ORIGINAL RESEARCH ARTICLE

TWEAK promotes migration and invasion in MEFs through a mechanism dependent on ERKs activation and Fibulin 3 down-regulation[†]

Running head: TWEAK induces migration through ERKs and Fibulin 3 regulation.

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Abstract

TWEAK regulates multiple physio-pathological processes in fibroblasts such as fibrosis. It also induces migration and invasion in tumors and it can activate p38 MAPK in various cell types. Moreover, p38α MAPK promotes migration and invasion in several cancer cells types and in mouse embryonic fibroblasts (MEFs). However, it remains unknown if TWEAK could promote migration in fibroblasts and whether p38α MAPK might play a role. Our results reveal that TWEAK activates ERKs, Akt and p38α/β MAPKs and reduces secreted Fibulin 3 in MEFs. TWEAK also increases migration and invasion in wt and p38α deficient MEFs, which indicates that p38α MAPK is not required to mediate these effects. In contrast, ERKs inhibition significantly decreases TWEAK-induced migration and Fibulin 3 knock-down mimics TWEAK effect. These results indicate that both ERKs activation and Fibulin 3 down-regulation would contribute to mediate TWEAK pro-migratory effect. In fact, the additional regulation of ERKs and/or p38β as a consequence of Fibulin 3 decrease might be also involved in the pro-migratory effect of TWEAK in MEFs. In conclusion, our studies uncover novel mechanisms by which TWEAK would favor tissue repair by promoting fibroblasts migration.

Keywords: TWEAK; migration; ERKs; Fibulin 3

Abbreviations: TWEAK, (TNF)-like weak inducer of apoptosis; MEFs, mouse embryonic fibroblasts; ERKs, extracellular regulated kinases; p38 MAPKs, p38 Mitogen activated Kinases; MMPs, matrix metalloproteinases

INTRODUCTION

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily, broadly expressed and with multiple biological activities, including cell migration and invasion (Burkly et al., 2007). TWEAK is expressed as a transmembrane-bound isoform (mTWEAK), but it can also be cleaved to generate a soluble form (sTWEAK) (Wiley and Winkles, 2003). Both isoforms act through binding to its receptor, fibroblast growth factor-inducible 14 (Fn14) (Brown et al., 2010; Han et al., 2003; Roos et al., 2010), leading to the activation of different signaling pathways. This includes both canonical and non-canonical NFκ-B pathways (Roos et al., 2010; Saitoh et al., 2003), as well as Akt, ERKs and JNKs routes (Dogra et al., 2007; Kumar et al., 2009). In addition, the modulation of TNF-α response by TWEAK has been described in several physio-pathological contexts. For example, we have recently demonstrated that TWEAK specifically reverses TNF-α-induced insulin resistance on glucose uptake through the activation of PP2A, which leads to JNK dephosphorylation in adipocytes (Vazquez-Carballo et al., 2013). Accordingly, sTWEAK decrease in severely obese patients may favor the proinflammatory activity elicited by TNFα (Maymó-Masip et al., 2013).

Although initial studies were focused on a potential role for TWEAK/Fn14 axis in apoptosis, it is currently well-accepted as an important mediator of regenerative processes in different tissues (Akahori et al., 2015). This includes the induction of fibrosis in response to damage in kidney, heart and liver (Chen et al., 2012; Novoyatleva et al., 2013; Ucero et al., 2013, Wilhelm et al., 2016). In fact, strong evidences demonstrate significant and physiologically relevant effects of TWEAK in fibroblasts. For example, it increases cardiac fibroblasts proliferation and collagen synthesis (Chen et al., 2012), as well as proliferation and inflammation in kidney (Ucero et al, 2013) and dermal fibroblasts (Chicheportiche et al., 2002).

TWEAK promotes migration and invasion in various types of tumors such as breast cancer (Asrani et el., 2013), lung cancer (Whitsett et al., 2012) and glioma (Cherry et al., 2015; Fortin et al., 2012; Tran et al., 2006) through mechanisms that involve Fn14 up-regulation. Canonical and non-canonical NF-κB pathways have been shown to play a role in mediating TWEAK-Fn14 effects in glioma (Cherry et al., 2015). To our knowledge, no studies have been performed to analyze whether TWEAK regulates migration and invasion in fibroblasts. The migratory response of fibroblasts to acute injury, together with the initial fibrosis and inflammation, are part of a beneficial wound-healing process, involved in tissue repair (Seki and Schwabe, 2015). In this

context, it is important to define the mechanisms that regulate these responses, paying special attention to fibroblasts. p38α MAPK promotes migration/invasion of MEFs and increases MMP2 and MMP9 activities (Arechederra et al., 2015; Priego et al., 2016). Moreover, MMPs inhibition impairs p38α-induced invasion, which supports the involvement of MMP2 and MMP9 in this process (Arechederra et al., 2015). MMP2 is also relevant for migration of fibroblasts in response to hyperglycemia (Janardhanan et al., 2015).

Fibulin 3 is an extra-cellular matrix glycoprotein that is expressed in several tissues, where it regulates different cellular functions (Timpl et al., 2003; McLaughlin et al., 2007). Fibulin 3 genetic disruption leads to early onset of aging-associated phenotypes affecting muscles and fat, among other organs (McLaughlin et al., 2007). Changes in Fibulin 3 levels are associated with different pathologies such as osteoarthritis, where its level is decreased in articular cartilage (Hasegawa et al., 2016). Fibulin 3 is also relevant in cancer, where it plays a dual role (Obaya et al., 2012). In some tumors, Fibulin 3 acts as a tumor suppressor, while in others, such as glioma or colorectal cancer, it promotes cell migration and invasion (Hu et al., 2009; Hu et al., 2012; Arechederra et al., 2015). In MEFs, Fibulin 3 inhibits migration, so that the p38α-mediated Fibulin 3 down-regulation favors this process (Arechederra et al., 2015).

