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

*ROLE IN MIGRATION AND INVASION**

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

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

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

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

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

According to this, in several tumor cells lines p38α mediates migration and invasion through regulation of cell motility, MMP expression, and/or activity (16, 22–23).

The function of p38γ and p38δ in cancer has not been well characterized. It has been recently shown that they can play a tumor suppressor role inhibiting cell migration, MMP2 secretion and tumor growth in MEFs (24). In contrast, p38δ-null mice are more resistant to skin tumor development (25) and colon cancer development is impaired in p38γ/δ-deficient mice (26).

Fibulins are a family of extracellular matrix (ECM) proteins (27–28). They are secreted glycoproteins characterized by the presence of a shared globular domain at the C terminus called "fibulin-like" domain (27–29). This domain is preceded by a series of epidermal growth factor (EGF)-like domains. These proteins play relevant roles in the assembly and stabilization of supramolecular ECM complexes and as a consequence they regulate essential cellular functions such as adhesion, migration, or proliferation, being involved in tissue organogenesis, vasculogenesis, fibrogenesis, and tumorigenesis (27–28, 30). In particular, Fibulin 3 (also referred to as EFEMP-1) is expressed in different tissues and it can play a dual role in cancer (30). In some tumors, such as glioma, it is overexpressed, promoting cell migration and invasion (31–32). Similarly, in pancreatic adenocarcinoma Fibulin 3 is up-regulated, which is associated with metastatic tumor growth (33). In contrast, fibulin 3 was shown to be down-regulated in non-small lung carcinoma, breast cancer, or coloncarcinoma, where it might behave as a tumor suppressor (34–37). In particular, in non-small lung carcinoma it has been recently demonstrated that Fibulin 3 inhibits epithelial to mesenchymal transition (EMT) and self-renewal of lung cancer stem cells (35).

In some of the tumors, fibulin 3 expression is down-regulated as a consequence of promoter methylation (34, 37). In fact, transcriptional regulation of fibulin 3 gene is a relevant mechanism controlling its expression through different regulatory sequences present at the 5′-end (38). However, it remains unknown the mechanism responsible for this epigenetic regulation of Fibulin 3 expression.

DNA methyltransferases (DNMTs) are responsible for DNA methylation (39). There are three enzymatically active mammalian DNMTs, DNMT1, DNMT3A, and DNMT3B. DNMT1-induced DNA methylation is associated with DNA replication (40), while DNMT3A/3B are thought to function as *de novo* DNA methyltransferases, and their levels can be regulated, being of relevance its post-transcriptional regulation (39). In particular, binding of HuR protein to the 3′-UTR of DNMT3B mRNA enhances its stability, increasing its protein levels (41). Microarrays analyses revealed that Fibulin 3 mRNA levels were up-regulated in p38$\alpha^{-/-}$ MEFs.[6] Based on that, together with the above described functions of Fibulin 3 and p38α in the control of migration and invasion, it could be hypothesized that p38α could act through Fibulin 3 to regulate these processes. Therefore, we explored in detail if p38α MAPK and other p38 isoforms were able to regulate Fibulin 3 expression in non-tumor cells (MEFs), the mechanisms involved, and its function. We also determined whether p38α was also able to regulate Fibulin 3 expression in the HCT116 colon carcinoma cell line and its impact on migration, invasion, and tumorigenesis in these cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—Wt and p38$\alpha^{-/-}$ mouse embryonic fibroblasts (MEFs) have been generated in our laboratory (21) and p38$\gamma^{-/-}$, p38$\delta^{-/-}$, and p38 $\delta/\gamma^{-/-}$ MEFs in Dr. Cuenda's laboratory and immortalized by passages. The human colorectal carcinoma HCT116 cell line was obtained from ATCC (CCL-247) and authenticated by microsatellite markers analysis. HCT116 cells with permanent p38α knock-down (different clones) were previously generated using a p38α shRNA inserted in pSuper.retro.puro vector (12) and were maintained with 2 μg/ml puromycine (Sigma-Aldrich P8833). As a control, cells transfected with the empty vector were also generated. MEFs were grown in DMEM medium and HCT116 cells in McCoy's (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) plus antibiotics at 37 °C, 5% CO_2 in a humidified atmosphere. p38α and/or p38β were inhibited with SB203580 (Calbiochem; 559389) at 5 μM (for p38α) or 10 μM (for p38α and β). DNA methylation was inhibited with 5-aza-2′-deoxycytidine (Sigma 3656) at 0.5–1 μM. Transcription was inhibited by treatment with actinomycin D (Sigma A9415) at 5 μg/ml.

RT-qPCR Analysis—After isolation of total RNA using RNeasy Mini Kit (Qiagen 74104), 1–5 μg RNA was reverse transcribed using SuperScript III RT kit (Qiagen, 18080) to generate cDNA. Then, Real Time PCR was performed using SYBR green (Roche) and specific primers: for human fibulin 3: forward 5′-TGGCGGCTTCCGTTGTTATCCA-3′ and reverse: 5′-TGGGGCAGTTCTCGGCACAT3-′; for mouse fibulin 3: forward 5′-GAATGTGATGCCAGCAACC-3′ and reverse 5′-TCACAGTTGAGTCTGTCACTGC-3′; for mouse dnmt3a: forward 5′-CGGCAGAATAGCCAAGTTCA-3′ and reverse 5′-GGGAAGCCAAACACCCTTT C-3′ and to normalize (endogenous control) primers for: human GAPDH: forward 5′-CATCGAAGGTGGAAGAGTGG-3′ and reverse: 5′-CATCAAGAAGGTGGTGAAGC-3′; and mouse GAPDH: forward: 5′-CATCAAGAAGGTGGTGAAGC-3′ and reverse: 5′-CATCGAAGGTGGAAGAGTTGG-3′. Quantification was performed through calculation of RQ ($2^{-\Delta\Delta Ct}$). Ct (threshold cycle) for a gene minus Ct for GAPDH = ΔCt and then, this is referred to wt control values (sample ΔCt-wt ΔCt = ΔΔCt) to calculate RQ value.

Pyrosequencing—Genomic DNA was extracted from 24h serum-deprived MEFs using the alkaline lysis method and modified by sodium bisulfite using BisulFlash DNA Modification Kit (EPIGENTEK, P-1026). The DNA region −28853253/−28853452 was amplified by PCR using the PyroMark PCR kit (Qiagen 978703) using the following primers: forward: 5′-CCTCCTGTGGCTGCTGCTGCAG-3′; reverse (biotinylated): 5′-CACTTTGACATGTCTCTTCTACCTCCA-3′. PCR cycles were as follows: 30 sec at 95 °C, 30 sec at 52 °C, and 30 sec at 72 °C (45 cycles). PCR products were converted into single-stranded DNA. One strand was isolated using streptavidin-Sepharose beads and was used as a template in the pyrosequen-

[6] A. Porras, unpublished results.

cing PCR reaction using two different primers: 5′-GCTGCCC-TCCCCTACGCACTCCTT-3′ for the analysis of the methylation status of five CpG sites and 5′-CCCGCAGGTAGGAGC-CCAAAGC-3′ for the analysis of seven additional CpG sites.

Cell Extracts Preparation and Western Blot Analysis—Cells were lysed in a buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM $NaVO_3$, and 20 mM NaF and centrifuged (at 13.000 rpm 10 min, 4 °C). Supernatants (total cell extracts) were stored at −80 °C. Protein concentration was determined by the Bradford method.

Western blot analysis was carried out as previously described (9) using total cell extracts or mediums from serum-deprived cells (to analyze Fibulin 3 secretion). Proteins were separated by electrophoresis using Anderson gels (or SDS-page gels) and transferred to nitrocellulose membranes that were probed with the following antibodies against: P-p38MAPK (9211) P-ERKs (9101), P-Ser 473 Akt (9271) and DNMT3A (2160) from Cell Signaling Technology, p38α MAPK (sc-535), Fibulin 3 (sc-99177), HuR (sc-20694) from Santa Cruz Biotechnology, β-actin (Sigma A5441).

Fibulin 3 and HuR Knock-down—Permanent fibulin 3 knock-down in MEFs was performed by infection with mouse fibulin 3 shRNAs Lentiviral Particles (75000 infectious units) containing a mixture of different shRNAs (Santa Cruz Biotechnology sc-44625-V) in the presence of 10 μg/ml Polybrene (Santa Cruz Biotechnology sc-134220) or a control shRNA for non-silenced cells. Similarly, permanent HuR knock-down in wt MEFs was performed by transfection of a mixture of plasmids containing different mouse HuR shRNAs (Santa Cruz Biotechnology sc-35620-sh) using Metafectene-Pro (Biontex T040–0.2) as previously described (21). Then, cells were selected with 1 μg/ml puromycin.

Permanent fibulin 3 knock-down in HCT116 cells was carried out using a human fibulin 3 shRNA (OriGene Technologies, TR30018) inserted in the pGFP-B-RS vector. Cells were transfected using Metafectene-Pro (Biontex T040-0.2). Different clones were selected with blasticidin (2 μg/ml, Invitrogen R210-01) and used for the experiments. As a control, cells transfected with the empty vector were also generated. Transient fibulin 3 knock-down was also performed using a second human fibulin 3 shRNA (Sigma NM-004105). Cell assays were initiated 48 h after transfection.

Re-expression of p38α and DNMT3A—To re-express p38α MAPK or DNMT3A in p38α−/− MEFs, transient transfections were performed using Metafectene-Pro (Biontex T040-0.2) and the following constructs: (i) p38α cDNA cloned into the EcoRI site of the pEFmlink expression vector (4); (ii) dnmt3a inserted in the pcDNA3 vector, as previously described (21). Cell assays were performed 48–72 h after transfection.

