *et al.* 2012a;Tong *et al.* 2011).

Por último, nuestros resultados demuestran que C3G inhibe la migración e invasión celular en MEFs. Datos de la literatura coinciden con nuestros resultados, observándose un incremento en la migración en los fibroblastos deficientes en C3G (Ohba et al. 2001) o una disminución en dicho proceso al sobreexpresar C3G en células de cáncer de mama (Dayma and Radha 2011). Sin embargo, nuestros resultados demuestran que C3G controla el proceso de migración a través de la regulación negativa de la actividad de p38α. Por ello, la ruta C3G/p38α no solo actúa modulando la muerte celular como previamente habíamos descrito(Gutierrez-Uzquiza et al. 2010; Maia et al. 2009), sino que también regula la migración e invasión celular en MEFs. Además, en este modelo C3G y Rap-1 presentan efectos opuestos. Rap-1 en presencia de p38α promueve la migración e invasión celular, lo que está de acuerdo con resultados de otros grupos (Kim *et al.* 2012b;Lin *et al.* 2010;McSherry *et al.* 2011).

CONCLUSIÓN FINAL

p38α MAPK protege de la muerte inducida por niveles moderados de estrés oxidativo a través de la regulación positiva de la respuesta antioxidante. Además, p38α favorece la migración e invasión celular por un mecanismo que implica la regulación negativa de fibulina 3, a través de metilación de secuencias reguladoras de su gen. Por último, C3G inhibe la migración e invasión celular a través de la regulación negativa de la actividad de p38α.

En el caso de un modelo tumoral, estas rutas podrían contribuir al control de la progresión tumoral y representarían dianas potenciales para el desarrollo de nuevas terapias anti-tumorales.

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APPENDIX

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p38α Mediates Cell Survival in Response to Oxidative Stress via Induction of Antioxidant Genes

EFFECT ON THE p70S6K PATHWAY[*] [S]

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Background: p38α MAPK is activated by stress stimuli, which can regulate cell death.
Results: In response to H_2O_2, p38α MAPK increases SOD and catalase levels, impairs ROS accumulation, and leads to cell survival.
Conclusion: p38α MAPK signals survival under moderate oxidative stress through up-regulation of antioxidant defenses.
Significance: To know how p38α regulates ROS levels is important for cell homeostasis.

We reveal a novel pro-survival role for mammalian p38α in response to H_2O_2, which involves an up-regulation of antioxidant defenses. The presence of p38α increases basal and H_2O_2-induced expression of the antioxidant enzymes: superoxide-dismutase 1 (SOD-1), SOD-2, and catalase through different mechanisms, which protects from reactive oxygen species (ROS) accumulation and prevents cell death. p38α was found to regulate (i) H_2O_2-induced SOD-2 expression through a direct regulation of transcription mediated by activating transcription factor 2 (ATF-2) and (ii) H_2O_2-induced catalase expression through regulation of protein stability and mRNA expression and/or stabilization. As a consequence, SOD and catalase activities are higher in WT MEFs. We also found that this p38α-dependent antioxidant response allows WT cells to maintain an efficient activation of the mTOR/p70S6K pathway. Accordingly, the loss of p38α leads to ROS accumulation in response to H_2O_2, which causes cell death and inactivation of mTOR/p70S6K signaling. This can be rescued by either p38α re-expression or treatment with the antioxidants, *N*-acetyl cysteine, or exogenously added catalase. Therefore, our results reveal a novel homeostatic role for p38α in response to oxidative stress, where ROS removal is favored by antioxidant enzymes up-regulation, allowing cell survival and mTOR/p70S6K activation.

The intracellular redox state is tightly regulated because it is essential for the control of cell fate. High levels of ROS[5] can lead to molecular damage and cell death, whereas low ROS levels can be essential second messengers (1). Pro-oxidant and antioxidant systems are involved in this regulation, preventing an excessive accumulation of ROS.

Different members of the MAPK family, such as ERKs, JNKs, and p38, can be activated by ROS (1). This activation leads to a great variety of biological responses, including cell death or survival. Hence, although the stress MAP kinases can induce apoptosis in response to oxidative stress (2, 3), differences in the duration and magnitude of the oxidative stress might be directly proportional to the state of activation of these kinases, and this might determine cell death or survival.

p38α MAPK plays an important role in the coordination of cellular stress responses to signals such as ROS. In fact, it is well known that p38α MAPK plays an important role in mediating apoptosis (4) and/or senescence induced by different stimuli, including ROS (5, 6). For example, ROS generated by oncogenic H-Ras induces apoptosis through p38α activation, inhibiting tumor initiation (7). In contrast, low levels of oxidative stress can also induce cell cycle arrest (8) or cell survival (9, 10) through p38. Initially, stress signaling mechanisms are pro-survival systems because they tend to repair damage before committing cells to death or senescence. Interestingly, p38α can mediate survival upon activation with H_2O_2 (10), and p38α and β can have pro-survival roles (11–13) such as during quiescence of dormant tumor cells (14). However, the precise mechanisms by which p38 signaling achieves cell survival are poorly understood.

We have previously demonstrated that Akt activity is negatively regulated by p38α (15), and recent data from Nogueira *et*

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[5] The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; ATF, activating transcription factor; MEF, mouse embryonic fibroblast; DCFH, 2′,7′-dichlorfluorescein-diacetate; NAC, *N*-acetyl cysteine; mTOR, mammalian target of rapamycin.