In the present study, we therefore aimed at studying the potential function of TWEAK as a regulator of migration/invasion of fibroblasts and the mechanisms involved. Genetically modified MEFs have been used to evaluate the implication of p38 MAPK and Fibulin 3.

MATHERIAL AND METHODS

Cell lines, culture and treatments

Immortalized wt and p38α-/- mouse embryonic fibroblasts (MEFs), with or without permanent fibulin 3 knock-down, previously generated in our laboratory were used (Gutierrez-Uzquiza et al., 2010; Arechederra et al., 2015). MEFs were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) plus antibiotics at 37ºC, 5% CO_2 in a humidified atmosphere. Fibulin 3 knock-down cells were maintained in the presence of 1µg/ml puromycin (Sigma-Aldrich P8833). For the experiments, puromycin was removed.

For signaling and functional assays, TWEAK (Peprotech 310-06) was used at 100ng/ml. To inhibit ERKs and evaluate its function in migration, cells were treated with PD98059 at 10μM (Calbiochem 513000).

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% NP40, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin,10 μg/ml leupeptin, 1 mM $NaVO_3$ and 20 mM NaF. Cell lysates were centrifuged at 13000 rpm during 10 min at 4ºC. Then, the supernatant fractions (total cell extracts) were stored at -80ºC. Protein concentration was determined by the Bradford method.

Western-blot analysis was carried out as previously described (Zuluaga et al., 2007) using total cell extracts or media from serum-deprived cells to analyze Fibulin 3 secretion (corresponding to equal amounts of cell lysates proteins). Proteins were separated by electrophoresis using Anderson gels (Nebreda et al., 1995) or SDS-page gels and transferred to nitrocellulose membranes that were probed with the following antibodies: P-p38MAPK (9211) P-ERKs (9101), P-Ser 473 Akt (9271) from Cell Signalling Technology, p38α MAPK (sc-535), Fibulin 3 (sc-99177 and sc-365224) from Santa Cruz Biotechnology and β-actin (Sigma A5441).

Wound healing assays

Confluent cells were pre-treated with mitomycin C (25μg/ml Sigma-Aldrich M0503) for 30 min to inhibit cell growth. Then, a straight scratch was performed and the medium was replaced by a fresh medium without serum. Cells were maintained for 6-24h at 37ºC in a humidified atmosphere and 5% CO_2. Migration was followed by phase-contrast microscopy (Eclipse TE300 Nikon microscope coupled to a digital sight DS-U2 camera) at different time points up to wound healing closure. Photographs were taken to quantify (using TScratch program) the percentage of wound healing closure at the different times.

Invasion assays

Invasion through collagen IV was assayed using collagen IV (1.5μg/cm^2, Corning 354233) coated transwells (8 μm filter, BD 353097). After a 6h pre-treatment with TWEAK (100ng/ml), 20000 cells were seeded in the upper chamber in a serum-free medium in the presence of TWEAK. Untreated cells were also used as a control. In the lower chamber, serum-free medium was added and cells were left in the

incubator for 24h at 37ºC and 5% CO_2. Then, medium and collagen IV from the upper chamber were removed and cells present in the filter (lower chamber) were fixed with 4% paraformaldehyde and stained with crystal violet 0.2% p/v (Sigma C-0775). Finally, cells were counted using a phase-contrast microscope (Eclipse TE300 Nikon microscope couple to a digital sight DS-U2 camera).

Zymography

To determine MMP2 and MMP9 activities, 80% confluent cells were serum-deprived for 24h. Then, culture medium was collected and used for an electrophoresis in 8%SDS polyacrilamide gels polymerized in the presence of 0.1% gelatin under non-reducing conditions. Gels were washed with 2.5%Triton X-100 (30min) to remove SDS, rinsed once with substrate buffer (0.2M NaCl, 5 mM $CaCl_2$, 1%Triton X-100, 0.02% NaN_3, 50mM Tris pH 7.5) and incubated in substrate buffer at 37ºC overnight to allow protein renaturation and MMP activation. To visualize MMPs, the gel was stained with Coomassie Brilliant Blue (BioRad 161-0400).

RT-qPCR analysis of fibulin 3 mRNA levels

After isolation of total RNA using NucleoSpin RNA Kit (Mackerey-Nagel, 740955), 1-5 µg RNA was reverse transcribed using Superscript III RT kit (Invitrogen, 180800-044) to generate cDNA. Then, Real Time PCR was performed using SYBR Green Master (Rox) (Roche 11929100) and the following specific primers: for mouse fibulin 3: forward 5´GAATGTGATGCCAGCAACC3´ and reverse 5´TCACAGTTGAGTCTGTCACTGC3´ and to normalize mouse primers for GAPDH: forward: 5'CATCGAAGGTGGAAGAGT3' and reverse: 5'CATCAAGAAGGTGGTGAA3'. 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.

Statistical analysis

Data are represented as the mean values ± SEM from 3-10 independent experiments. Statistical significance was tested by one-way ANOVA followed by unpaired Student's test with Bonferroni adjustment. When two treatments were performed simultaneously (figures 3 and 5) a two-way ANOVA analysis was done, followed by a Tukey multiple-comparison post-test to compare all pairs of data. To evaluate statistically significant differences between two groups, a two-tailed paired Student's t-test was used.