Wound Healing Assays—Confluent cells were pre-treated with mitomycin C (25 μg/ml, Sigma-Aldrich M0503) for 30 min to inhibit cell growth. Then, a straight scratch was performed and the medium replaced by a fresh one without serum (for MEFs) or with 2% FBS (±HGF for HCT116 cells). Cells were maintained for 12–72 h at 37 °C and 5% CO_2. Migration was followed by a phase-contrast microscope (Eclipse TE300 Nikon coupled to a digital camera) at different time points. Photographs were taken to quantify (using TScratch program) the percentage of wound healing closure at the different times.

Invasion Assays—Invasion through matrigel was assayed using matrigel (444 μg/cm^2) (BD Biosciences, 356234)-coated transwells (8 μm filter, BD 353097). Cells (50,000) were seeded in the upper chamber in a serum-free medium. In the lower chamber, FBS (10%) or HGF (40 ng/ml) was added to the medium to act as a chemoattractant. Then, cells were left in the incubator for 24 h at 37 °C. Medium and matrigel from the upper chamber were removed, and cells present in the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet 0.2% p/v (Sigma-Aldrich C-0775). Cells were counted using a phase-contrast microscope.

Zymography—To determine MMP-2 and MMP-9 activities, 80% confluent cells were serum-deprived for 24–48 h and the culture medium was used for an electrophoresis in 8% SDS-polyacrylamide gels polymerized in the presence of 0.1% gelatin under non-reducing conditions. Gels were washed with 2.5% Triton X-100 (30 min) to remove SDS, rinsed with substrate buffer (0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN3, 50 mM Tris pH 7.5) and incubated in this buffer at 37 °C overnight to allow protein renaturation and MMP activation. To visualize gelatin degradation, the gel was stained with Coomassie Brilliant Blue (Bio-Rad, 161-0400).

Focus Formation Assays—To measure anchorage-dependent growth, 300 cells (MEFs or HCT116 cells) were seeded in a 10-cm dish. After 10–13 days for MEFs or 8–10 days for HCT116 cells, foci were stained with a 0.2% crystal violet solution. The total number of foci was quantified using Image J program and their size using OpenCFU program. The size of colonies was measured as volume applying the equation $4/3\pi r3$, where r is the radius of foci.

Growth in Soft Agar—To measure anchorage-independent growth, cells were cultured in 24-well dishes containing two agar layers. Cells (3×10^3) were resuspended in 0.7% agar (BD, 214530) diluted in complete medium (2×) and poured onto a 0.5% layer of agar (diluted in medium). Fresh medium was added to the top layer every 3 days. After 2 weeks, colonies were stained with 0.005% crystal violet and counted using a dissecting microscope.

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

Statistical Analysis—Data are represented as the mean values ± S.E. The comparisons were done between two experimental groups. An unpaired Student's *t* test was used.

RESULTS

p38 MAPKs Down-regulate Fibulin 3 Expression—Using Affymetrix gene Chips, we identified several mRNAs that were up-regulated in p38α knock-out MEFs growing with serum as compared with wt (data not shown). Among them, fibulin 3

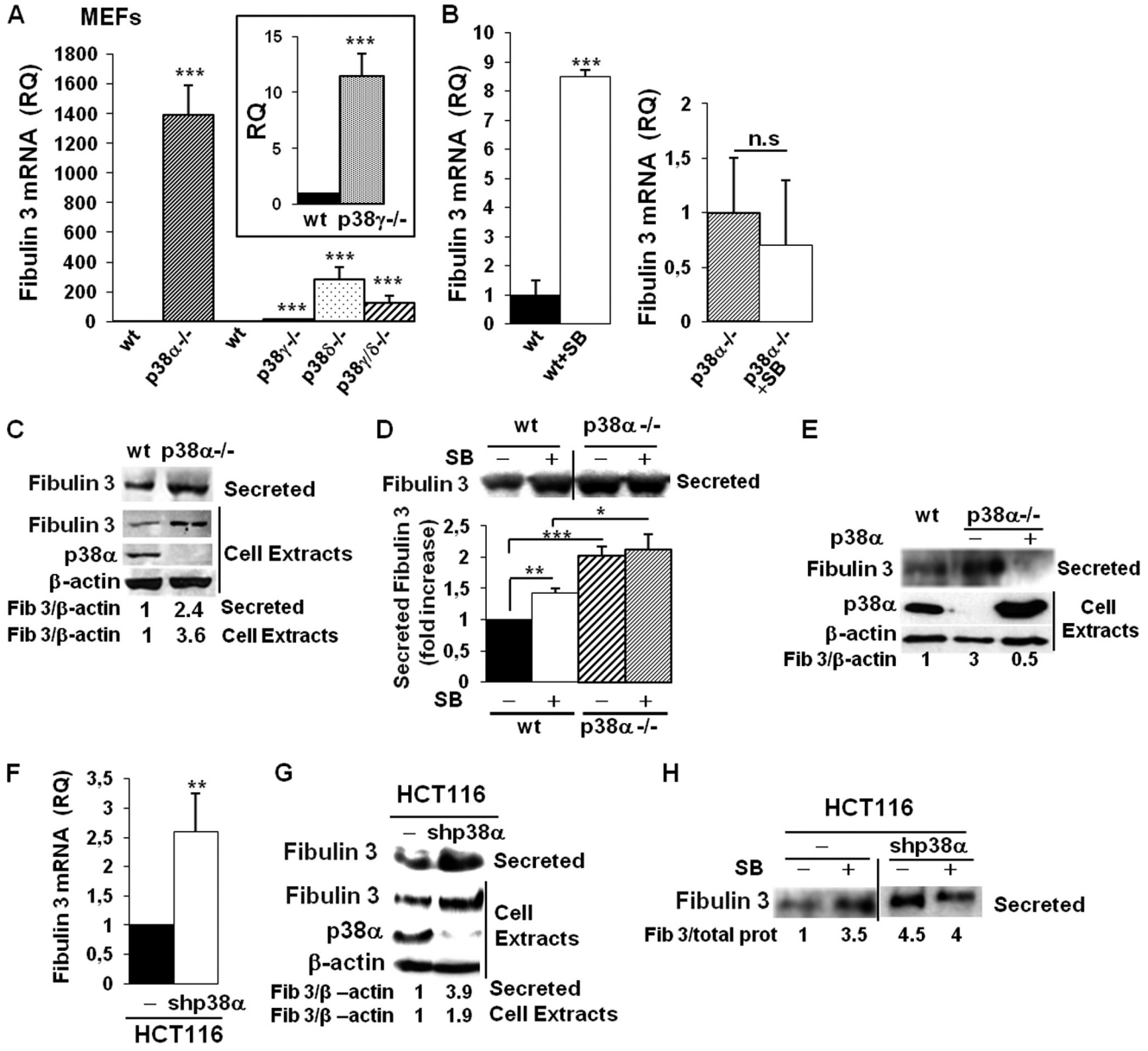

FIGURE 1. **p38 MAPKs are negative regulators of Fibulin 3 expression.** Cells (MEFs wt, p38$\alpha^{-/-}$, p38$\gamma^{-/-}$, p38$\delta^{-/-}$, and p38 $\gamma/\delta^{-/-}$ or HCT116 cells (non-silenced (−) and p38α knock-down (shp38α))) were maintained in the absence of serum for 24 h and then, total RNA or proteins were isolated. *A* and *B*, analysis by RT-qPCR of fibulin 3 mRNA levels in MEFs. Results represent the mean ± S.E. of RQ values ($n = 3$–8). ***, $p < 0.001$ as compared with wt cells. *B*, effect of SB203580 (5 μM) in wt (*left histogram*) and p38$\alpha^{-/-}$ (*right histogram*) MEFs. ***, $p < 0.001$, SB203580 treated wt MEFs as compared with untreated wt cells. *C–E*, 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. Histograms show the mean ± S.E. of the densitometric analyses of the blots normalized with β-actin. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared with wt cells. *D*, effect of SB203580 (5 μM) in wt and p38$\alpha^{-/-}$ MEFs. *E*, effect of p38α re-expression in p38$\alpha^{-/-}$ MEFs. *F*, analysis by RT-qPCR of fibulin 3 mRNA levels in HCT116 cells. Results represent the mean ± S.E. of RQ values ($n = 5$). **, $p < 0.005$ as compared with non-silenced cells. *G* and *H*, Western blot analysis of Fibulin3 protein levels in the culture medium or in cell extracts as indicated, normalized with β-actin. p38α was used as a control of its expression. *H*, effect of SB203580 (5 μM) in Fibulin 3 secretion to the culture medium referred to total protein levels.

mRNA levels were highly up-regulated. This was validated and quantified by RT-qPCR (Fig. 1*A*) using different MEFs cell lines (wt and p38$\alpha^{-/-}$). 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 Fig. 1*B*, treatment of wt MEFs with SB203580 led to an up-regulation of fibulin 3 mRNA, while it had no effect on p38$\alpha^{-/-}$ cells, even when using a higher SB203580 concentration (data not shown). This indicates that p38α, but not p38β, is responsible for fibulin 3 regulation. We also analyzed if other p38MAPKs isoforms could also regulate fibulin 3 expression. Results from Fig. 1*A* show increased levels of fibulin 3 mRNA in MEFs lacking p38γ, p38δ or both as compared with wt cells, although lower than in p38$\alpha^{-/-}$ MEFs. Therefore, we decided to focus our studies on the analysis of the function of p38α in the regulation of Fibulin 3 expression. According to mRNA data, we found increased levels of the secreted and intracellular Fibulin 3 protein in MEFs deficient in p38α (Fig. 1, *C* and *D*). Similarly, treatment of wt MEFs with SB203580 led to the up-regulation of secreted Fibulin 3 protein levels (Fig. 1*D*). Moreover, transient re-expression of p38α into p38$\alpha^{-/-}$ MEFs abolished Fibulin 3 up-regulation (Fig. 1*E*). All

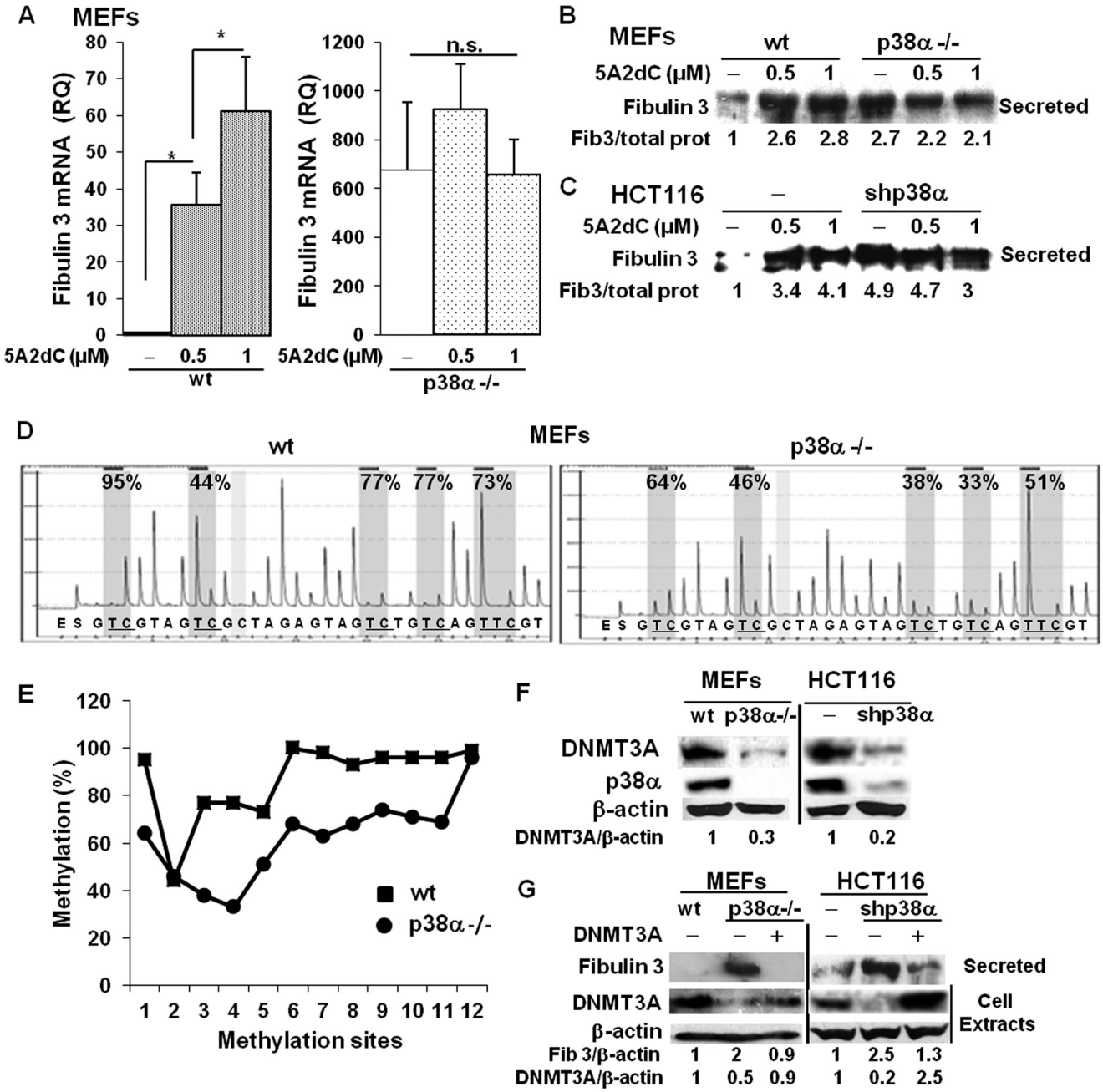

FIGURE 2. **p38α leads to hypermethylation of fibulin 3 gene regulatory sequences.** Cells (MEFs or HCT116 cells) were maintained in the absence of serum for 24 h, and then, total RNA, DNA or proteins were isolated. When indicated (*A*, *B*, and *C*), cells were maintained with 5-aza-2′-deoxicytidine (5A2dC) (0.5 μM or 1 μM) for 48 h (24 h pretreatment plus 24 h treatment in the absence of serum). *A*, analysis by RT-qPCR of fibulin 3 mRNA levels in MEFs (wt and p38$\alpha^{-/-}$). *, $p < 0.05$. *B* and *C*, Western blot analysis of Fibulin 3 protein levels in the culture medium in MEFs (wt and p38$\alpha^{-/-}$) and HCT116 cells (non-silenced (−) and p38α knock-down), respectively. Densitometric analyses of the blots normalized with total protein levels are shown. *D* and *E*, percentages of methylation of CpG sites from fibulin 3 gene regulatory sequences in wt and p38$\alpha^{-/-}$ MEFs. *D*, representative pyrosequencing diagrams showing the methylation status of 5 CpG sites. *E*, graphic showing the percentages of methylation of the 12 analyzed CpG sites. *F*, Western blot analysis of DNMT3A protein levels normalized with β-actin. p38α was used as a control of its expression. *G*, changes in Fibulin 3 protein levels upon DNMT3A expression in p38$\alpha^{-/-}$ MEFs and in p38α knock-down HCT116 cells. Western blot analysis of DNMT3A (in cell extracts) secreted Fibulin 3 normalized with β-actin.

these data indicate that p38 MAPKs, mainly p38α, are negative regulators of fibulin 3 expression.

As fibulin 3 expression is deregulated in different tumors, including colorectal cancer (30, 37), we tested whether p38α could down-regulate fibulin 3 expression in the colon-carcinoma HCT116 cell line. Fig. 1*F* shows an increase in fibulin 3 mRNA levels in HCT116 cells with permanent p38α knock-down. Similarly, the levels of the secreted and intracellular Fibulin 3 protein were higher in p38α knock-down cells (Fig. 1*G*). Moreover, inhibition of p38α with SB203580, as an alternative experimental approach, also increased Fibulin 3 secretion in cells expressing p38α (Fig. 1*H*). Therefore, p38α is also a negative regulator of fibulin 3 in HCT116 cells.

p38α MAPK Induces the Hyper-methylation of Regulatory Sequences of Fibulin 3 Gene—Fibulin 3 expression can be repressed through promoter methylation in some tumors (34,

37) leading to changes in their invasive capacity (34). So, we wondered whether p38α might down-regulate fibulin 3 through promoter methylation. To analyze it, we first studied the effect of the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5A2dC). As shown in Fig. 2*A*, fibulin 3 mRNA levels highly increased in wt MEFs treated with 5A2dC, while they remained unchanged in p38α$^{-/-}$ MEFs. Accordingly, 5A2dC treatment induced an increase in the levels of the secreted Fibulin 3 protein both, in wt MEFs (Fig. 2*B*) and in non-silenced HCT116 cells (Fig. 2*C*), while it had no effect, either in p38α knock-out MEFs (Fig. 2*B*) or knock-down HCT116 cells (Fig. 2*C*). These results suggest that p38α might down-regulate fibulin 3 expression through a mechanism dependent on DNA methylation. To demonstrate it, we analyzed methylation status of fibulin 3 gene regulatory sequences in wt and p38α knock-out MEFs. In particular, the 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. The average methylation percentage of 10 out of 12 CpG sites was higher in wt as compared with p38α$^{-/-}$ MEFs (Fig. 2, *D* and *E*). These results strongly indicate that p38α represses fibulin 3 expression through hypermethylation of 5′-UTR regulatory sequences.

DNA methylation results from the activity of DNMTs (39). DNMT3A/3B protein levels can be regulated, being relevant its post-transcriptional regulation (39). In particular, binding of HuR protein to the 3′-UTR of DNMT3B mRNA enhances its stability, increasing its protein levels in colorectal RKO cells (41). p38α MAPK can phosphorylate HuR, which enhances its binding to certain mRNAs such as p21mRNA, increasing its protein levels (42). This raised the possibility that p38α MAPK could be regulating DNMT3A/3B protein levels through a HuR-dependent mechanism. So, we analyzed DNMT3A protein levels in wt and p38α$^{-/-}$ MEFs and in non-silenced and p38α knock-down HCT116 cells. We found a significant decrease in DNMT3A protein levels in both, p38α$^{-/-}$ MEFs and p38α knock-down HCT116 cells, as compared with cells expressing p38α (Fig. 2*F*). These results indicate that p38α MAPK positively regulates DNMT3A protein levels, which inversely correlates with Fibulin 3 levels, suggesting that DNMT3A would be responsible for the hypermethylation of fibulin 3 gene regulatory sequences and the subsequent down-regulation of Fibulin 3. To prove it, DNMT3A was re-introduced in p38α deficient MEFs and in p38α knock-down HCT116 cells by transfection of a dnmt3a construct, which led to a strong decrease in Fibulin 3 levels (Fig. 2*G*).