RESULTS

Effect of TWEAK on MEFs migration and invasion

We first evaluated if TWEAK was able to activate in MEFs signaling pathways that control cell migration, such as ERKs and p38 MAPK, which are known to be activated by TWEAK in various cell types (Dogra et al., 2007; Kumar et al., 2009; Ucero et al., 2013). TWEAK led to an increase in the levels of P-Akt and P-ERKs after 5-15 min that was maintained up to 24h with some variations along the time (Fig. 1A and 1B). P-p38 MAPK levels were slightly increased by TWEAK at short-times (10-15 min), 3h and 24h (Fig. 1A and 1B). Based on our previous published data showing that p38α promotes migration of MEFs (Arechederra et al., 2015), the effect of TWEAK on the activation of these signaling pathways was also studied in p38α deficient MEFs (Fig. 1). Akt was activated after 5 min and this activation was maintained along the time with a maximum at 6-24h. TWEAK also increased P-ERKs levels in p38α-/- cells, but to a lesser extent (Fig. 1A). In addition, another p38 isoform, different from p38α, was activated by TWEAK in p38α-/- MEFs with a peak of activation at 1-6h (Fig. 1A). As shown in figure 1A and 1B, the maximum activation of Akt and ERKs in wt and p38α-/- MEFs was achieved after 24h of treatment with TWEAK. However, it should be noticed that P-Akt and P-ERKs levels were higher in cells lacking p38α due to the negative regulation of these pathways by p38α (Porras et al., 2004; Zuluaga et al., 2007). No significant variations in total levels of Akt, ERKs or p38 MAPKs were observed under the tested conditions (Suppl. Fig. 1), which supports the notion that TWEAK is just inducing the activation of these pathways.

We next analyzed the effect of TWEAK on migration and invasion in wt and p38α-/- MEFs. As shown in figure 2A, wound healing assays revealed that TWEAK was able to increase migration in both wt and p38α deficient MEFs at 8h. However, at 24h this pro-migratory effect of TWEAK was only detectable in cells lacking p38α, probably as a consequence of its lower basal migratory capacity. Thus, at 24h non-treated wt MEFs have already closed the wound. Similar results were obtained in migration experiments developed in Boyden chambers (Suppl. Fig. 2). All these data indicate that the pro-migratory effect of TWEAK does not require p38α. Moreover, this effect of TWEAK is mediated by its receptor, Fn14, as migration is reduced upon permanent Fn14 knock-down (Suppl. Fig. 3A and 3C). Similarly, TWEAK-induced

activation of Akt, ERKs and p38 MAPKs is reduced in Fn14 silenced cells. However, it should be noticed that Fn14 protein levels are lower in p38α-deficient MEFs. A small decrease in TWEAK mRNA levels was also observed in p38α-deficient MEFs (Suppl. Fig. 3D), but it is unclear its relevance.

We also found that TWEAK enhanced invasion through collagen in wt and p38α-/- MEFs (Fig. 2B), although the effect was higher in p38α deficient cells, probably as a consequence of its lower basal invasive capacity. This correlates with TWEAK-induced increase in MMP9 activity in both wt and p38α deficient MEFs (Fig. 2C). It is remarkable that MMP9 activity was almost undetectable in untreated p38α deficient cells, but it was strongly up-regulated by TWEAK. Surprisingly, MMP2 activity was slightly reduced upon TWEAK treatment, although it remained very high under all experimental conditions.

TWEAK enhances MEFs migration through a mechanism dependent on ERKs, but not mediated by the PI3K pathway

As Akt and ERKs were activated by TWEAK in MEFs and these pathways control migration in different cell types, their function in TWEAK-induced migration was evaluated. Inhibition of PI3K with LY294002 prevented Akt phosphorylation, but it had no significant effect on TWEAK-induced migration, either in wt or p38α deficient MEFs (Suppl. Fig. 4). However, it decreased basal migration in wt cells at 24h. This indicates that PI3K is not mediating TWEAK pro-migratory function. In contrast, inhibition of the ERKs pathway with PD98059 (Fig. 3A) reduced TWEAK-induced migration in both wt and p38α-/- MEFs at 8h (Fig. 3B) and, even at 24h, in cells lacking p38α. These data indicate that ERKs are involved in mediating the pro-migratory effect of TWEAK in MEFs.

Role of Fibulin 3 in TWEAK-induced migration and invasion of MEFs

We have shown that ERKs inhibition decreased the pro-migratory effect of TWEAK, but it did not abolish it (Fig. 3B and 3C). Therefore, it is likely that other signaling pathways may contribute to mediate TWEAK effects. We previously demonstrated that p38α deficient MEFs over-expressed Fibulin 3, which inhibits migration (Arechederra et al., 2015). Hence, we evaluated whether TWEAK could also increase migration through the control of Fibulin 3 expression and/or secretion. We first analyzed secreted Fibulin 3 protein levels and we found that TWEAK decreased them in both, wt and p38α-/-, MEFs (Fig. 4A), although they were higher in p38α-deficient

MEFs, according to our previous results (Arechederra et al., 2015). To determine if these changes were due to a transcriptional regulation, we quantified fibulin 3 mRNA levels (Fig. 4B). However, we did not find significant changes in response to TWEAK, either in wt or p38α deficient MEFs. We then determined intracellular protein levels and we found some differences in the pattern of bands present in the Western-blots (Fig. 4C). The higher molecular weight forms, which are known to be glycosilated and prone to be secreted (Djokic et al., 2013), were decreased upon TWEAK treatment. This was more evident when electrophoresis was performed under non-reducing conditions (data not shown). All this suggests that Fibulin 3 secretion could be altered in response to TWEAK. In contrast, inhibition of either protein synthesis with cycloheximide or proteasomal degradation with MG-132 had no significant effect (data not shown).