Additionally, we analyzed how p38α controls DNMT3A protein levels. We found higher levels of dnmt3a mRNA in wt than in p38α$^{-/-}$ MEFs (Fig. 3*A*). Upon inhibition of transcription with actinomycin D, those levels highly decreased in p38α$^{-/-}$ MEFs at 2h and to a significant less extent in wt cells (Fig. 3*B*), which suggests that dnmt3a mRNA is stabilized by p38α. As HuR could be involved in dnmt3a mRNA stabilization, we first measured total HuR protein levels and found them down-regulated in p38α$^{-/-}$ MEFs and p38α knock-down HCT116 cells (Fig. 3*C*). Moreover, HuR knock-down decreased DNMT3A protein levels in wt MEFs (Fig. 3*D*). Therefore, our results demonstrate that p38α acting through HuR stabilizes dnmt3a

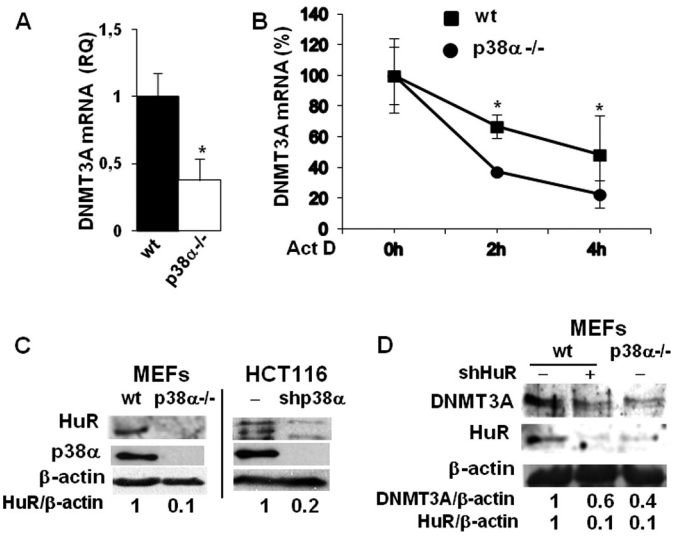

FIGURE 3. **DNMT3A is regulated by a p38α-HuR dependent pathway.** MEFs (wt and p38α$^{-/-}$) and HCT 116 cells (non-silenced (−) and with p38α knock-down (shp38α)) were maintained in the absence of serum for 24h (*A*, *C*, and *D*), or for 2–4 h (*B*), in the presence or absence of actinomycin D (Act D), as indicated. Then, total RNA or proteins were isolated. *A* and *B*, analysis of dnmt3a mRNA levels by RT-qPCR. Results represent the mean ± S.E. of RQ values ($n = 3$) (*A*) or the percentage of the control value, considered as 100% (*B*). *, $p < 0.05$, as compared with wt cells. *B*, effect of transcription inhibition by Act D on dnmt3a mRNA levels. *C*, effect of p38α expression on HuR protein levels analyzed by Western blot. *D*, effect of HuR knock-down (in wt MEFs) on DNMT3A protein levels analyzed by Western blot. In both cases, blots were normalized with β-actin.

mRNA, leading to increased DNMT3A protein levels, which in turn would down-regulate Fibulin 3 via hypermethylation of regulatory sequences of the gene.

Fibulin-3 Knock-down Increases Migration and Invasion of MEFs—Fibulin 3 has been shown to play a role in migration and invasion in several tumors, either promoting or inhibiting cell invasiveness depending on the tumor type (30). p38α can mediate cell migration and invasion (8, 16), so it would be possible that p38α, through down-regulation of fibulin 3, could favor cell migration and invasion. To analyze it, fibulin 3 was permanently knocked-down in MEFs (Fig. 4*A*). As expected, wound healing assays revealed a faster migration of wt MEFs as compared with p38α$^{-/-}$ cells (Fig. 4*B*). 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. 4*B*). Moreover, fibulin 3 knock-down promoted invasion through matrigel of p38α knock-out MEFs, so that the number of invading cells was similar to the one found in wt MEFs (Fig. 4*C*). 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 slightly increased (Fig. 4*C*). These results indicate that fibulin 3 acts as an inhibitor of cell migration and invasion in MEFs as it happens in certain tumors, such as non-small lung carcinoma (34–35). However, this effect was stronger in p38α$^{-/-}$ MEFs, where the levels of secreted Fibulin 3 are much more higher. Moreover, the p38α pro-migratory/invasive effect might be, at least in part, mediated by Fibulin 3 down-regulation in MEFs.

As MMPs are relevant for extracellular matrix degradation during cell migration/invasion (43–44), we evaluated whether the increased invasion observed in fibulin 3 knock-down MEFs was

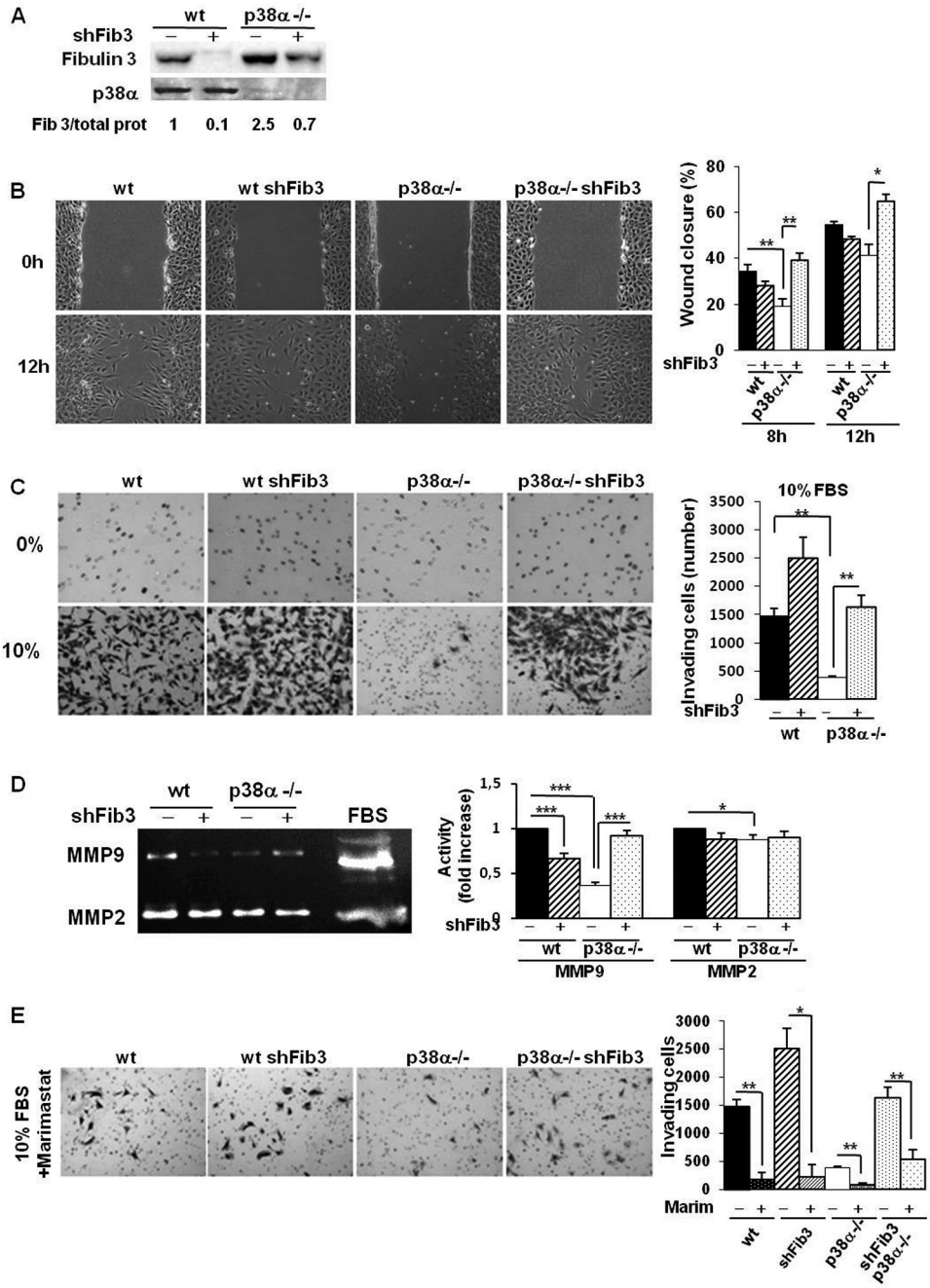

FIGURE 4. **Fibulin 3 knock-down enhances migration and invasion of MEFs.** *A*, Western blot analysis of Fibulin 3 protein levels in the culture medium from MEFs (wt and p38$\alpha^{-/-}$, with (shFib3) or without fibulin 3 knock-down) maintained in the absence of serum for the last 24 h. p38α was used as a control. Fibulin 3 quantification referred to total protein levels is shown. *B*, wound healing assay. Cells were maintained in the absence of serum and allowed to migrate. Representative images from phase contrast microscope after 0 and 12 h of migration. Histograms show the mean ± S.E. of the percentage of wound closure at 8 and 12 h. *, $p < 0.05$; **, $p < 0.01$. *C* and *E*, invasion through matrigel using FBS (10%) as a chemoattractant, either in the absence (*C*) or presence (*E*) of the MMP inhibitor, marimastat (marim). Representative images of invading cells after staining with crystal violet (phase contrast microscope). Histograms show the mean value ± S.E. of the number of invading cells ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *D*, zymographic analysis of MMP2/9 activities using gelatin as the substrate and FBS as a control. Representative zymogram. Histograms show the mean ± S.E. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value ($n = 6$). *, $p < 0.05$; ***, $p < 0.001$.