We next evaluated if Fibulin 3 down-regulation could be involved in TWEAK-induced migration using MEFs where fibulin 3 gene was stably knocked-down (Fig. 5A). We found that TWEAK was unable to further increase migration in Fibulin 3 silenced cells (Fig. 5B and Suppl. Fig. 2). This effect was particularly significant in MEFs lacking p38α, where Fibulin 3 knock-down enhanced migration, preventing any additional pro-migratory effect of TWEAK, either at 8 or 24h. Similarly, invasion through collagen was increased in Fibulin 3 knock-down (wt and p38α-/-) MEFs and TWEAK had no further effect (Fig. 5C). These data suggest that the Fibulin 3 down-regulation induced by TWEAK could play a role in mediating TWEAK effects on migration and invasion in MEFs. To confirm it, we evaluated whether the addition of exogenous recombinant Fibulin 3 protein was able to prevent TWEAK-induced migration in wound healing assays. We found that Fibulin 3 reduced migration in both untreated and TWEAK treated cells, but the effect was only significant in wt MEFs treated with either Fibulin 3 alone (for 6h) or in combination with TWEAK (for 8h) (Suppl. Fig.5). These results suggest that Fibulin 3 is only partially responsible for TWEAK effects.

Altogether our data indicate that both TWEAK-induced Fibulin 3 down-regulation and ERKs activation would be involved in promoting migration. On the other hand, we previously demonstrated that the enhanced migration induced by Fibulin 3 knock-down in p38α-/- MEFs was mediated by p38β hyperactivation (Arechederra et al., 2015). Hence, we determined p38α/β MAPK and ERKs activities in Fibulin 3 knock-down MEFs in response to TWEAK. As expected, P-p38 levels increased in untreated Fibulin 3 knock-down p38α-/- MEFs (Fig. 5) and slightly decreased after 24h of treatment with TWEAK. On the other hand, P-ERKs levels significantly increased upon Fibulin 3 knock-down in untreated wt MEFs, but not in p38α-/- cells, where they were

already up-regulated. Additionally, P-ERKs levels were further increased in response to a 24h TWEAK treatment, except in Fibulin 3 knock-down wt MEFs. p38β knock-down reduced P-p38 levels, as expected (Suppl. Fig. 6), but curiously, it also down-regulated the activation of ERKs induced by Fibulin 3 knock-down .

All these data suggest that the activation of ERKs and p38β, induced either directly by TWEAK or by Fibulin 3 down-regulation, would contribute to mediate the pro-migratory effect of TWEAK in MEFs.

Discussion

TWEAK regulates different cellular functions (Burkly et al., 2007), including migration and invasion in several types of tumors (Asrani et el., 2013; Cherry et al., 2015; Fortin et al., 2012; Tran et al., 2006; Whitsett et al., 2012). In fibroblasts, TWEAK activates proliferation, differentiation and collagen synthesis in specific contexts (Chen et al., 2012). TWEAK also decreases collagen synthesis under certain conditions (Ucero et al., 2013). However, it remained unknown whether it could regulate fibroblasts migratory capacity as well. Our data demonstrate for the first time that TWEAK promotes migration and invasion in MEFs through a mechanism that involves ERKs activation and Fibulin 3 down-regulation, but does not require p38α MAPK. In fact, in MEFs expressing p38α, TWEAK initially (at 8h) increased migration in wound healing assays, but not at later times (24h). In contrast, in MEFs lacking p38α, TWEAK enhanced migration at 8 and 24h. This might be a consequence of the higher basal migratory capacity of wt cells due to: i) p38α pro-migratory function, ii) lower basal levels of Fibulin 3, an inhibitor of migration, and iii) a lower ERKs activity in response to TWEAK. Moreover, in the absence of p38α, Fibulin 3 down-regulation enhances p38β (Arechederra et al., 2015) and ERKs activation. These changes in p38β and ERKs activities, together with the initial ERKs activation induced by TWEAK would contribute to increase migration and invasion. Moreover, the long-term ERKs activation might be a consequence of p38β activation, based on the reduced ERKs phosphorylation observed upon p38β knock-down. Therefore, we propose a model where TWEAK activates ERKs, Akt and p38α/β MAPKs and reduces secreted Fibulin 3 (Figure 7). The direct activation of ERKs and/or p38β by TWEAK and the additional regulation of these pathways by Fibulin 3 might determine the pro-migratory and invasive effect of TWEAK in MEFs.

Our results also reveal that TWEAK-induced down-regulation of secreted Fibulin 3 is not dependent on either transcriptional (Fig. 4B) or translational

mechanisms (data not shown) or on proteasomal degradation differences (data not shown). In contrast, based on the TWEAK-induced decrease in high molecular weight Fibulin 3 forms, which are known to be secreted (Djokic et al., 2013), Fibulin 3 secretion might be reduced in response to TWEAK. Moreover, Fibulin 3 can be cleaved by different MMPs such as MMP9 (Djokic et al., 2013), raising the possibility that the increase in MMP9 activity induced by TWEAK might contribute to decrease secreted Fibulin 3 protein levels.

Previously, it was demonstrated that TWEAK-induced ERKs activation in renal fibroblasts mediated proliferation, which might contribute to promote renal fibrosis (Ucero et al., 2013). Here, we additionally show that ERKs activation by TWEAK promotes migration and invasion of MEFs under non-proliferative conditions, which agrees with the pro-migratory function of ERKs in MEFs (Muñoz-Félix et al., 2016). Moreover, MMP9 activity is increased by TWEAK in MEFs, as previously described in neonatal rat cardiac fibroblasts, where this increase together with the enhanced expression of collagen contributed to induce myocardial fibrosis (Chen et al., 2012). The importance of TWEAK/Fn14 axis in cardiac fibrosis is also supported by other studies (Novoyatleva et al., 2013). Our data also suggest that TWEAK might play a role in tissue regeneration by increasing migration and invasion of fibroblasts. This agrees with the previously described pro-regenerative function of TWEAK in muscle under ischemic and non-ischemic conditions (Akahori et al., 2015).