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 Fig. 4*D*, MMP2 and -9 activities were lower in p38$\alpha^{-/-}$ 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 (Figs. 4*D* and 5*D*), 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. 4*E*). This suggests that other MMPs, different from MMP2 and -9, might be responsible for the increased invasion of fibulin 3 knock-down wt MEFs.

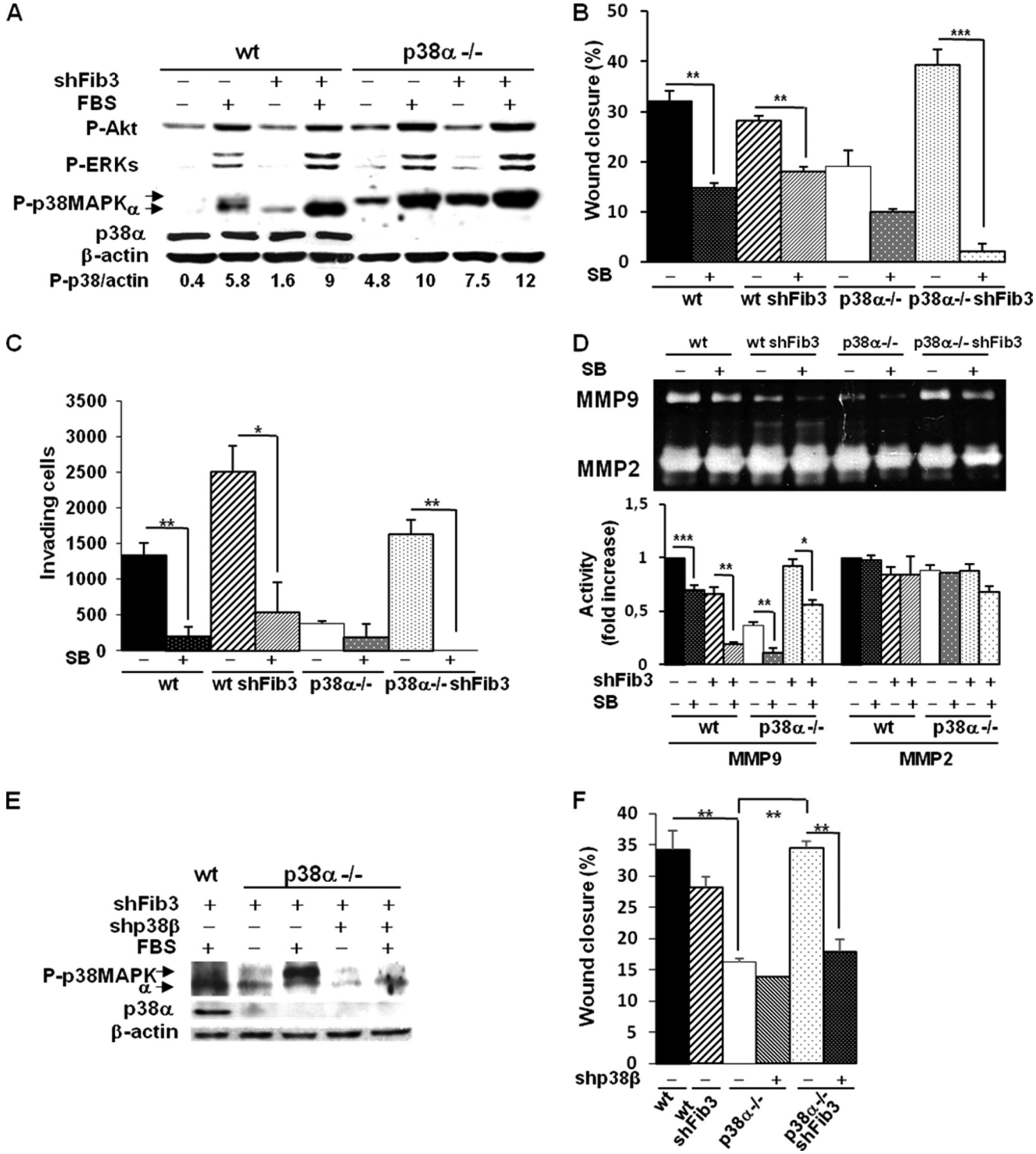

FIGURE 5. **Analysis of the function of p38α/β in migration and invasion of fibulin 3 knock-down MEFs.** *A*, Western blot analysis of P-Akt, P-ERKs, and P-p38 MAPK levels normalized with β-actin. p38α was used as a control of its expression. P-p38/β-actin ratio derived from the densitometric analysis is shown (arbitrary units). *B*, effect of p38α/β inhibition by SB203580 (10 μM) on wound healing closure. Histograms show the mean value ± S.E. of the percentage of wound closure at 8h. **, $p < 0.01$; ***, $p < 0.001$. *C*, effect of p38α/β inhibition by SB203580 (10 μM) on invasion through matrigel. FBS (10%) was used as a chemoattractant. Histograms show the mean value ± S.E. of the number of invading cells ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *D*, zymographic analysis of MMP2/9 activities using gelatin as the substrate. Representative zymogram. Histograms show the mean ± S.E. of the densitometric analyses of gelatinase areas expressed as fold increase of the control value ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E*, effect of p38β knock-down on P-p38 MAPK levels analyzed by Western blot analysis and normalized with β-actin. p38α was used as a control of its expression. *F*, effect of p38β knock-down on wound healing closure. Histograms show the mean value ± S.E. of the percentage of wound closure at 8 h ($n = 3$). **, $p < 0.01$.

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 Fig. 5*A*, 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

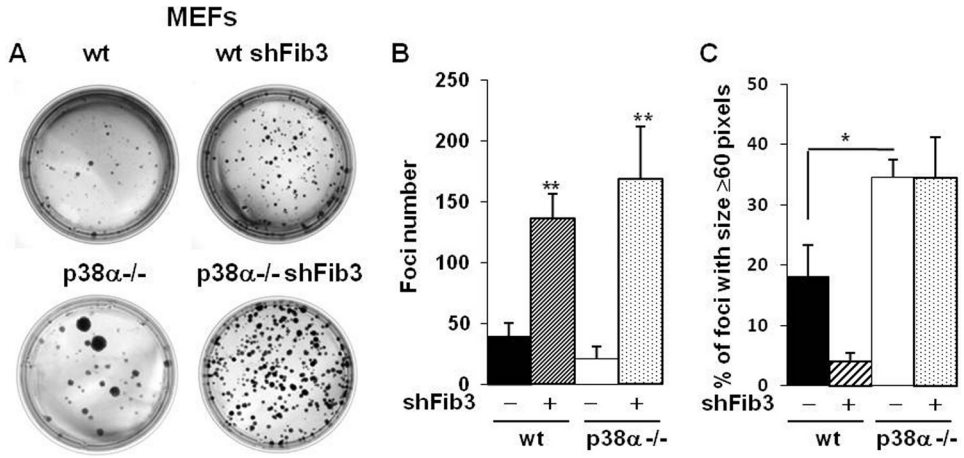

FIGURE 6. **Fibulin 3 knock-down enhances focus formation.** *A*, representative images of anchorage-dependent growth of MEFs (wt and p38$\alpha^{-/-}$, with (shFib3) or without fibulin 3 knock-down) at 13 days. *B* and *C*, number and size of foci, respectively. Histograms show the mean ± S.E. of foci number (*B*) and the percentage of those with a size ≥60 pixels. *, $p < 0.05$; **, $p < 0.01$.

was enhanced in wt MEFs, while in p38$\alpha^{-/-}$ cells there was a strong increase in the phosphorylation of another p38 MAPK isoform (potentially, p38β) with a lower mobility (Fig. 5*A*). 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 Fig. 5, *B* and *C*, inhibition of p38α/β impaired migration and invasion of fibulin 3 knock-down cells and wt MEFs. This partially correlates with the decrease in MMP9 activity upon treatment with SB203580 (Fig. 5*D*). Moreover, transient knock-down of p38β in fibulin 3 knock-down p38$\alpha^{-/-}$ MEFs abolished p38 hyperactivation (Fig. 5*E*), as well as migration in wound healing assays (Fig. 5*F*). All these data indicate that the enhanced activation of p38α in wt and p38β in p38$\alpha^{-/-}$ cells induced by fibulin 3 knock-down is necessary for migration and invasion of these cells.

As fibulin 3 knock-down increases the invasive capacity of MEFs, mainly that of p38$\alpha^{-/-}$ cells, we further analyzed the behavior of these cells. They grew faster than wt and p38$\alpha^{-/-}$ MEFs (data not shown). In addition, anchorage-dependent growth assays revealed an enhanced foci formation upon fibulin 3 knock-down (wt and p38$\alpha^{-/-}$ MEFs) (Fig. 6, *A* and *B*). In contrast, as shown in Fig. 6*C*, foci size was only increased in p38α knock-out cells (with or without fibulin 3 knock-down). All these data indicate 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 (45). However, fibulin 3 knock-down MEFs were unable to grow in soft agar or to induce tumors in xenograft assays (data not shown), which suggests that fibulin 3 knock-down is not sufficient to induce transformation, but it may collaborate with other genes as it happens in lung carcinoma (34–35).