On the other hand, as TWEAK exerts a pro-migratory and invasive effect in several types of tumors (Asrani et el., 2013; Cherry et al., 2015; Fortin et al., 2012; Tran et al., 2006; Whitsett et al., 2012), the identification of the signaling pathways regulating these processes in MEFs could also have an impact in cancer. In fact, we have previously demonstrated that Fibulin 3 down-regulation in colorectal carcinoma HCT116 cells decreases migration, invasion and tumor growth (Arechederra et al, 2015).

Taking all this into account, it is essential to establish all TWEAK actions, as well as to determine the signaling pathways involved in order to identify potential new targets for therapeutic interventions in tissue regeneration and fibrotic processes. The identification of the herein described new functions and pathways regulated by TWEAK in MEFs will contribute to it.

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Figure legends

Figure 1-Effect of TWEAK on the activation of AKt, ERKs and p38 MAPK in MEFs. MEFs (wt and p38α-/-) were serum-starved for 16h and then treated with TWEAK for the indicated periods of time. Western-blot analysis of P-Akt, P-ERKs and P-p38MAPK normalized with β-actin. Total levels of p38α were determined as a control of p38α expression. **(**A) Time-course study (5min-24h). Representative western-blots are shown. P-Akt/β-actin, P-ERKs/β-actin and P-p38MAPK/β-actin ratios derived from the densitometric analyses, expressed as the fold increase of those from untreated cells from the same genotype. (B) Effect of TWEAK at 24h. Left panel, representative western-blot; right panel, histogram showing the mean value ± S.E.M. of P-Akt/β-actin, P-ERKs/β-actin and P-p38MAPK/β-actin ratios derived from the densitometric analyses and expressed as the percentage of those from untreated wt values (100%) (n=4). *p<0.05 compared as indicated (One-way ANOVA). Arrows indicate the band corresponding to p38β isoform.

Figure 2-TWEAK increases migration and invasion of wt and p38α deficient MEFs. MEFs (wt and p38α-/-) were used. (A) Wound healing assay. MEFs pretreated with mitomycin C were maintained in the absence of serum, untreated or treated with TWEAK. Left panel, representative microscopy images of migrating cells at 0, 8 and 24h. Right panel, histogram showing the mean value ± S.E.M. of the percentage of wound closure (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared as indicated (One-way ANOVA). (B) Invasion through collagen. Untreated or TWEAK pre-treated cells (for 6h) were seeded in the upper chamber in a serum-free medium with or without TWEAK, as indicated. Left panel, representative microscopy images of invading cells. Right panel, histogram showing the mean value ± S.E.M. of the number of invading cells expressed as the percentage of that from untreated wt cells (100%) (n=4). *p<0.05 compared as indicated (One-way ANOVA). (C) Zymographic analysis of MMP2/9 activities. Left panel, representative zymogram. Right panel, the histogram shows the mean ± S.E.M. of MMP2 and MMP9 activities expressed as the percentage of those from untreated wt cells (100%) (n=6) *p<0.05, **p<0.01, ***p<0.01 compared as indicated (One-way ANOVA).

Figure 3-Inhibition of ERKs decreases TWEAK-induced migration. MEFs (wt and p38α-/-) were maintained in the absence of serum, untreated or treated with TWEAK and/or PD98059, as indicated. (A) Western-blot analysis of P-ERKs levels in response to TWEAK (24h) showing the inhibitory effect of PD98059 (at 10μM and 20μM). Data were normalized with β-actin. (B) and (C) Wound healing assay in MEFs pretreated with mitomycin C. (B) Representative microscopy images of migrating cells at 0, 8 and 24h. (C) Histogram showing the mean value ± S.E.M. of the percentage of wound closure (n=6). *p<0.05, **p<0.01 compared as indicated (Two-way ANOVA).

Figure 4-TWEAK reduces the levels of secreted Fibulin 3 protein. Analysis of Fibulin 3 expression and secretion by serum-deprived MEFs (wt and p38α-/-) maintained untreated or treated with TWEAK for 24h. (A) Fibulin 3 protein levels

secreted to the medium. Upper panel, representative western-blot showing secreted Fibulin 3 and cell extracts β-actin (loading control). Lower panel, histogram showing the mean value ± S.E.M. of Fibulin 3/total amount of proteins ratio derived from the densitometric analysis. Data are expressed as the percentage untreated wt cells value (100%) (n=3). *p<0.05, compared as indicated. (B) Analysis by RT-qPCR of fibulin 3 mRNA levels. Results represent the mean ± S.E.M. of RQ values (n=3-8). ***p<0.001 wt versus p38α-/- cells (One-Way ANOVA). (C) Western-blot analysis of intracellular Fibulin 3 protein levels normalized with β-actin. 100 KDa and 55 KDa molecular weight bands are marked with arrows and their intensities have been quantified by densitometric analysis and normalized with β-actin. Fibulin 3 100KDa/β-actin and Fibulin 3 55KDa/β-actin ratios are shown. Other high molecular weight bands are labeled with an asterisk (*).

Figure 5. Effect of Fibulin3 knock-down on TWEAK-induced migration and invasion of MEFs. (A) Western-blot analysis of Fibulin 3 protein levels secreted to the medium by serum-deprived MEFs (wt and p38α-/-), with (shFib3) or without Fibulin 3 knock-down. (B) Wound healing assay. Left panels, representative microscopy images of migrating cells at 0, 8 and 24h. Right panels, histograms showing the mean value ± S.E.M. of the percentage of wound closure (n=10). *p<0.05, **p<0.01 compared as indicated (Two-way ANOVA). (C) Invasion through collagen. Untreated or TWEAK pre-treated cells (for 6h) were seeded in the upper chamber in a serum-free medium with or without TWEAK, as indicated. Left panel, representative microscopy images of invading cells. Right panel, histogram showing the mean value ± S.E.M. of the number of invading cells (n=4). *p<0.05 compared as indicated (Two-way ANOVA).