Fibulin 3 Knock-down Inhibits Migration and Invasion of HCT116 Cells—In colorectal cancer, a down-regulation of fibulin 3 gene expression by promoter methylation was shown to occur in advanced stages, which correlated with the induction of metastasis (37). We have shown here that fibulin 3 expression is repressed in non-silenced HCT116 cells as compared with p38α knock-down cells and this can be prevented by DNA demethylation. So, we evaluated the function of Fibulin 3 in migration and invasion in HCT116 cells through gene silencing (Fig. 7*A*). As shown in Fig. 7*B*, non-silenced HCT116 cells migrated faster than p38α knock-down cells, either in the absence or presence of HGF. Fibulin 3 knock-down impaired migration of non-silenced cells and slightly reduced that of p38α knock-down cells. This was confirmed using another shRNA against fibulin 3 (sh2Fib3 in Fig. 7, *C* and *D*). Similarly, fibulin 3 silencing blocked basal and HGF-induced invasion through matrigel (Fig. 8*A*). Accordingly, MMP2 and 9 activities were lower in fibulin 3 knock-down cells (Fig. 8*B*), which correlated with its reduced invasive capacity. To evaluate the relevance of MMPs in the invasion capacity of fibulin 3 knock-down HCT116 cells, the effect of the MMP inhibitor, marimastat, was assessed. As shown in Fig. 8*C*, HGF-induced invasion was impaired by marimastat treatment. Therefore, the changes in the activity of MMPs might mediate the pro-invasive effect of fibulin 3 in HCT116 cells.

It should be noticed that although Fibulin 3 levels were lower in non-silenced cells, as compared with p38α knock-down cells, its knock-down inhibited both migration and invasion. In fact, Fibulin 3 appears to be a positive regulator of cell migration and invasion in non-silenced and p38α knock-down HCT116 cells, but this effect is more prominent when p38α is expressed. This correlates with the levels of p38α/β phosphorylation (Fig. 8*D*). Thus, in non-silenced HCT116 cells, HGF induced the activation of p38α MAPK and another isoform with a lower mobility (probably p38β), which was the only one activated in p38α knock-down cells (Fig. 8*D*). This p38 MAPKs activation was highly reduced in fibulin 3 knock-down cells, which might account for the decreased migration. In fact, inhibition of p38α/β with SB203580 had a similar effect to that of fibulin 3 knock-down (data not shown). ERKs and Akt activation was also decreased in fibulin 3 knock-down cells (data not shown), but its relevance in the migration of these cells appears to be unclear.

Together these results indicate that Fibulin 3 promotes migration and invasion of HCT116 cells through a mechanism that requires p38α and/or p38β activation. At the same time, p38α limits Fibulin 3 expression, which could represent a negative feed-back loop. So, we next wanted to determine the function of Fibulin 3 in the regulation of the tumorigenic capacity of

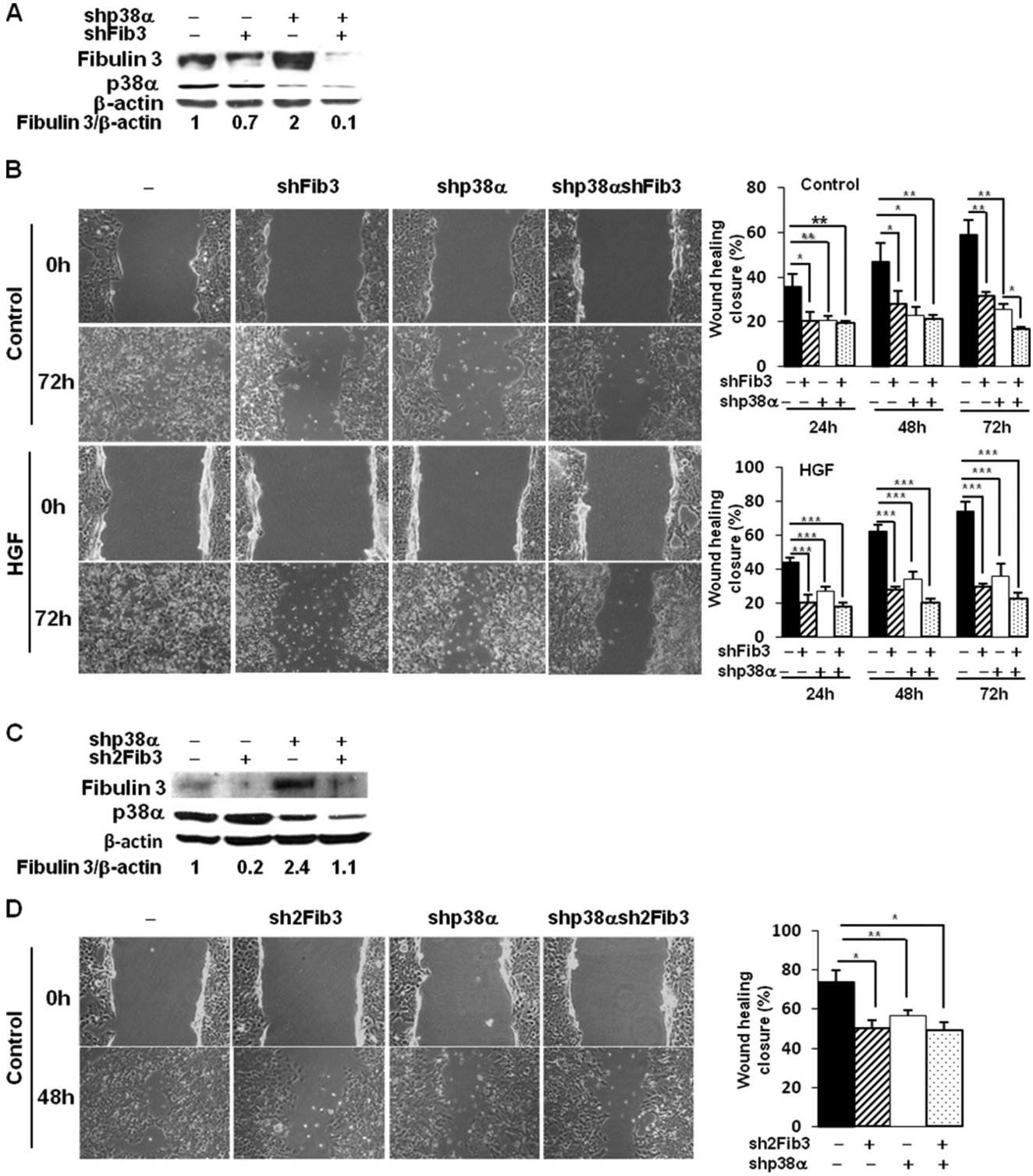

FIGURE 7. **Effect of fibulin 3 knock-down on migration of HCT116 cells.** HCT116 cells with a permanent (shFib3) or transient (sh2Fib3) fibulin 3 knock-down using two different human fibulin 3 shRNAs were used. *A* and *C*, Western blot analysis of Fibulin 3 protein levels in the culture medium from HCT116 cells (non-silenced (−) and p38α knock-down (shp38α), with (shFib3 (in *A*) and sh2Fib3 (in *C*)) or without fibulin 3 knock-down) normalized with β-actin (in cell extracts) and its quantification. p38α was used as a control of its expression. *B* and *D*, wound healing assay. Cells were pretreated with mitomycin and maintained with 2% serum, with or without 40 ng/ml of HGF (control), as indicated. *Left panels*, representative images from phase contrast microscope at 0 and 72 h (in *B*) or at 0 and 48 h (in *D*) of migration. *Right panels*, histograms showing the mean value ± S.E. of the percentage of wound closure at 24, 48, and 72 h (in *B*) or at 48 h (in *D*) with or without HGF ($n = 4$), as indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

these cells. As shown in Fig. 9*A* (*left panel*), fibulin 3 knock-down significantly reduced the number of foci in both non-silenced and p38α knock-down cells, although the effect was more prominent in non-silenced HCT116 cells. In addition, the foci size was smaller (Fig. 9*A*, *right panel*). The number of foci was also reduced by p38α knock-down, but differences were