Figure 6-Effect of TWEAK on ERKs and p38 MAPKs activation in Fibulin 3 knock-down MEFs. MEFs (wt and p38α-/-, with (shFib3) or without Fibulin 3 knock-down) were serum-starved for 16h and then treated with TWEAK for 24h. Representative western-blot analysis of P-ERKs and P-p38MAPKs normalized with β-actin. P-ERKs/β-actin and P-p38MAPKs/β-actin ratios derived from the densitometric analysis are expressed as the fold increase of that of untreated wt MEFs (for ERKs and p38α) or untreated p38α-/- MEFs (for p38β). Arrows indicate the band corresponding to either p38α or p38β isoform.

Figure 7-TWEAK promotes migration and invasion in MEFs through a mechanism involving down-regulation of Fibulin 3 and ERKs activation. Models showing that TWEAK increases migration and invasion through down-regulation of secreted Fibulin 3 protein levels and ERKs activation (directly or through modulation of Fibulin 3). p38α MAPK has no significant effect, while p38β MAPK could contribute to TWEAK effects in p38α-/- MEFs. The direct activation of ERKs and/or p38β by TWEAK and the additional regulation of these pathways by Fibulin 3 might determine the pro-migratory and invasive effect of TWEAK in MEFs.

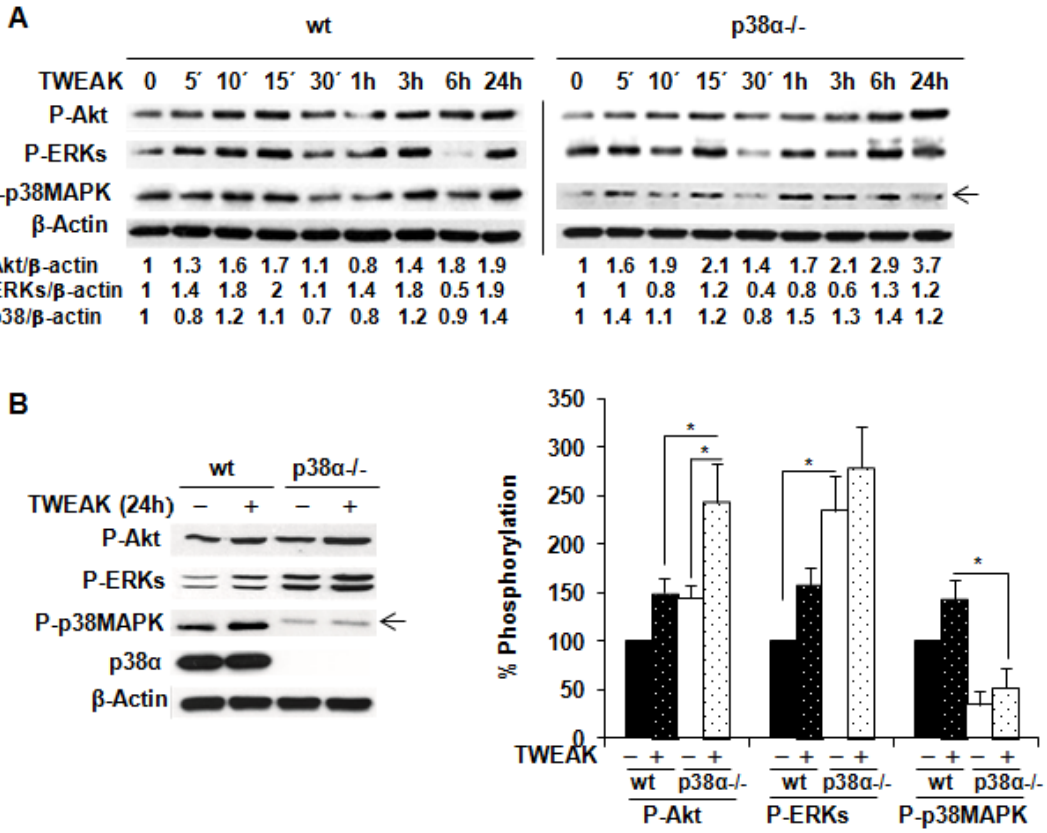
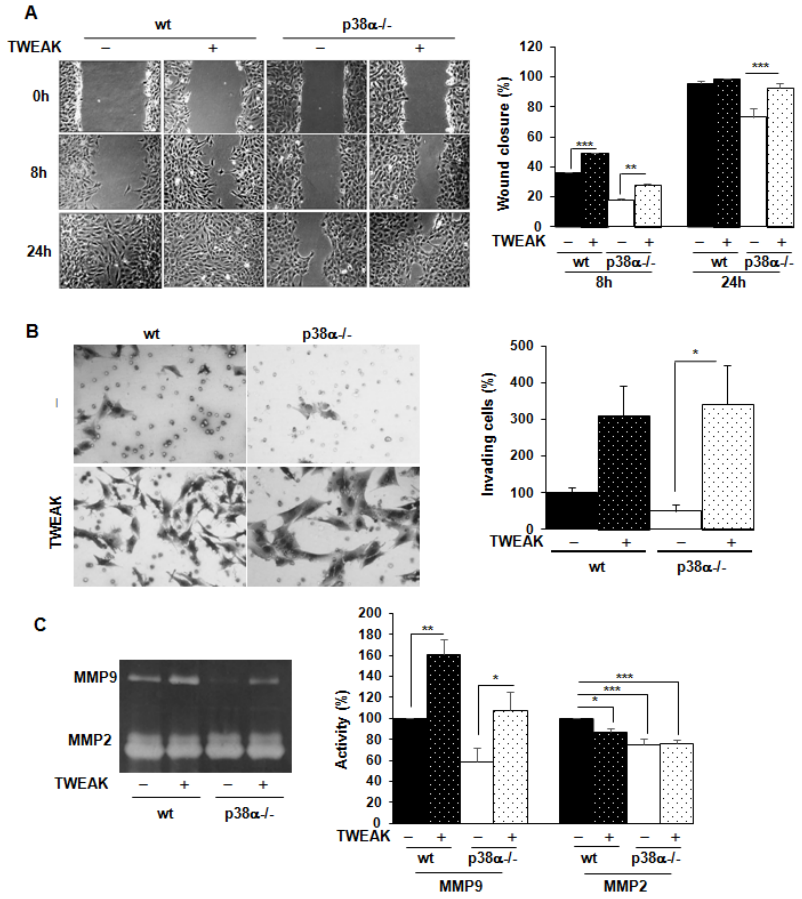