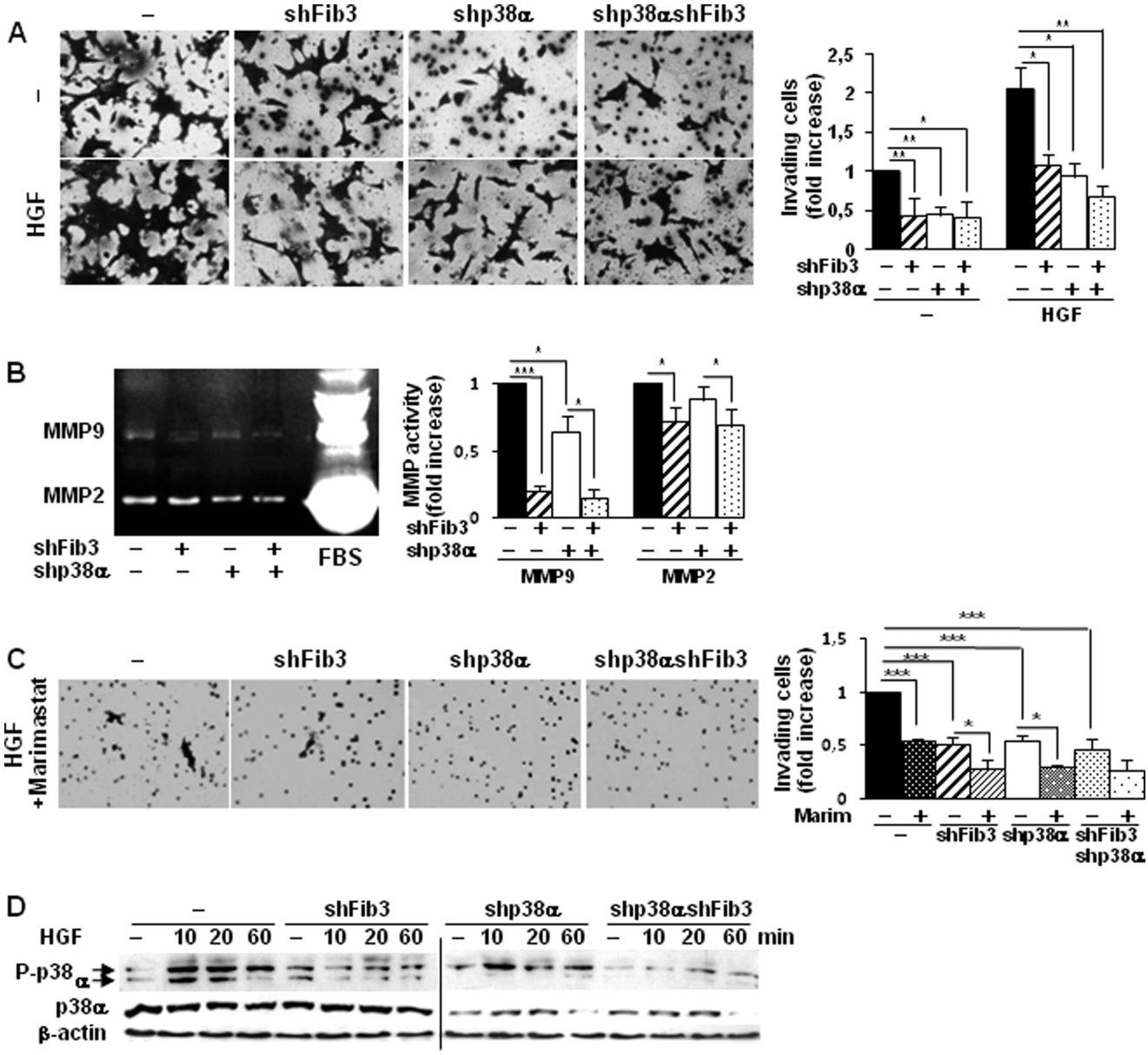

FIGURE 8. **Effect of fibulin 3 knock-down on invasion of HCT116 cells.** Role of MMPs. *A*, invasion through matrigel of HCT116 cells (non-silenced (−) and p38α knock-down (shp38α), with (shFib3) or without fibulin 3 knock-down) using HGF as a chemoattractant. *Left panel*, representative images of invading cells after staining with crystal violet (phase contrast microscope). *Right panel*, histograms show the mean value ± S.E. of the number of invading cells expressed as fold increase of control values ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *B*, zymographic analysis of MMP2/9 activities using gelatin as the substrate and FBS as a control. *Left panel*, representative zymogram. *Right panel*, histograms show the mean ± S.E. of the densitometric analyses of gelatinase areas ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$. *C*, effect of the MMP inhibitor, marimastat (marim) on invasion using HGF as a chemoattractant. *, $p < 0.05$; ***, $p < 0.001$. *D*, Western blot analysis of P-p38 MAPK and p38α levels in cell extracts from HCT116 cells (untreated or treated with HGF for different times) normalized with β-actin.

not statistically significant. This effect of fibulin 3 knock-down was confirmed using another shRNA (sh2Fib3, Fig. 9*B*). To further understand the function of Fibulin 3 in the tumorigenic capacity of HCT116 cells, xenografts assays in nude mice were performed. As shown in Fig. 9*C*, non-silenced HCT116 cells led to tumor formation 14 days after injection and the tumor size progressively increased over time. Tumors sizes were significantly decreased by fibulin 3 knock-down in non-silenced cells, but not in p38α knock-down cells, where the size of the tumors was highly reduced, independently of fibulin 3 silencing (Fig. 9*C*). All this indicates that Fibulin 3 promotes tumor growth in HCT116 cells through a mechanism dependent on p38α. Moreover, in these cells, p38α is a potent promoter of tumor growth. Nevertheless, to further confirm the role of Fibulin 3 and p38α in tumorigenesis, soft agar assays were performed using also SB203580 to inhibit p38α/β. As shown in Fig. 9*D*, SB203580 treatment and, either fibulin 3 or p38α knock-down, highly reduced anchorage independent cell growth, but only in cells expressing p38α. Accordingly, SB203580 had no further effect when fibulin 3 or p38α were knocked-down. These data are in agreement with data derived from xenografts assays, although the effect of p38α knock-down is lower, probably due to differences in the microenviroment and/or the influence of the angiogenic processes.

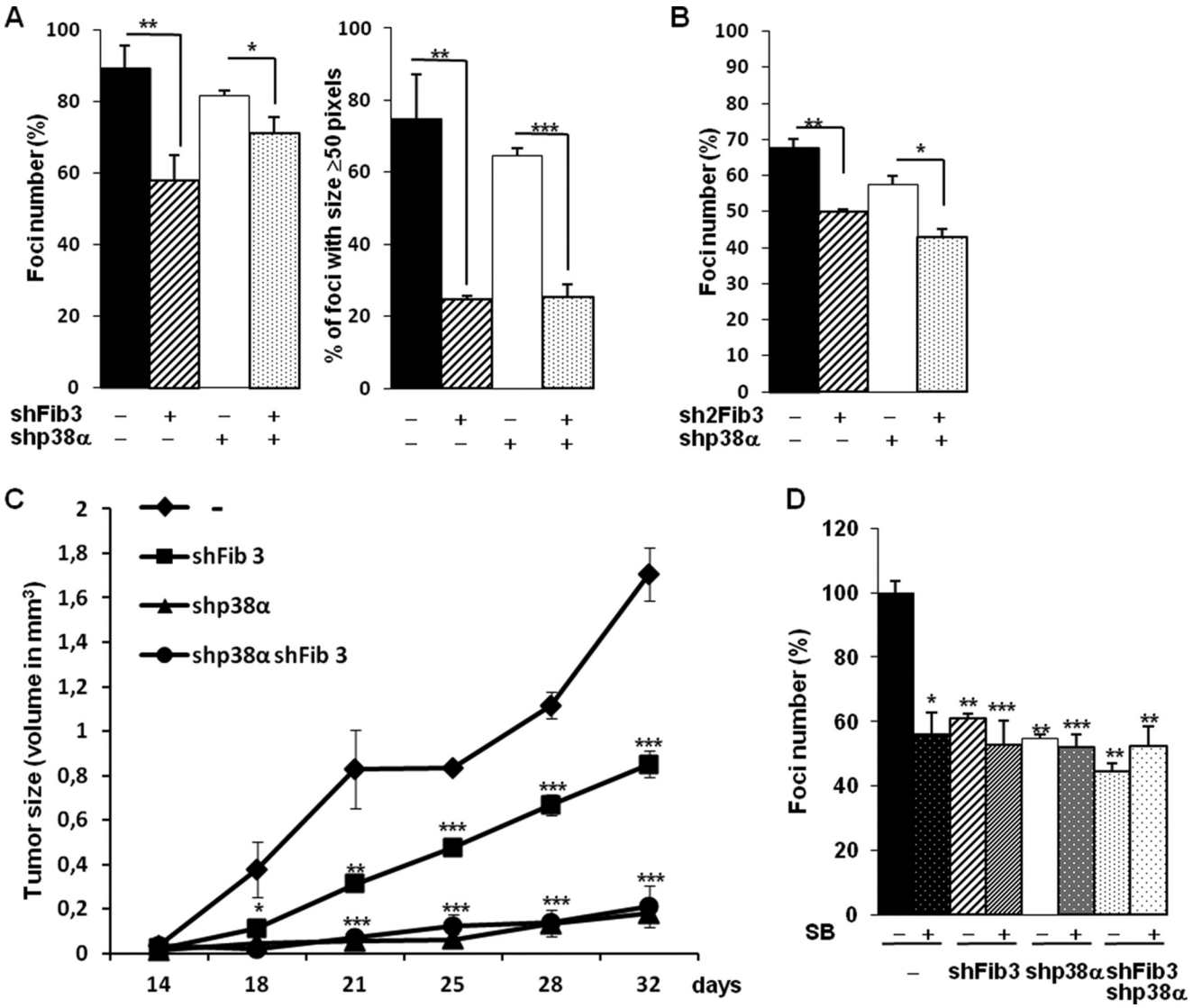

FIGURE 9. **Fibulin 3 and p38α knock-down inhibit tumorigenesis of HCT116 cells.** *In vitro* and *in vivo* growth of HCT116 cells (non-silenced (−) and p38α knock-down (shp38α), with (shFib3 (in *A*) and sh2Fib3 (in *B*)) or without fibulin 3 knock-down) was analyzed. HCT116 cells with a permanent (shFib3) or transient (sh2Fib3) fibulin 3 knock-down using two different human fibulin 3 shRNAs were used. *A* and *B*, anchorage dependent growth of HCT116 cells at 10 days (*A*) or at 3 days (*B*). Histograms show the mean ± S.E. of foci number (expressed as the percentage of that of non-silenced cells at 14 d) and the percentage of those with a size ≥50 pixels. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, xenograft assay. Immunodeficient nude mice were injected subcutaneously with HCT116 cells. Histograms show the mean value ± S.E. of tumor volume at the indicated time points ($n = 6$). **, $p < 0.01$; ***, $p < 0.001$. *D*, anchorage independent growth of HCT116 cells at 14 days, in the absence or presence of SB203580 (5 μM) as indicated. Histograms show the mean value ± S.E. of the foci number expressed as the percentage of that from non-silenced cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared with non-silenced cells.