Figure 1



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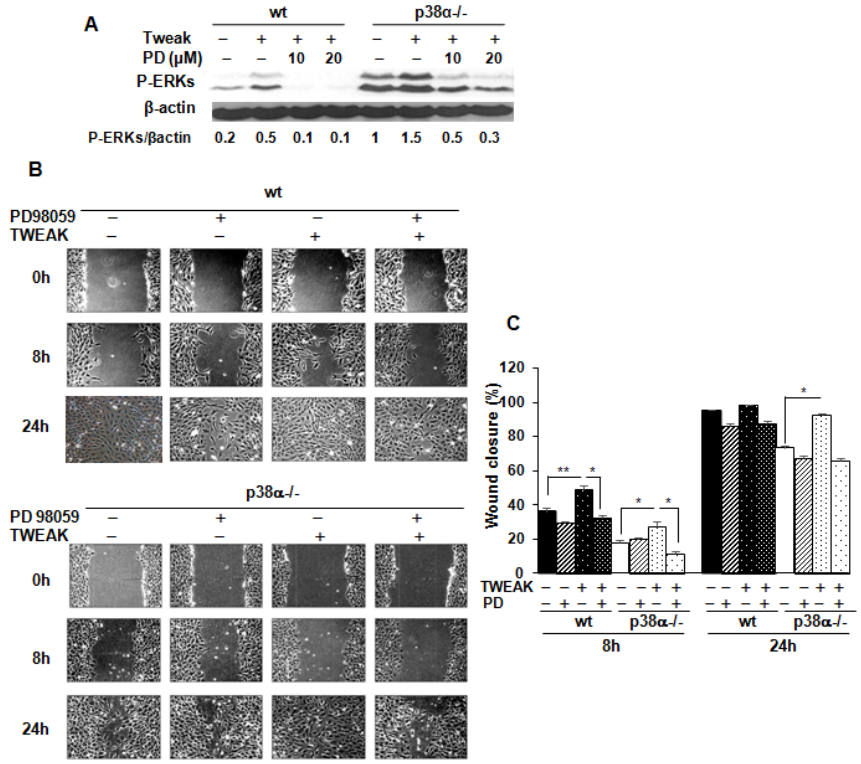