DISCUSSION

Data presented here uncover Fibulin 3 as a new target of p38 MAPK, which participates in the regulation of migration and invasion in MEFs and HCT116 cells. p38α, p38γ, and p38δ regulate Fibulin 3 expression, but the effect of p38α is more dramatic, so it has been characterized. We described for the first time that p38α down-regulates Fibulin 3 expression through hyper-methylation of fibulin 3 gene regulatory sequences, leading to changes in migration and invasion. Moreover, p38α would do so through the up-regulation of DNMT3A protein levels. According to this, re-introduction of DNMT3A in $p38\alpha^{-/-}$ MEFs and p38α knock-down HCT116 cells down-regulates Fibulin 3. The up-regulation of DNMT3A by p38 is in agreement with the previously shown p38-mediated increase in methyltransferase activity in response to anandamide (46).

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 (41). Therefore, we hypothesized that p38α would stabilize dnmt3a mRNA through HuR phosphorylation, as it happens with p21 mRNA (42). In that case, p38α MAPK would phosphorylate HuR, leading to cytoplasmic accumulation of HuR and enhancement of its binding to the 3′-UTR of the mRNA. Similarly, p38 MAPK mediated cytoplasmic accumulation of HuR stabilizes survival motor neuron mRNA (47). Other studies also support this hypothesis, but they involve the participation of additional proteins. For example, p38 MAPK via MK2 regulates the stability of other mRNAs such as TNF mRNA through regulation of HuR and tristetraprolin (TTP) (48). MK2 phosphorylates TTP, decreasing its affinity to the AU-rich element and its ability to replace

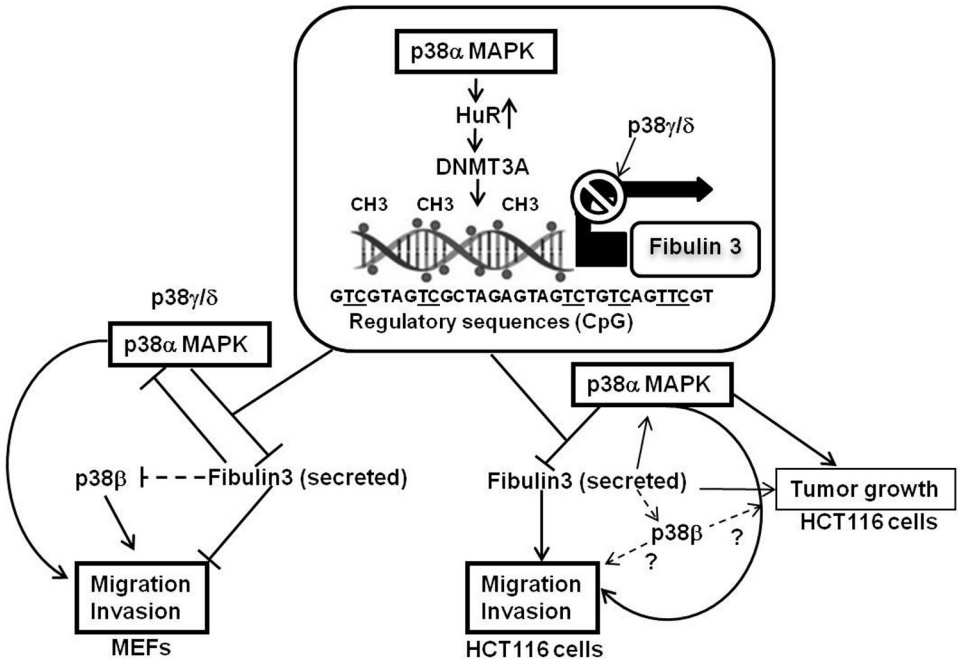

FIGURE 10. **p38 MAPK down-regulates fibulin 3 expression leading to regulation of migration/invasion and tumor growth.** Model showing that p38α, γ and δ decrease fibulin 3 transcription, leading to low levels of secreted fibulin 3. p38α-HuR-mediated DNMT3A up-regulation might be responsible for hypermethylation of regulatory sequences of fibulin 3 gene and its silencing. In MEFs, Fibulin 3 negatively regulates migration and invasion through p38α/p38β inhibition. In HCT116 cells, Fibulin 3 promotes migration/invasion and tumor growth through p38α activation, although p38β could contribute to it. At the same time, p38α limits fibulin 3 expression as a negative feed-back loop.

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 (49). In this line, our results support the involvement of HuR in the p38α-mediated up-regulation of DNMT3A through dnmt3a mRNA stabilization. Accordingly, HuR knock-down highly decreases DNMT3A protein levels in wt MEFs.

It is important to highlight the relevance of fibulin 3 gene silencing induced by hypermethylation of its regulatory sequences in cancer (34, 37). This down-regulation of fibulin 3 is associated with poor prognosis in some tumors such as non-small cell lung cancinoma (34–35). However, the mechanisms controlling fibulin 3 gene hypermethylation remain unknown. Therefore, the finding of p38α MAPK as a novel regulator of this process in normal (MEFs) and tumor cells opens new perspectives to fully characterize how fibulin 3 expression is controlled under physiological conditions and in cancer.

Although p38α MAPK down-regulates Fibulin 3 in both MEFs and HCT116 cells, the role played by Fibulin 3 in the control of migration and invasion is different in the two cell types. This agrees with previous data from the literature. For example, in glioma and pancreatic adenocarcinoma, Fibulin 3 is overexpressed, promoting migration and invasion (31–33). In contrast, in non-small cell lung cancer cell lines, Fibulin 3 is a negative regulator of invasiveness, so in those cell lines where fibulin 3 is silenced by promoter methylation, cells became highly invasive and expressed higher levels of MMP2 and 7 (34). Wnt/β-catenin pathway activation also contributes to invasion (50). Fibulin 3 down-regulation also promotes epithelial to mesenchymal transition and self-renewal of lung cancer stem cells (35). On the other hand, although Fibulin 3 down-regulation in colorectal cancer was previously correlated with lymph node metastasis and poor survival (37), in the HCT116 colon carcinoma cell line we have demonstrated that fibulin 3 silencing decreases migration, invasion, and tumor growth.

MMPs appear to play a role in both, MEFs and HCT116 cells. In particular, there is a good correlation between MMP2/9 activities and the invasive capacity of HCT116 cells, suggesting their involvement, which was supported by the impairment of invasion by a broad spectrum MMP inhibitor. Moreover, p38α is a positive regulator of these MMPs, as described in other tumor cell lines (16).

Curiously, in the two cell models that we have studied, Fibulin 3 regulates p38α and/or p38β activity, but in an opposite way, and their effects appear to be dependent on this regulation. In MEFs, Fibulin 3 down-regulates p38α and p38β activation, which would limit migration and invasion. In contrast, in HCT116 cells, Fibulin 3 enhances p38α/β activation, favoring cell migration/invasion. So, the low level of Fibulin 3 produced by HCT116 cells expressing p38α is enough to promote migration and invasion through p38α. In the A549 lung carcinoma cell line, p38 is also activated by Fibulin 3, but its function has not been characterized (51).

Although there is not a straightforward explanation to the distinct regulation of p38 activation by Fibulin 3 in MEFs and HCT116 cells, there are a great number of differences between these two cell models that might account for this discrepancy. MEFs are non-tumoral embryonic mesenchymal cells from a murine origin, while HCT116 cells are epithelial tumor cells from a human origin. These differences can support an opposite response mainly based on the following reasons: (i) epithelial and mesenchymal cells express different proteins (*i.e.* E-cadherin and N-cadherin, respectively) and (ii) HCT116, as

other tumor cells, present several genetic alterations. Thus, it should be highlighted that the colon carcinoma HCT116 cell line bears a mutation in codon 13 of the ras gene, which leads to the up-regulation of a number of signaling pathways such as Ras/ERKs, PI3K/Akt or even p38 MAPKs. In addition, TGF-β1 and β2 are expressed by these cells, which would dysregulate additional pathways and gene expression.

It is also noticeable the role played by p38α promoting tumor growth of HCT116 cells *in vitro* and *in vivo*. This is in contrast with its pro-apoptotic function, previously identified in HCT116 cells treated with cisplatin (12) and with its tumor suppressor role in other tumor cell lines (8). However, in agreement with our results, increased levels of phosphorylated p38α have been also correlated with malignancy in various cancers (8) such as head and neck carcinoma (52), where p38α promotes tumor growth *in vitro* and *in vivo*. In addition, in a mouse model of colitis-associated tumor induction, p38α deficiency decreases cell proliferation and survival of colon tumors (53). Furthermore, the role of Fibulin 3 favoring tumor growth of non-silenced HCT116 cells also supports this pro-tumorigenic function of p38α and it correlates with p38α activation levels.

In conclusion, 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 p38α-HuR-mediated up-regulation of DNMT3A. Depending on the cellular context, Fibulin 3 acts as either a positive or a negative regulator of migration and invasion (Fig. 10) through mechanisms involving p38α/β. In addition, Fibulin 3 also promotes tumor growth of HCT116 cells through a mechanism dependent on p38α, which acts as a potent promoter of tumor growth. At the same time, p38α limits fibulin 3 expression, which could represent a negative feed-back loop (Fig. 10).

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