Figure 3

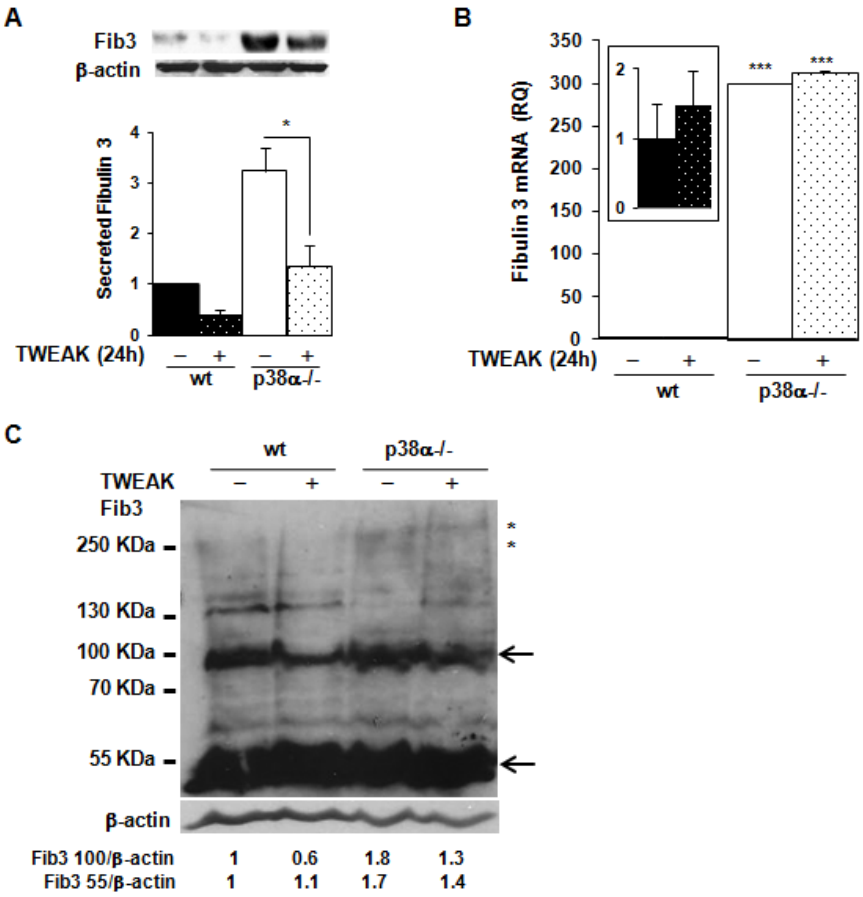

Figure 4

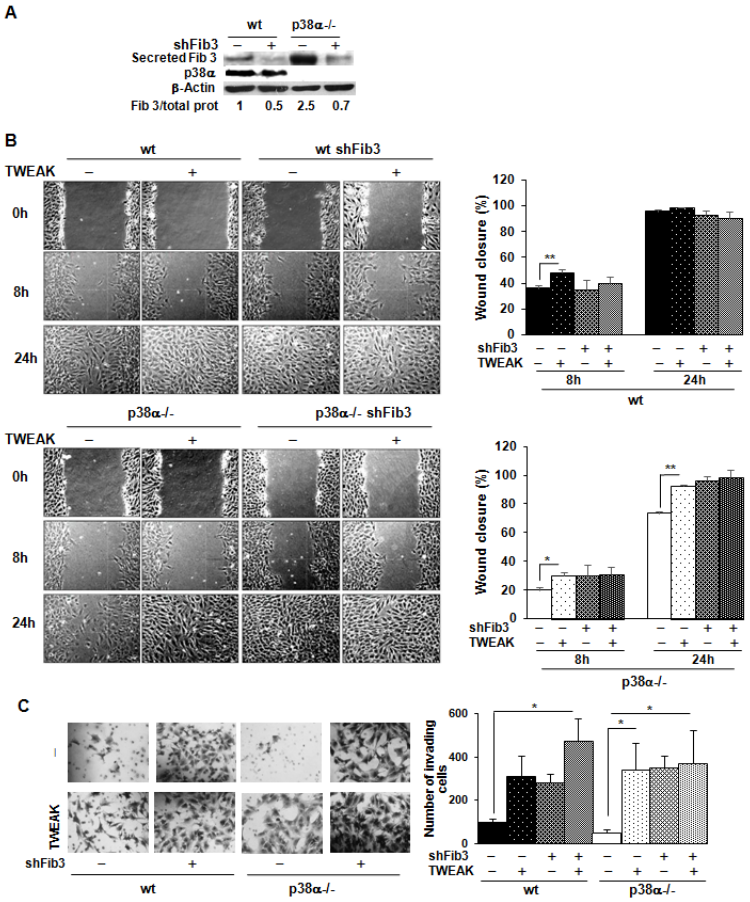

Figure 5

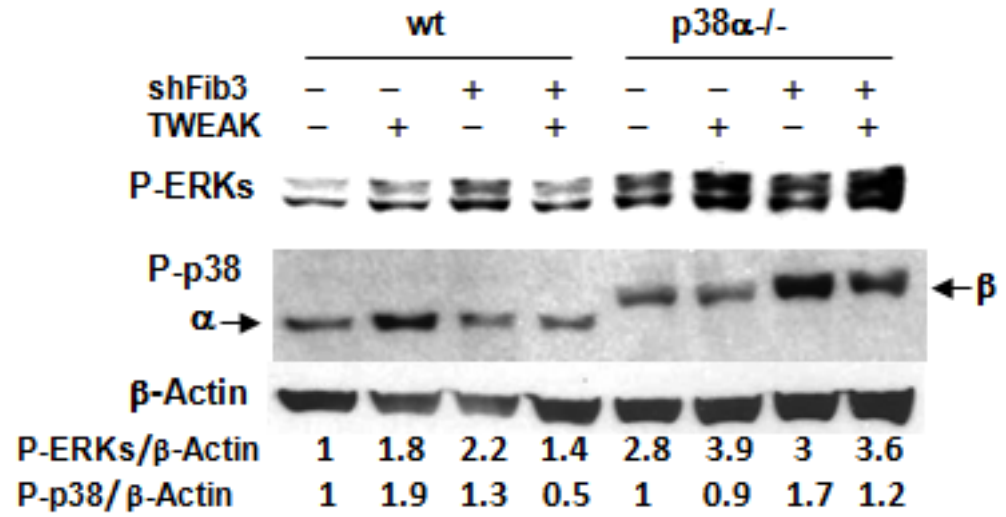

Figure 6

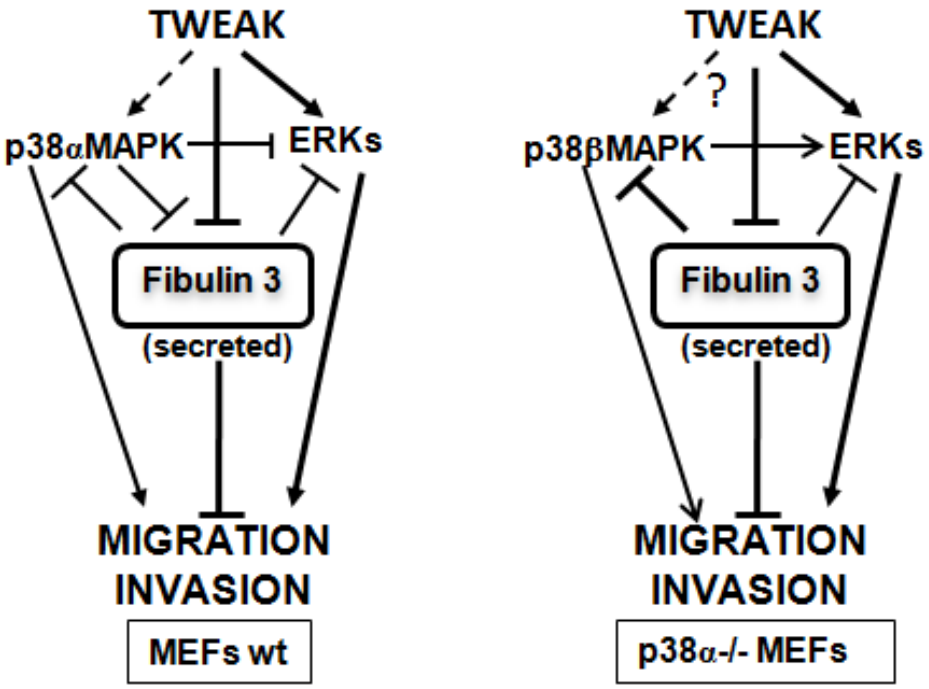

Figure 7